Asef mediates HGF protective effects against LPS-induced lung injury and endothelial barrier dysfunction

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Meng F, Meliton A, Moldobaeva N, Mutlu G, Kawasaki Y, Akiyama T, Birukova AA. Asef mediates HGF protective effects against LPS-induced lung injury and endothelial barrier dysfunction. Am J Physiol Lung Cell Mol Physiol 308: L452–L463, 2015. First published December 24, 2014; doi:10.1152/ajplung.00170.2014.—Increased vascular endothelial permeability and inflammation are major pathological mechanisms of pulmonary edema and its life-threatening complication, the acute respiratory distress syndrome (ARDS). We have previously described potent protective effects of hepatocyte growth factor (HGF) against thrombin-induced hyperpermeability and identified the Rac pathway as a key mechanism of HGF-mediated endothelial barrier protection. However, anti-inflammatory effects of HGF are less understood. This study examined effects of HGF on the pulmonary endothelial cell (EC) inflammatory activation and barrier dysfunction caused by the gram-negative bacterial pathogen lipopolysaccharide (LPS). We tested involvement of the novel Rac-specific guanine nucleotide exchange factor Asef in the HGF anti-inflammatory effects. HGF protected the pulmonary EC monolayer against LPS-induced hyperpermeability, disruption of monolayer integrity, activation of NF-κB signaling, expression of adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, and production of IL-8. These effects were critically dependent on Asef. Small-interfering RNA-induced downregulation of Asef attenuated HGF protective effects against LPS-induced EC barrier failure. Protective effects of HGF against LPS-induced lung inflammation and vascular leak were also diminished in Asef knockout mice. Taken together, these results demonstrate potent anti-inflammatory effects by HGF and delineate a key role of Asef in the mediation of the HGF barrier protective and anti-inflammatory effects. Modulation of Asef activity may have important implications in therapeutic strategies aimed at the treatment of sepsis and acute lung injury/ARDS-induced gram-negative bacterial pathogens.

hepatocyte growth factor; guanine nucleotide exchange factors; cytoskeleton; pulmonary endothelium; inflammation; permeability; vascular leak

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) is often associated with sepsis and remains a major cause of morbidity and mortality with an overall mortality rate of 30–40% (26, 33). Increased capillary endothelial permeability and reduced alveolar liquid clearance capacity are major pathological mechanisms of pulmonary edema and ARDS. Mechanisms of endothelial cell (EC) permeability involve dynamic cytoskeletal changes, assembly and disassembly of cell-cell junctions, and signaling cross talk between various cytoskeletal compart-

ments, such as actin networks and microtubules (24, 27). Interestingly, alterations in cell cytoskeleton also play an important role in the modulation of inflammatory responses. In vascular endothelium, inflammatory mediators increase expression of cell adhesion molecules [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM), and E-selectin], which trigger adhesion and tissue transmigration of activated neutrophils (13, 40, 45). These events escalate general lung inflammation. However, little is known about intracellular processes, which determine lung EC barrier preservation and reduce inflammation in acute lung injury (ALI), and effective barrier-protective substances for ALI/ARDS treatment remain to be identified.

Hepatocyte growth factor (HGF) is a prosurvival mediator that regulates different biological processes, including the maintenance of vascular barrier integrity, and appears at increased concentrations in lung circulation under pathological conditions such as ALI, sepsis, lung inflammation, and ventilator-induced lung injury (25, 34, 51). Novel therapeutic strategies using HGF have been suggested for cardiovascular diseases (1, 43). Increased HGF levels have been detected in inflamed lungs and are suggested to serve as a compensatory mechanism to help protect lung vascular integrity in ALI conditions and attenuate devastating consequences of lung inflammation and tissue injury (46). HGF binding to c-Met receptor stimulates receptor tyrosine kinase activity and recruitment of multiple SH2 domain-containing signaling molecules (32, 36). In turn, HGF-induced activation of Rac-GTPase leads to endothelial barrier protection via enhancement of the peripheral actin cytoskeleton and increased interactions between adherens junction proteins α/β-catenin and VE-cadherin (3, 23).

