ANGIOPOIETIN-1 REQUIRES P190RHOGAP TO PROTECT AGAINST VASCULAR LEAKAGE IN VIVO

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Running Title: p190RhoGAP is necessary for Ang-1 anti-permeability

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SUMMARY

Angiopoietin-1 (Ang-1), a ligand of the endothelial-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, PI3K. This induces activation of one endogenous GTPase, Rac1, and inhibition of another, RhoA. Loss of either part of this dual effect abrogates Ang-1’s cytoskeletal and anti-permeability actions, suggesting that coordinated GTPase regulation is necessary for the vessel-sealing effects of Ang-1. p190RhoGAP, 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 p190RhoGAP to do so. Treatment with p190RhoGAP siRNA 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, p190RhoGAP 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.

INTRODUCTION

Angiopoietin-1 (Ang-1) is a 498 a.a. secreted glycoprotein whose germline depletion leads to several cardiovascular defects that result in embryonic lethality(1,2). Though 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). Though necessary for developmental angiogenesis to occur, Ang-1 expression and Tie-2 phosphorylation persist into adulthood in organs not considered angiogenically active (4). Secreting angiogenically active (4), suggesting a non-angiogenic role in the mature vasculature. In fact, Ang-1 has been shown to protect adult blood vessels against plasma leakage due to VEGF 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 multi-organ 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 (GEFs) 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, whereas 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 RhoA (13,14). Rho activity is regulated, in part, by the inhibitory GTPase activating protein, p190RhoGAP (15). Rac1 downregulates RhoA activity through p190RhoGAP 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 (1) to elucidate a novel signaling pathway connecting Ang-1 to Rac1 and RhoA; (2) to characterize Ang-1 mediated endothelial cytoskeletal changes; and (3) 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 p190RhoGAP in the pulmonary permeability response to Ang-1 and endotoxin.

**EXPERIMENTAL PROCEDURES**

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

**Chemicals:** Human recombinant Ang-1, CD14 and LPS-binding protein (LPB) were purchased from R&D systems (Minneapolis, MN).

**Animals:** FVB adult mice (20-25g) were used in accordance with an IACUC-approved protocol. LY294002 is from Cell Signaling Technology (Beverly, MA). Other reagents were obtained from Sigma (St Louis, MO).

**Cell Culture:** Human microvascular endothelial cells from lung (HMVEC-L) (Cambrex Bio Science 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 hours. 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/L NaF, and 1 mmol/L Na3VO4 and prepared for Western analysis as described before (7). Primary antibodies were anti-phospho-Akt Ab (Ser 473), anti-Akt Ab, and anti-phospho-myosin light chain 2 (Ser19) Ab (Cell Signaling Technology); anti-GAPDH Ab (Chemicon, Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience, Piscataway, NJ) and SuperSignal WestDura (Pierce) chemiluminescence substrates were used to detect primary Abs. All Western blots were performed in quadruplicate unless otherwise noted. Representative examples are shown. Densitometry (ImageJ, NIH) was used to compare results with unpaired t-test.

**Immunoprecipitation:** 200 mcg total protein from HMVEC-L lysed in Triton buffer were incubated with anti-Tie2-Ab (clone Ab33, Upstate, Lake Placid, NY) or p190RhoGAP Ab...
(Transduction laboratory, Lexington, KY) for 3 h, followed by incubation with protein A sepharose (Zymed, San Francisco, CA) 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), anti-Tie-2 Ab, or anti-p190RhoGAP Ab.

**PI3K activity assay:** After signal starvation, HMVEC-L were treated with vehicle or Ang-1 (100 ng/ml) for 15 min. Total and phosphorylated p85 subunits of PI3K were quantified using a commercial enzyme-linked immunosorbent assay according to the manufacturer’s instruction (Active Motif, Carlsbad, CA).

**Rac1 and Rho activity assay:** These were performed and quantified using the commercially available kit according to the manufacturer’s instruction (Cytoskeleton, Denver, CO). Briefly, lysates from cells treated with Ang-1 (100ng/ml) or inhibitor were incubated for 60 min with a 40 mcl slurry of a GST-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 Data Supplement—Methods).

