Isthmin is a novel vascular permeability inducer that functions through cell-surface GRP78-mediated Src activation

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Aims
Isthmin (ISM) is a recently identified 60 kDa secreted angiogenesis inhibitor. Two cell-surface receptors for ISM have been defined, the high-affinity glucose-regulated protein 78 kDa (GRP78) and the low-affinity αvβ5 integrin. As αvβ5 integrin plays an important role in pulmonary vascular permeability (VP) and ISM is highly expressed in mouse lung, we sought to clarify the role of ISM in VP.

Methods and results
Recombinant ISM (rISM) dose-dependently enhances endothelial monolayer permeability in vitro and local dermal VP when administered intradermally in mice. Systemic rISM administration through intravenous injection leads to profound lung vascular hyperpermeability but not in other organs. Mechanistic investigations using molecular, biochemical approaches and specific chemical inhibitors revealed that ISM-GRP78 interaction triggers a direct interaction between GRP78 and Src, leading to Src activation and subsequent phosphorylation of adherens junction proteins and loss of junctional proteins from inter-endothelial junctions, resulting in enhanced VP. Dynamic studies of Src activation, VP and apoptosis revealed that ISM induces VP directly via Src activation while apoptosis contributes indirectly only after prolonged treatment. Furthermore, ISM is significantly up-regulated in lipopolysaccharide (LPS)-treated mouse lung. Blocking cell-surface GRP78 by systemic infusion of anti-GRP78 antibody significantly attenuates pulmonary vascular hyperpermeability in LPS-induced acute lung injury (ALI) in mice.

Conclusion
ISM is a novel VP inducer that functions through cell-surface GRP78-mediated Src activation as well as induction of apoptosis. It induces a direct GRP78-Src interaction, leading to cytoplasmic Src activation. ISM contributes to pulmonary vascular hyperpermeability of LPS-induced ALI in mice.

Keywords
Isthmin • GRP78 • Vascular permeability • Src • Acute lung injury

1. Introduction
Isthmin (ISM) is a recently discovered angiogenesis inhibitor that acts on endothelial cells (ECs) through receptors αvβ5 integrin and glucose-regulated protein 78 kDa (GRP78).1,2 It is a 60 kDa secreted protein first identified as part of the Fgf8-synexpression group in the Xenopus midbrain–hindbrain boundary.3 ISM contains a centrally located thrombospondin type 1 repeat (TSR) and a C-terminal adhesion-associated domain in MUC4 and other proteins (AMOP) domain. The TSR domain is known to be involved in cell–cell and cell–matrix interactions,4 while the AMOP domain is speculated to be involved in cell adhesion due to its presence in cell-adhesion molecules.5 We have previously shown that ISM influence both physiological and pathological angiogenesis.1 Systemic administration of recombinant ISM inhibits tumour growth mainly by inducing apoptosis in both tumour ECs and tumour cells that express high cell-surface GRP78.2 Importantly, ISM triggers apoptosis in target cells through its two cell-surface receptors in two independent signaling pathways.3

The endothelium constitutes a single layer of ECs that serves as the barrier between the circulating blood and the surrounding tissues, maintaining homeostasis and regulating vascular permeability (VP).6 It regulates the passage of circulating cells, plasma proteins and fluid...
to/from the interstitial space to supply tissues with nutrients and clearing waste products. This regulation is performed by the coordinated action of permeability inducers and inhibitors. Opening up of the endothelial barrier is a critical step in mediating physiological processes such as immune surveillance, inflammation as well as cancer, atherosclerosis, and other pathological conditions.\textsuperscript{7–9}

We have previously reported that ISM functions as an integrin αvβ5 antagonist, possibly disrupting cell–matrix interactions.\textsuperscript{10} Integrin αvβ5 is involved in the regulation of pulmonary VP in an acute lung injury (ALI) model.\textsuperscript{11} Using neutralizing antibodies, it was shown that blocking αvβ5 integrin led to inhibition of VEGF-induced pulmonary permeability.

Intercellular junctions are important regulators of VP. They are made up of cell-adhesion molecules that mediate direct cell–cell interactions, bind cells into tissues, and help in the maintenance of tissue integrity and cell–cell communication. In ECs, two types of junctions are present, the adherens junctions (AJ) and the tight junctions (TJ). However, unlike epithelial cells, the AJ and TJ in ECs are less defined. Along the sent, the adherens junctions (AJ) and the tight junctions (TJ). However, the major players of the AJ are the cadherins; in unlike epithelial cells, the AJ and TJ in ECs are less defined. Along the sents, the adherens junctions (AJ) and the tight junctions (TJ).