Our previous studies demonstrated the involvement of the Dbl family member Rac-specific guanine nucleotide exchange factor (GEF) Tiam1 in the EC barrier protection induced by several agonists, including HGF (3, 10, 11, 37). However, Tiam1 downregulation did not cause complete suppression of HGF protective effects, suggesting activation of additional mechanisms. Another Rac/Cdc42-specific GEF, APC-stimulated guanine nucleotide exchange factor (Asef), has been originally identified in cancer cells. Asef contains Dbl homology domain exhibiting GEF activity, plekstrin homology domain which determines the subcellular localization and activity by interacting with phosphatidylinositol phosphate, Src homology (SH) 3 autoinhibitory domain, and a region that binds tumor suppressor adenomatous polyposis coli protein (APC) (19). Asef-dependent Rac and Cdc42 signaling has been im-

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licated in regulation of actin cytoskeleton dynamics in epithelial and neuronal cells (20).

This study investigated the role of Asef in control of lung endothelial barrier. With the use of comprehensive evaluation of lung barrier function, including biochemical assays, imaging studies, molecular inhibition approaches, and a genetic animal model, this study investigated the role of Asef in HGF-mediated vascular barrier protection against lung inflammation and injury induced by bacterial pathogens.

MATERIALS AND METHODS

Cell culture and reagents. Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza (Allendale, NJ), propagated according to the manufacturer’s recommendations, and used for experiments at passages five to seven. Human HGF was obtained from R&D Systems (Minneapolis, MN). Cell-permeable c-Met kinase inhibitor, N-[3-fluoro-4-(7-methoxy-4-quinolinyl)-phenyl]-1-(2-hydroxy-2-methylpropyl)-5-methyl-3-oxo-phenyl-2,3-dihydro-1H-pyrazole carboxamide, also known as carboxamide, was purchased from EMD Millipore (Billerica, MA). Carboxamide is a cell-permeable quinoline compound that acts as a potent inhibitor of HGF receptor c-Met (IC_{50} = 4 nM) and used as selective c-Met inhibitor in endothelial cells. Reagents for immunofluorescence were purchased form Molecular Probes (Eugene, OR). Antibodies against NF-κB and IκBα were obtained from Cell Signaling (Beverly, MA); Asef, VE-cadherin, ICAM-1, and VCAM-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Unless specified, biochemical reagents including LPS were obtained from Sigma (St. Louis, MO).

Measurement of endothelial permeability. The cellular barrier properties were analyzed by measurements of transendothelial electrical resistance across confluent human pulmonary artery endothelial monolayers using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as previously described (2, 4). Express micromolecule permeability testing assay (XPerT) was recently developed in our group (14) and currently available from Millipore (catalog no. 17–10398; Vascular Permeability Imaging Assay). This assay is based on high-affinity binding of avidin-conjugated FITC-labeled tracer to the biotinylated extracellular matrix proteins immobilized on the bottom of culture dishes after the EC barrier is compromised by treatment with a barrier-disruptive agonist. XPerT permeability assays were performed in 96-well plates. Visualization of EC monolayer permeability was performed in HPAEC plated on glass cover slips coated with biotinylated gelatin followed by agonist stimulation, incubation with FITC-avidin tracer, fluorescence microscopy, and imaging analysis as previously described (14, 42).

Asef knockdown in human pulmonary EC culture. To deplete endogenous Asef, an Asef-specific set of three Stealth Select small-interfering RNA (siRNA) duplexes was purchased from Invitrogen (Carlsbad, CA) in ready-to-use, desalted, deprotected, annealed double-strand form. Nonspecific RNA (Dharmacon, Lafayette, CO) was used as a control treatment. Transfection of EC with siRNA was performed as previously described (7). After 72 h of transfection, cells were used for experiments or harvested for Western blot verification of specific protein depletion.