**In vitro permeability by FITC-albumin flux:** This assay was conducted as previously described (7) (see Data Supplement—Methods).

**siRNA Transfection:** Control small interfering RNA (siRNA) (Ambion, Austin, TX) or siRNA directed to human p190RhoGAP (5’-GGAUUGUGUGGAAUGUAAG-3’ and 5’-CUUACAUUCACACAAUCC-3’) was transfected using SilentFect Lipid reagent (Bio-Rad) according to the manufacturer’s instructions. The cells were used for each experiment 3 days after transfection. Transfection efficiency was 90-100%. Down-regulation of p190RhoGAP 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 (Balazs, et al., manuscript in preparation). Generation of lentiviral vectors was accomplished by a five-plasmid transfection procedure (17) (see Data Supplement—Methods).

**In vivo permeability assay:** Mice (8-12 weeks old, female FVB strain) were pretreated with Ang-1 (10 mcg, ip). 8 h after the first Ang-1 injection, the second dose of Ang-1 (10 mcg, ip) and LPS (100 µg, ip) were co-injected. Lung permeability was assessed 16 h after the second injection as described previously (7) (see Data Supplement—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 control siRNA or 10 µg p190RhoGAP siRNA in 2 ml delivery solution injected into the tail vein over 7 seconds. The method of siRNA delivery is known to suppress 80-90% gene expression in multiple organs, including the lung (18). High-pressure hydrodynamic delivery does not adversely affect serum chemistries or result in end-organ injury (19). Animals were allowed to recover for 96h prior to administration of Ang-1 or endotoxin.

**p190RhoGAP knockdown at this time point was confirmed by lysing organs in RIPA buffer and performing Western analysis as outlined above.**

**Statistical analysis:** Results are reported as mean ± SEM. Comparisons between continuous variables were performed using unpaired two-sided t-test.

**RESULTS**

**Ang-1 activates Rac1 and inhibits RhoA**

Using pull-down assays, we examined whether Ang-1 affects the activities of Rac1 and RhoA in human lung microvascular ECs. Rac1 activity was increased 200% (p<0.05) 15
minutes and 250% (p<0.05 vs. control) 30 minutes following treatment with Ang-1 (Fig 1A, upper panel) while RhoA activity was decreased 50% (p<0.05) 30 minutes after Ang-1 addition (Fig 1A, lower panel). After establishing that Ang-1 activates Tie-2, PI3K, and Akt in HMVEC-L (Suppl A-C), we found that the PI3K inhibitor LY294002 (10µM) blocked Ang-1-induced activation of Rac1 (p<0.05, Fig 1B).

Ang-1 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 p190RhoGAP activation by 60% (p<0.05, Fig 1D). Suppression of p190RhoGAP by siRNA (Fig 1Ea) did not affect Ang-1-induced Rac1 activation (Fig 1Eb, upper panel), but did abolish the inhibitory effect of Ang-1 on RhoA (Fig 1Eb 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 (a) that Ang-1 activates endothelial Rac1 and p190RhoGAP to inhibit RhoA and (b) that p190RhoGAP acts downstream of activated Rac1.

Enhancement of endothelial junctions by Ang-1 requires PI3K and Rac1, not p190RhoGAP

Centripetal force exerted on the actin cytoskeleton can increase EC permeability. This is resisted by homologous binding between VE-cadherin, the transmembrane proteins that maintain adherens junctions between neighboring ECs. The cytoskeletal distribution of actin and myosin distinguishes these states—centralized 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 (ROCK) (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 (Suppl D). By immunofluorescence microscopy, we observed an increase in cortical actin, peripheral MLC-p, and junctional VE-cadherin compared to control confluent cells (Fig 2Aa-h). As expected, LY294002 reversed these effects of Ang-1, leading to thick central stress fiber bundles containing actin and MLC-P and intercellular gaps with attenuated VE-cadherin staining (Fig 2Ai-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-c) that were not present in control-virus-infected cells (Fig 2Ba). In the presence of control virus, Ang-1 retained the ability to augment junctional VE-cadherin staining (Fig 2Ba). However, this effect of Ang-1 was markedly diminished in Rac1T17N- and RhoAG14V-treated cells, resulting in gap formation (Fig 2Be-f). These results show that inhibition of endogenous Rac1 or activation of RhoA is sufficient to prevent Ang-1 mediated junctional fortification.