2. Methods

2.1 Cell lines and culture conditions

ECs used are human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cells (HMVEC-dermal) (Lonza), and human pulmonary microvascular endothelial cells (HMVEC-Lung) (ScienCell). HUVECs were isolated from fresh umbilical cords with informed consent from donors, according to the Declaration of Helsinki and approved by the local Domain-Specific Review Board (DSRB) (protocol C/00/553). All ECs were cultured in EndoGro-LS (Millipore) supplemented with FBS and gentamycin (Sigma Aldrich) on pre-coated culture dishes/slides. All experiments were performed with cells from Passages 4–8.

2.2 Animals

Animal care and experimentation was carried out following protocol approved by the local institutional animal care and use committee, which conform to NIH guidelines (IACUC protocol 066/12). Only female Balb/c mice were used in the experiments.

2.3 Recombinant proteins

rISM and rGRP78 were expressed in Escherichia coli and purified using Ni-NTA affinity chromatography followed by reverse-phase HPLC and confirmed to be endotoxin free. rSRC was purchased from Millipore.

2.4 In vitro EC monolayer permeability assay (FITC-dextran flux)

FITC-Dextran (average MW 2 000 000, Sigma Aldrich) flux across post-confluent EC monolayers was measured. Briefly, cells were seeded on pre-coated 1 μm transwell inserts (BD Biosciences) and grown to confluence. They were then starved in starvation media (EndoGro-LS without supplements + 2% FBS) for 3 h before subjected to different doses of ISM treatment. After treatment, FITC-dextran solution (0.4 mg/mL) was added to the upper chamber, and the inserts were shifted to another well with fresh media. Five minutes later, 0.1 mL of solution from the lower chamber was transferred to a 96-well plate, and fluorescence intensity at 485/530 nm was measured as permeability. In some experiments, the monolayer was treated with the pan-caspase inhibitor Z-VAD-fmk (Millipore), the Src kinase inhibitor PP1 (Sigma Aldrich), neutralization antibodies to αvβ5 (P1F76), or GRP78 (E-4, Santa Cruz Biotechnology) for 2 h prior to ISM treatment.

2.5 In vivo permeability assay

Local dermal permeability assays were performed on 8- to 10-week-old Balb/c mice as previously described.\textsuperscript{16} Details are described in the Supplementary material online, methods.

2.6 Immunohistochemistry and immunocytochemistry

Tissue sections were stained with anti-VE-cadherin (Santa Cruz Biotechnology). For immunocytochemistry (ICC), confluent HUVEC monolayers were starved for 3 h prior to ISM treatment (1 μM) for various periods. The cells were then fixed and probed with primary antibodies (anti-pY99 and anti-β-catenin (Santa Cruz Biotechnology); anti-VE-cadherin (Lifespan Biosciences)). Fluorescence-conjugated secondary antibodies were used followed by DAPI counter stain. Images were obtained using Zeiss LSM-510 Meta Confoal Microscope. Fluorescent intensities were quantified by ImageJ software (mean intensity of 20 junctions per field).

2.7 Immunoprecipitation and immunoblotting

Standard immunoprecipitation (IP) protocol was used. Cells were lysed with RIPA buffer containing protease inhibitors (Roche) and 2 mM phosphatase inhibitor sodium orthovanadate (Sigma Aldrich). Skin samples were lysed using urea lysis buffer containing protease and phosphatase inhibitors. About 0.5–1 mg protein lysate was used in IP.

Standard western blots were performed. In IP experiments, following binding with the primary antibody, residual lysate was collected and probed for β-actin for protein loading control. Band intensities were quantified using ImageJ software. Antibodies used are listed in the Supplementary material online, methods.

2.8 Protein co-localization by confocal microscopy

GRP78-mCherry and Src-GFP expression plasmids were transiently transfection into HUVECs. Forty-eight hours later, cells were treated with 1 μM rISM for another 24 h, fixed and stained with DAPI. Images were obtained using Zeiss LSM-510 Meta confocal microscope. Co-localization between GRP78-mCherry and Src-GFP was analysed by Imaris software and Pearson’s correlation coefficient (R\textsubscript{p}) calculated.\textsuperscript{17,18}

2.9 Proximity ligation assay

Cells were incubated for 24 h in 2% FBS media with or without 1 μM ISM. Interactions between GRP78 and Src, GRP78 and ISM as well as Src and ISM were determined using respective antibodies and analysed for proximity ligation assay (PLA) signal using Duolink in situ kit according to manufacturer’s instruction (Olink Bioscience). A more detailed description is available in the Supplementary material online, methods.
2.10 LPS-induced ALI in mice