Immunofluorescence. Endothelial monolayers plated on glass cover slips were subjected to immunofluorescence staining as described previously (5). Texas red phalloidin was used to visualize F-actin. VE-cadherin antibody was used to visualize adherens junctions. Slides were analyzed using a Nikon video imaging system (Nikon Instech, Tokyo, Japan). Images were processed with Image J (National Institute of Health, Bethesda, MD) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) software.

Immunoblotting. After stimulation with agonist of interest, cells were lysed, and protein extracts were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with specific antibodies. Immunoreactive proteins were detected with the enhanced chemiluminescent detection system according to the manufacturer’s protocol (Amersham, Little Chalfont, UK). Equal protein loading was verified by reprobing membranes with antibody to β-actin or β-tubulin.

Neutrophil migration and ELISAs. Neutrophil chemotaxis was measured in a 96-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD) as described previously (28). Concentration of interleukin-8 (IL-8) and soluble ICAM-1 was measured in HPAEC conditioned media using an ELISA kit available from R&D Systems.

In vivo model of ALL. All animal care and treatment procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Generation of Asef^{−/−} mice is described elsewhere (18). Hetero- and homozygous Asef^{−/−} mice were fertile and indistinguishable from their wild-type littermates based on growth rates, external appearance, and pathological examination of internal organs. Absence of Asef appeared to be compatible with normal physiological functioning of mice except for modest impairment of retinal angiogenesis in neonatal mice and reduced angiogenic potential in adult mice (18). Adult male Asef^{−/−} mice and matching wild-type controls, 8–10 wk old, with average weight 20–25 g were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg). HGF (10 μg/kg) was first administered intravenously via jugular vein followed by intratracheal instillation of bacterial LPS (0.7 mg/kg; Escherichia coli O55:B5, it) or saline (15 min after onset of HGF injection). Second HGF injection to maintain HGF circulating levels was performed 5 h after LPS challenge. After 24 h, animals were killed under anesthesia.

Evaluation of lung injury parameters. After the experiment, bronchoalveolar lavage (BAL) was performed using 1 ml of sterile Hanks’ balanced saline buffer. The BAL protein concentration was determined by the BCATM Protein Assay kit (Thermo Scientific, Pittsburgh, PA). BAL inflammatory cell counting was performed using a standard hemacytometer technique (6, 16). Total lung myeloperoxidase (MPO) content was determined from homogenized lungs as described elsewhere (29). For analysis of LPS-induced lung vascular leak, Evans blue dye (30 ml/kg) was injected into the external jugular vein 2 h before termination of the experiment. Measurement of Evans blue accumulation in the lung tissue was performed by spectrofluorimetric analysis of lung tissue lysates according to the protocol described previously (30, 31). For histological assessment of lung injury, the lungs were harvested without lavage collection and fixed in 10% formaldehyde. After fixation, the lungs were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Sections were evaluated at ×40 magnification.

Statistical analysis. Results are presented as means ± SD of three to six independent experiments. Stimulated samples were compared with controls by unpaired Student’s t-test. For multiple-group comparisons, a one-way ANOVA, followed by the post hoc Tukey test, were used. P < 0.05 was considered statistically significant.

RESULTS

HGF attenuates endothelial hyperpermeability induced by LPS. Effects of HGF on LPS-induced lung EC monolayer permeability for macromolecules associated with septic inflammation were analyzed using an express permeability testing assay developed by our group and described in MATERIALS AND METHODS (14). LPS significantly increased EC monolayer permeability for FITC-labeled avidin, whereas HGF attenuated LPS barrier disruptive effects (Fig. 1, A and B). HGF protective
**Xpert permeability:**