Knockdown of p190RhoGAP siRNA did not attenuate junctional VE-cadherin staining nor did it promote intercellular gap formation (Suppl Fa). Moreover, Ang-1 retained its ability to increase junctional VE-cadherin staining despite p190RhoGAP knockdown (Suppl Fb), suggesting that p190RhoGAP is dispensable for Ang-1 induced structural effects in otherwise unstimulated cells.

p190RhoGAP is necessary for Ang-1 to block endotoxin-induced structural disruption

Endotoxin treatment (100ng/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 p190RhoGAP 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 minutes of endotoxin exposure (100 ng/ml) scattered and attenuated junctional VE-cadherin staining and promoted gap formation compared to vehicle-treated cells (Fig 3Ca-b). Ang-1 prevented these effects, and cell-cell junctions appeared normal (Fig 3Cc). Rac1T17N (Fig 3Dc) or p190RhoGAP siRNA (Fig 3Ce) 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 p190RhoGAP to augment junctions weakened by endotoxin-mediated RhoA activation.

Ang-1 blocks endotoxin-induced hyperpermeability through PI3K, Rac1, and p190RhoGAP

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, p190RhoGAP siRNA had little effect on basal permeability, but did prevent the protective effect of Ang-1 (Fig 4C).

Inhibition of p190RhoGAP abolishes the protective effect of systemic Ang-1 against endotoxemic vascular leak in vivo

Since p190RhoGAP is necessary for Ang-1 to inhibit several effects of endotoxin, including RhoA activation (Fig 3B), intercellular gap formation (Fig 3C), and in vitro permeability (Fig 4C), we next addressed the importance of this pathway in vivo. Such validation is important because in vitro assays lack crucial elements such as unidirectional laminar flow, variable hydrostatic pressure, interacting cell types (e.g., neutrophils and vascular smooth muscle 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 mcg i.p.) produced an eight-fold increase in dye extravasation compared to 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 to control lung sections and those taken from animals pre-treated with Ang-1 (Fig 5B).

Delivery of p190RhoGAP siRNA (10 mcg i.v.) effectively reduced p190RhoGAP expression in the lung (Fig 5Ca) and was sufficient to block the anti-permeability effect of Ang-1 (Fig 5Cb). Moreover, histological sections confirmed that p190RhoGAP knockdown abrogated the ability of Ang-1 to block endotoxin-mediated interstitial edema and leukocyte infiltration (Fig 5D). These results validate our earlier in vitro findings in the context of the whole lung in healthy adult rodents and also establish the critical contribution of p190RhoGAP 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 p190RhoGAP, and inhibit RhoA activity. Ang-1 is able to block the structural rearrangements and hyperpermeability induced by endotoxin, but requires p190RhoGAP to do so. To demonstrate its importance more conclusively, expression of p190RhoGAP was inhibited in vivo using siRNA. A schematic summarizing the dichotomous actions of Ang-1 and endotoxin on the Rac1/RhoA balance is presented in Figure 6.

Inhibition of either Rac1 or p190RhoGAP 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 p190RhoGAP to attenuate pro-permeability signaling in vivo has not yet been described. Although p190RhoGAP suppression does not alter baseline cytoskeletal structure or in vitro permeability (or in vivo permeability, 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 p190RhoGAP by Ang-1. This is consistent with the regulatory role described for p190RhoGAP in other cell types (23). Therefore, p190RhoGAP although p190RhoGAP 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 p190RhoGAP, 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). In this last respect, our in vivo results were also notable for the ability of Ang-1 to block endotoxin-induced infiltration of leukocytes into the lung parenchyma. Ang-1 downregulates 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 nature of Ang-1 may arise both due to decreased adhesion molecule expression in ECs and 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 p190RhoGAP siRNA is that p190RhoGAP 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 p190RhoGAP 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
REFERENCES