LPS from *E. coli* (serotype O111:B4; Sigma Aldrich) was delivered intratraceally as previously described, after the mice were anesthetized with 2% isoflurane (Baxter), while control mice received saline in a similar fashion. Lung permeability was assessed by injecting Evans Blue dye 20 min before sacrificing the animals by CO2 asphyxiation and by dye extraction from the lungs as described above. Bronchoalveolar lavage (BAL) fluid was collected by flushing the lungs with saline 24 h post LPS challenge. Lungs were harvested for protein analysis by western blotting as well as fixed for immunochemistry (IHC). For antibody neutralization study, 5 mg/kg of anti-GRP78 (Santa Cruz Biotechnology) or isotype control (Novus Biologicals) was injected intraperitoneally 24 h before LPS challenge. Lung permeability was then determined.

2.11 Statistical analysis

Statistical analyses between two groups were performed by Student’s *t*-test and between multiple groups using one-way ANOVA. The sample size is indicated in the respective figure legends. A P-value of <0.05 was considered significant in all cases (*P* < 0.05). Error bars represent SD for in vitro experiments and SEM for in vivo experiments.

3. Results

3.1 ISM induces EC monolayer permeability in a dose- and time-dependent manner

To examine whether ISM influences EC permeability, ECs were grown to confluence on multi-well inserts and treated with recombinant ISM. ISM-induced HUVEC monolayer permeability in a dose-dependent manner as measured by FITC-dextran (average MW 2 000 000) flux across the post-confluent monolayers over a period of 16 h (Figure 1A). Significant increase in dextran flux was observed at 100 nM. Using a concentration of 1 μM, a time-course analysis was performed. ISM increased FITC-Dextran flux at time points ≥ 2 h following exposure, with time-dependent increase in permeability up to 16 h (end of experiment) (Figure 1D). Similarly, ISM also induced dose- and time-dependent increases in dermal microvascular (HMVEC-Dermal) and lung microvascular (HMVEC-Lung) monolayer permeability (Figure 1B, C, E, and F). As ISM is highly expressed in mouse lung, it may play a role in regulating pulmonary VP (see Supplementary material online, Figure S1).

3.2 ISM induces VP in vivo

To test whether ISM induces VP in vivo, a Miles assay was adopted. Balb/c mice systemically injected with Evans Blue dye were subjected to intradermal injections of different doses of ISM and saline controls. ISM enhanced local dermal permeability in a dose-dependent manner within 15 min of intradermal injection (Figure 2A and B). To study whether ISM triggers VP systemically, we administered ISM via tail vein and analysed dye extravasation in multiple organs of mice (Figure 2C–E). Organs were harvested at 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h post ISM administration, and dye extraction was carried out. Significant dye leakage from lung was first observed at 15 min after ISM administration and peaked at 1 h, by which time there was also a significant increase in stomach permeability (Figure 2C and data not shown).

**Figure 1** ISM enhances endothelial monolayer permeability in vitro. (A–C) ISM induces permeability in a dose-dependent manner (*n* = 3). HUVEC, HMVEC-Dermal, and HMVEC-Lung monolayers were treated with increasing concentrations of ISM or medium alone for 16 h. The permeability of medium alone is taken as 1. (D–F) ISM-induced permeability increases over time (*n* = 3). Endothelial monolayers were treated with different doses of ISM for 16 h in the presence of 2% serum. *Significantly increased comparing with control at *P* < 0.05 by one-way ANOVA.
Subsequently, pulmonary permeability gradually declined and returned to basal level by 24 h (Figure 2C). ISM did not induce any observable VP in kidney, liver, or heart in all the time points investigated (Figure 2E and data not shown). These results indicate that ISM not only induces dermal permeability following a local delivery, it also induces pulmonary VP when systemically introduced into the blood circulation.