![Graph showing FITC Fluorescence, AU](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>FITC Fluorescence, AU</th>
</tr>
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<tbody>
<tr>
<td>Veh</td>
<td>2000</td>
</tr>
<tr>
<td>LPS</td>
<td>6000</td>
</tr>
<tr>
<td>HGF + LPS</td>
<td>8000</td>
</tr>
<tr>
<td>cMet-i</td>
<td>10000</td>
</tr>
<tr>
<td>cMet-i + LPS</td>
<td>10000</td>
</tr>
<tr>
<td>cMet-i/HGF + LPS</td>
<td>10000</td>
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</table>

**FITC-Avidin**

![Images of FITC-Avidin](image)

**Vehicle**

**LPS**

**HGF + LPS**

**cMet-i/HGF + LPS**

**Inhibition of LPS-induced permeability**

![Graph showing Normalized Resistance](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normalized Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
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</tr>
<tr>
<td>HGF + LPS</td>
<td>0.7</td>
</tr>
<tr>
<td>cMet-i</td>
<td>0.8</td>
</tr>
<tr>
<td>cMet-i + LPS</td>
<td>0.9</td>
</tr>
<tr>
<td>cMet-i/HGF + LPS</td>
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</table>

**Inhibition of LPS-induced permeability (%) of max response**

- LPS: 100%
- HGF + LPS: 25%
- cMet-i/HGF + LPS: 25%

*Significant difference*
effect was abolished by cell pretreatment with the pharmacological inhibitor of c-Met receptor, carboxamide. The results show no effect of inhibitor alone (without HGF administration) on basal and LPS-induced EC permeability. Visualization of permeability sites in the LPS-challenged EC monolayers showed penetration of fluorescent probe via weakened cell–cell junctions and LPS-induced paracellular gaps. HGF pretreatment of EC monolayers before LPS challenge markedly decreased penetration of FITC-labeled avidin through intercellular junctions (Fig. 1B). Effects of HGF on EC dysfunction induced by LPS were further examined using the measurements of transendothelial electrical resistance. HPAEC were pretreated with HGF followed by incubation with LPS for up to 15 h. HGF dramatically attenuated disruptive effects of LPS (Fig. 1C).

**HGF suppresses LPS-induced EC monolayer disruption.** Effects of HGF on LPS-induced EC monolayer disruption were next examined by analysis of actin cytoskeleton remodeling and changes in adherens junction integrity. LPS induced formation of actin stress fibers and cell contraction associated with appearance of paracellular gaps after 5 h of LPS treatment, the time point corresponding to a pronounced increase in EC monolayer permeability. These changes were reduced by EC pretreatment with HGF (Fig. 2, A and B). Immunofluorescence analysis of adherens junction remodeling confirmed disruption of cell–cell contacts in response to LPS. In turn, HGF pretreatment preserved the continuous adherens junction pattern in LPS-challenged EC (Fig. 2A). Pretreatment with c-Met inhibitor abrogated HGF protective effects against LPS-induced disruption of EC monolayer integrity. These results reflect potent protective effects of HGF against EC barrier dysfunction caused by endotoxin derived from gram-negative bacteria.

**HGF inhibits LPS-induced activation of inflammatory signaling.** Attenuation of LPS-induced barrier disruptive and inflammatory signaling by HGF was further examined. LPS stimulation of pulmonary EC triggered the canonical inflammatory pathway and induced degradation of IκBα inhibitory subunit leading to activation of NF-κB signaling. These effects were inhibited by HGF (Fig. 3A). LPS-induced activation of NF-κB-dependent inflammatory gene expression requires nuclear translocation of NF-κB p65 subunit. Subcellular fractionation assays (Fig. 3B) showed nuclear translocation of NF-κB after LPS challenge that was attenuated by HGF.