FIGURE LEGENDS

Figure 1: Ang-1 has opposite effects on Rac1 and RhoA through p190RhoGAP. (A) Ang-1 activates Rac1 and inactivates 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 PAK pull-down assay and detected by immunoblotting with anti-Rac antibody. Similarly, the GTP-bound active form of RhoA was collected by rhotekin pull-down 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 Rac1T17N was performed as described in Methods. HMVEC-L were incubated with Ang-1, and Rac and Rho activity were measured as described above. (D) Ang-1 induces phosphorylation of p190RhoGAP in a Rac1-dependent fashion. p190RhoGAP phosphorylation was detected in vehicle (Cont) and Ang-1 (100ng/ml) treated HMVEC-L as described in Methods (left panel). HMVEC-L transfected with Rac1T17N lentivirus were treated with control (Cont) or Ang-1 (100ng/ml) for 30 min (right panel). (E) p190RhoGAP is not required for Ang-1 mediated Rac1 activation but is necessary for Ang-1 to suppress RhoA. p190RhoGAP knockdown does not block Ang-1-induced Rac1 activation but does block Ang-1-induced RhoA inactivation. siRNA against p190RhoGAP was transfected as described in Methods. (a) p190RhoGAP expression in HMVEC-L transfected with siRNA. GAPDH is blotted as a loading control. (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.

Figure 2: Ang-1-induced fortification of cell boundaries requires PI3K activation, Rac1 activation, and RhoA suppression. (A) Ang-1 causes peripheral MLC-P and cortical actin rearrangement in a PI3K-dependent manner. Confluent monolayers of HMVEC-L were incubated with vehicle (control) or Ang-1 with or without PI3K inhibitor, LY294002 (10 µM), in 0.25% FBS EBM-2 for 30 min. The cells were then fixed and stained for F-actin (red, a,e,i), MLC-P (green, b,f,j), nucleus (blue) and VE-cadherin (green, d,h,l). Shown are control (a-d), Ang-1 (e-h), Ang-1 plus LY294002 (i-l). White arrows indicate intercellular gap formation. Scale bar, 5µm. (B) Lentiviral delivery of Rac1T17N (dominant negative Rac1) or RhoAG14V (constitutively active RhoA) was performed as described in the Methods. After reaching confluency, HMVEC-L were incubated with vehicle (Control, a-c) or Ang-1 (d-f) in 0.25% FBS/EBM-2 for 30 min. The cells were then fixed and stained for VE-cadherin. Shown are cells transfected with control virus (a,d), Rac1T17N (b,e), or RhoAG14V (c,f). Scale bar, 5µm.

Figure 3: Ang-1 reverses endotoxin-induced Rac1 and RhoA signaling and requires active Rac1 and p190RhoGAP to block endotoxin-induced endothelial structural distortion. (A) HMVEC-L were stimulated with endotoxin (LPS 100ng/ml) for 30 min with or without Ang-1. Cells were lysed and Rac and Rho activity were measured as described in Methods. * p<0.01, ** p<0.05. Mean ± SEM of four experiments. (B) p190RhoGAP siRNA was performed as described in Methods. HMVEC-L were then stimulated with endotoxin (LPS 100ng/ml) for 30 min with or without Ang-1 and Rho activity was measured. (C) Delivery of Rac1T17N lentivirus or transfection with p190RhoGAP siRNA was performed as described in Methods. After reaching confluency, HMVEC-L were incubated with vehicle (a), endotoxin alone (LPS 100ng/ml, b), or endotoxin and Ang-1 (c-e) in 0.25% FBS/EBM-2 for 30 min. The cells were then fixed and stained for VE-cadherin. Shown are cells transfected with control virus (a-c), Rac1T17N (d), or p190RhoGAP siRNA (e) (Note: control siRNA cells treated with endotoxin and Ang-1 were indistinguishable from (c)). White arrows indicate intercellular gaps (identified by actin and nuclear staining, not shown). Scale bar, 5µm.

Figure 4: Inhibition of PI3K, Rac1, or p190RhoGAP is sufficient to abrogate the protective effect of Ang-1 against endotoxin on endothelial permeability. (A) HMVEC-L were grown to confluence on Transwell membranes coated with 0.5% gelatin. Cells were treated with vehicles, Ang-1 (100 ng/ml), LPS, LPS with Ang-1, or LPS with Ang-1 and the PI3K inhibitor, LY290042 (10 µM). Permeability was
evaluated after 4 h as described in Methods. Pa values are expressed as percentage of control cells (See Methods for calculation), * p<0.01. (B) HMVEC-L were transfected with Rac1T17N lentivirus and subjected to the permeability assay as described above. *p<0.01. (C) HMVEC-L were transfected with p190RhoGAP siRNA and subjected to the permeability assay as described above. *p<0.01. Mean ± SEM of four experiments.