**Activation of vascular endothelium by inflammatory agents stimulates neutrophil adhesion to the vascular EC lining followed by neutrophil transmigration through the EC monolayer and neutrophil recruitment to the inflamed lung tissue.** The following studies evaluated effects of HGF on EC inflammatory activation. Western blot experiments showed the time-dependent, LPS-induced expression of EC surface adhesion molecules ICAM-1 and VCAM-1, which are involved in neutrophil adhesion. This LPS effect was attenuated by HGF pretreatment (Fig. 3C). In complementary studies, a content of soluble ICAM-1 was measured in HPAEC conditioned media. Similarly to analysis of ICAM-1 expression, LPS treatment caused elevation of soluble ICAM-1, whereas HGF inhibited this increase (Fig. 3D). We next measured IL-8 content in preconditioned medium collected from control and stimulated EC. LPS induced IL-8 expression by pulmonary EC, which was abolished by HGF pretreatment (Fig. 3E). Collectively, these data suggest potent protective effects of HGF against LPS-induced activation of inflammatory signaling in pulmonary endothelium.

**Asef mediates HGF-induced protection of EC barrier.** Previous works demonstrated the role of HGF-activated Rac1 signaling in EC barrier enhancement. The following experiments tested involvement of novel Rac1-specific guanine nucleotide exchange factor Asef in the HGF-induced down-regulation of inflammatory signaling. siRNA-induced Asef knockdown did not significantly change the permeability response to LPS alone but attenuated protective effects of HGF against LPS-induced permeability (Fig. 4A). EC treatment with Asef-specific siRNA resulted in 80–90% reduction of Asef endogenous protein content, as shown below (Fig. 5A).

The role of Asef in the HGF barrier protective effects was further assessed by analysis of cytoskeletal remodeling in control and Asef-depleted HPAEC stimulated with LPS with or without HGF pretreatment. Similarly to nontransfected EC (Fig. 2), HGF pretreatment attenuated LPS-induced stress fiber formation and disruption of monolayer integrity in cells transfected with nonspecific RNA. These effects of HGF were inhibited by Asef knockdown (Fig. 4B).

Asef knockdown also abolished protective effects of HGF against LPS-induced activation of NF-κB signaling (Fig. 5, A and B). siRNA-induced Asef protein knockdown was confirmed by Western blot with Asef-specific antibody (Fig. 5A, bottom). Moreover, Asef knockdown attenuated the protective effect of HGF against LPS-induced ICAM-1 and VCAM-1 expression (Fig. 5C) and release of soluble ICAM-1 (Fig. 5D), a hallmark of inflammatory activation of endothelial cells.

**Asef mediates protective effects of HGF in vivo.** The studies in pulmonary EC culture described above demonstrate a critical role for Asef as a key mediator of HGF-induced signaling. The role of Asef in the HGF-induced lung protection was further investigated in the model of ALI induced by intratracheal instillation of LPS (9, 50). *Asef*−/− mice (18) and matching wild-type controls were injected with HGF or vehicle (iv) followed by LPS intratracheal administration in the next 10–15 min. The HGF group also received a second HGF

Fig. 1. Effect of hepatocyte growth factor (HGF) on lipopolysaccharide (LPS)-induced endothelial cell (EC) permeability. A and B: human pulmonary artery endothelial cells (HPAEC) grown in 96-well plates (A) or on glass cover slips (B) with immobilized biotinylated gelatin (0.25 mg/ml) were treated with vehicle or HGF (50 ng/ml, 15 min) with or without pretreatment with cell-permeable c-Met kinase inhibitor (cMet-i, 50 nM carboxamide, 30 min), followed by challenge with LPS (300 ng/ml, 5 h) and addition of FITC-avidin (25 μg/ml, 3 min). Unbound FITC-avidin was removed, and FITC fluorescence was measured. XPerT, express micromolecule permeability testing assay. Data are expressed as means ± SD of 4 independent experiments; *P < 0.05 vs. LPS alone. C: HPAEC plated on microelectrodes were treated with HGF (50 ng/ml, 15 min) with or without pretreatment with c-Met kinase inhibitor (50 nM carboxamide, 30 min), followed by stimulation with LPS (300 μg/ml) as shown by arrows. Transendothelial electrical resistance (TER) was monitored over 15 h. Permeability data are expressed as means ± SD of 6 independent experiments; *P < 0.05.
A