Figure 5: Ang-1 blocks LPS-induced pulmonary hyperpermeability in vivo in a p190RhoGAP-dependent fashion. (A) Lung permeability (Measured in absorbance units, see Methods) was determined in control mice (vehicle ip), endotoxin (LPS 100 µg ip) or endotoxin plus Ang-1 (10 µg ip x 2 doses). (B) H and E stained 40x 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) In vivo delivery of p190RhoGAP siRNA. (a) p190RhoGAP protein is reduced in mouse lung after hydrodynamic delivery of specific siRNA but not after delivery of control siRNA. (b) In mice treated with control siRNA, endotoxin-induced permeability was unaffected as was the rescue ability of Ang-1; p190RhoGAP knockdown blocked the anti-permeability effect of Ang-1 in vivo. p190siRNA treatment in the absence of endotoxin caused no change in basal lung permeability compared to control siRNA (data not shown). Vascular permeability (D) H and E stained 40x photomicrographs of lungs taken from animals treated as in (C) above. In the presence of p190RhoGAP knockdown, Ang-1 can no longer inhibit endotoxin-induced edema and inflammation. *p<0.05. Mean ± SEM of four experiments.

Figure 6: Proposed Ang-1 barrier-protective signaling. The lower right scheme illustrates the counteracting effects of Rac1 and RhoA on actin, myosin light chain (MLC-P), and VE-cadherin at endothelial junctions. Either the Rac1 or RhoA limb is activated with a particular stimulus. p190RhoGAP links Rac1 activation to RhoA inhibition to coordinate these cytoskeletal regulators. We demonstrated that excess Ang-1 (upper left cell) shifts the balance between Rac1 and RhoA towards Rac1, leading to enhanced peripheral MLC-P, cortical actin rearrangement (red lines), and augmentation of the junctional VE-cadherin (green bars). Endotoxin, on the other hand, activates RhoA and shifts the balance away from Rac1, destabilizing cell architecture with red central actin stress fibers and “unzipped” green VE-cadherin resulting in interendothelial gaps and subsequent permeability. Upon coincubation of Ang-1 and endotoxin, the barrier protective effect of Ang-1 predominates by directly augmenting Rac1 activity as well as by suppressing RhoA activity through p190RhoGAP. We have shown that loss of PI3K, Rac1, or p190RhoGAP is sufficient to abrogate the protective effect of Ang-1 against endotoxin.
**Figure 1**

A

- GTP-Rac1
- Total Rac1
- GTP-RhoA
- Total RhoA

Ang-1 0 15 30 (min)

B

- GTP-Rac1
- Total Rac1

Cont Ang-1 Ang-1 +LY

C

- GTP-Rac1
- Total Rac1
- GTP-RhoA
- Total RhoA

Cont Ang-1

D

IP: p190
IB: P-Tyr

p190

Rac1T17N

Cont Ang-1 Cont Ang-1

Relative ratio p-p190/total p190

E

a

- p190
- GAPDH

Control p190 siRNA siRNA

b

- p190siRNA

GTP-Rac1 Total Rac1

GTP-RhoA Total RhoA

Cont Ang-1
Figure 2

Control

Ang-1

Ang-1 +LY

Control

Actin MLC-P Merge VE-cad

Control Ang-1 +LY

Control Rac1T17N RhoAG14V

Figure 2
Figure 3

A

GTP-RhoA
Total RhoA
GTP-Rac1
Total Rac1

LPS   -        +      +
Ang-1 -        -       +

B

GTP-RhoA
Total RhoA
GTP-Rac1
Total Rac1

LPS   -        +      +
Ang-1 -        -       +

C

Control
LPS
LPS + Ang-1

Rac1T17N
+ LPS + Ang-1

p190siRNA
+ LPS + Ang-1
Figure 5