F-Actin | VE-Cadherin | Merged

Vehicle

LPS

HGF+LPS

cMet-i/HGF+LPS

B

Paracellular gaps

Gap area, % of total area

Veh | LPS | HGF +LPS | cMet-i/HGF +LPS

*
in the bronchoalveolar lavage fluid (BALF). Compared with wild-type mice but failed to induce comparable protective effects in Asef knockouts. The protective effects of HGF against LPS-induced lung vascular leak in wild-type and Asef \(-/-\) mice were further assessed by measurement of Evans blue leakage into the lung tissue. LPS induced noticeable Evans blue dye accumulation in the lung parenchyma that was significantly decreased by HGF in wild-type controls (Fig. 7A). Protective effects of HGF were suppressed in the Asef \(-/-\) mice. These results were further confirmed by quantitative analysis of actin cytoskeletal rearrangement and adherens junctions remodeling performed by immunofluorescence staining with Texas red phalloidin and VE-cadherin, respectively. Paracellular gaps are marked by arrows. B: quantitative analysis of paracellular gap formation in control and treated HPAEC. Data are expressed as mean ± SD of 4 independent experiments; \(*P < 0.05\) vs. LPS alone.

Fig. 3. Effects of HGF on LPS-induced inflammatory signaling. A and B: HPAEC were treated with vehicle or HGF (50 ng/ml, 15 min) with or without pretreatment with carboxamide (50 nM cMet-i, 30 min), followed by stimulation with LPS (300 ng/ml) for 1 h. A: degradation of IκBα was detected using antibodies against nonphosphorylated protein. Equal protein loading was confirmed by determination of β-tubulin content in total cell lysates. B: fractionation assay was performed, and the content of NF-κB in the nuclear fraction was determined by Western blot analysis with specific antibodies. Determination of NF-κB content in corresponding total cell lysates and lamin B content in nuclear fractions were used to ensure equal loading. C and D: HPAEC were pretreated with HGF (50 ng/ml, 15 min) with or without carboxamide (50 nM cMet-i, 30 min), followed by stimulation with LPS (300 ng/ml) for 6 h. C: intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 expression was detected by Western blot with corresponding antibody. β-Tubulin staining was used as a normalization control. Soluble ICAM-1 (D) and IL-8 production (E) in EC preconditioned medium was evaluated by ELISA. \(*P < 0.05\) vs. LPS alone.
A

B

F-Actin

VE-Cadherin

Merged

nsRNA: LPS

nsRNA: HGF+LPS

si-Asef: LPS

si-Asef: HGF+LPS

LPS

HGF+LPS

LPS

HGF+LPS

Asef AND HGF-MEDIATED PROTECTION OF LUNG FUNCTION

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00170.2014 • www.ajplung.org
sis of Evans blue-labeled albumin extravasation in the lung preparations (Fig. 7B). In concurrence with cell culture studies, HGF treatment inhibited LPS-induced ICAM-1 expression in the lung, as detected by Western blot analysis of lung tissue homogenates from wild-type mice. This effect was abolished in Asef−/− mice (Fig. 7C).

Taken together, these results demonstrate the role for Asef in the mediation of HGF effects and further support anti-inflammatory and barrier-protective effects of HGF against septic inflammation and vascular endothelial barrier dysfunction in cell culture and the animal models of LPS-induced lung injury.

DISCUSSION

HGF is produced by endothelial cells, alveolar macrophages, fibroblasts, bronchial cells, and even by activated alveolar neutrophils (22). HGF levels are elevated in lung fluid of patients with ALI, suggesting the role of HGF in lung repair under inflammatory conditions (44). This study shows that, in
addition to protecting against agonist-induced EC permeability via downregulation of Rho-mediated barrier disruptive signaling (3), HGF is capable of suppressing the LPS-induced endothelial inflammatory activation and barrier disruption.

Rac-specific GEF Asef has been recently described in epithelial cell lines and shown to be involved in HGF-induced epithelial cell adhesion and migration (35). Asef expression increased the renal tubules as a cellular response to injury necessary for cell proliferation and repair of renal tubules (12). Molecular inhibition of Asef decreased the rates of basic fibroblast growth factor (bFGF)- and vascular endothelial growth factor (VEGF)-induced endothelial cell migration and partially suppressed angiogenesis (18). The role of Asef in modulation of inflammation has been unknown.

This study shows a novel mechanism of HGF anti-inflammatory effect via Asef-dependent attenuation of EC barrier disruption, expression of adhesion molecules ICAM-1 and VCAM-1, and production of interleukin-8. This mechanism is further supported by experiments with Asef knockdown in human pulmonary EC and Asef−/− mice. Asef knockouts developed more severe lung injury in response to LPS, whereas barrier protective and anti-inflammatory effects of HGF in the LPS model were suppressed. In support of the key role of Asef in the HGF-induced protective mechanism described in this study, Asef ablation also caused nearly complete inhibition of Rac activation in response to VEGF and bFGF in cells isolated from Asef−/− mice, leading to impaired cell migration and tube formation, although expression of other Rac-specific GEFs was not affected (18).

A role of Rac-Rho cross talk in control of lung inflammation and injury is not well understood. LPS-induced activation of Rho signaling occurs in parallel to stimulation of canonical inflammatory cascades (15, 41, 48, 49). One mechanism of LPS-induced Rho activation involves release from microtu-
bulges and activation of Rho-specific activator GEF-H1 (21). In turn, activation of the Rho pathway further enhances LPS-induced activation of NF-kB and stress kinases. This amplification of inflammatory signaling may be ceased by pharmacologic inhibition of Rho kinase activity (8, 39, 47, 48). Alternatively, molecular inhibition of GEF-H1 in vitro and gene ablation in vivo attenuated LPS-induced EC permeability and suppressed LPS-induced upregulation of ICAM-1, IL-8, and neutrophil adhesion (21). The precise mechanism of Asef-dependent attenuation of inflammation requires further investigation. However, based on our present and published data, and Asef function as Rac activator, we speculate that Asef activation may shift the balance between Rac and Rho activities toward Rac, leading to the reduction of Rho pathway input into the LPS-induced inflammatory response.

Our results demonstrate potent, but incomplete, inhibition of HGF anti-inflammatory and barrier protective effects by Asef knockdown in EC culture and in Asef knockout mice. In concordance with this observation, retinal angiogenesis was also modestly impaired in Asef−/− mice, which, similarly to our observations, did not show other significant abnormalities. These phenotypic features of Asef−/− mice raise the possibility...
that Asef function might be backed by other members of Dbl family guanine nucleotide exchange factors. Indeed, HGF may activate other Rac specific GEF Tiam1, shown to stimulate Rac activity and cortical actin remodeling, enhance endothelial cell adhesions junctions, and strengthen the endothelial barrier (3, 38). Therefore, activation of Tiam1 by HGF may complement anti-inflammatory effects of Asef. This signaling cross talk warrants further investigation.

Involvement of multiple GEFs in Rac I GTPase activation by various factors may be dictated by necessity for space- and time-dependent orchestration of Rac activity in response to agonist-induced or mechanical stimulation. Therefore, Asef and other GEFs such as Tiam1 may be involved in different steps of HGF-induced Rac regulation. Alternatively, activation of two or more GEFs by HGF may provide fine tuning of the levels of Rac activation by specific barrier protective agonists, mechanical signals, or disruption of monolayer integrity. Further studies are warranted to investigate the GEF interplay in the HGF-induced preservation of EC barrier and attenuation of inflammatory activation.

In summary, this study shows a novel mechanism of anti-inflammatory HGF effects on the LPS-induced endothelial inflammatory activation and lung injury mediated by HGF-activated Rac-specific GEF Asef. These results suggest that targeted activation of Rac-specific GEFs such as Asef may attenuate lung injury induced by bacterial pathogens and accelerate restoration of lung function.

GRANTS
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