### 3.3 ISM triggers tyrosine phosphorylation and loss of AJ proteins from cell–cell junctions

Increased tyrosine phosphorylation of the intercellular adhesion molecules have been implicated in increased paracellular permeability. Upon exposure to permeability regulators, monolayers undergo changes in tyrosine phosphorylation of junctional proteins, especially AJ proteins. To understand how ISM induces permeability, tyrosine phosphorylation of post-confluent EC monolayer was analysed using immunocytochemistry. Following serum starvation, ECs showed minimal tyrosine phosphorylation of junctional molecules, corresponding to tight intact boundaries. Exposure to ISM for 2 h led to a considerable increase in tyrosine phosphorylation in EC monolayer, particularly in cell–cell junctions (Figure 3A, thick white arrows). The AJs of ECs are predominantly composed of vascular endothelial cadherin (VE-cadherin). The integrity of the AJs is critical for maintaining the endothelial barrier and VP. Disruption of VE-cadherin homophilic interactions leads to loosening of cell–cell adhesions and an increase in paracellular permeability. VEGF/VPF is known to induce permeability by causing loss of VE-cadherin from cell junctions.

In post-confluent HUVEC monolayers, VE-cadherin is localized to cellular junctions with the formation of strong homophilic adhesions. Exposure to ISM reduced VE-cadherin staining at cellular junctions and VE-cadherin staining became discontinuous and occasionally disappeared from cellular boundaries (Figure 3A, middle panels). Loss of VE-cadherin from cell–cell junctions is time dependent, corresponding to the time-dependent increase in permeability (see Supplementary material online, Figure S2A). ‘Gaps’ of VE-cadherin cell boundary appeared in the monolayer upon ISM exposure (Figure 3A, thin white arrows). In addition, β-catenin, the cytoplasmic partner of VE-cadherin, was also lost from the junctions (Figure 3A, bottom panels).

Corresponding to the loss of VE-cadherin and β-catenin from intercellular junctions, ISM induced a time-dependent increase in phosphorylated Y658 of VE-cadherin starting from 3 h post exposure, with significant increase observed at 6 and 16 h post ISM treatment (Figure 3B). It is known that VE-cadherin cytoplasmic domain is phosphorylated at Y658 and Y731 by Src upon VEGF stimulation. ISM also induced VE-cadherin phosphorylation at Y658 and Y731 in a dose-dependent manner (Figure 3B). Furthermore, exposure to ISM led to a dose-dependent increase in phosphorylation of the cytoplasmic partners of VE-cadherin, β-catenin, and p120 catenin, at 6 h post ISM exposure, a time-point when significant increase in VP was observed (Figure 3C). Similarly, induction of dermal VP by ISM is accompanied with enhanced VE-cadherin and β-catenin phosphorylation in mouse
Thus, ISM induces permeability by triggering tyrosine phosphorylation of adherent junctional proteins and loss of these junctional proteins from cell–cell junctions.

### 3.4 ISM induces VP through Src activation as well as apoptosis induction

One of the EC-surface receptors for ISM, integrin αvβ5, plays a critical role in pulmonary VP. Meanwhile, the cytoplasmic Src kinase is a known mediator of integrin activity and VP. We therefore investigated whether Src is involved in ISM-induced VP. In cultured ECs, ISM potently activated Src (detected by the level of phosphorylated Src416) (Figure 4A). Meanwhile, activation of FAK, marked by phosphorylated FAK397, another integrin activity mediator, was not detected (data not shown). When Src activation was inhibited by the chemical inhibitor PP1 at 6 h post treatment, a time point when apoptosis has yet to fully manifest, ≏90% attenuation of ISM-induced permeability was observed. In comparison, at 16 h post ISM treatment, a time point when ISM induced apoptosis is well manifested, blocking Src activation reduced ISM-induced permeability only by ≏60% (Figure 4B).

To resolve whether ISM-induced VP represents a passive secondary effect of cells dying due to apoptosis and creating gaps, we compared the in vitro dynamics of ISM-induced permeability, Src activation, and apoptosis. Significant permeability can be observed at 2 h post ISM treatment (Figure 1). Meanwhile, Src activation can also be observed within 2 h post ISM induction and pY416Src peaks at 6 h after which it gradually declines to basal level by 24 h (Figure 4A). In comparison, ISM-induced apoptosis took much longer to manifest with little apoptosis observed at 6 h post treatment when measured by DNA fragmentation (Figure 4E). Apoptosis only became significant at 16 h post treatment and reached higher level at 24 h. Hence, at early time points post ISM treatment, ISM induced permeability through Src activation rather than apoptosis induction.

Furthermore, in vivo ISM induced dermal and pulmonary VP both within 15 min after exposure (Figure 2). At this time point, Src has been activated in the local dermis (Figure 4A) while apoptosis is unlikely to have manifested. These results indicate that ISM induces VP through Src activation independent from apoptosis.

On the other hand, at later time points (16 h or later) post ISM treatment, apoptosis indeed contributes to a significant portion of ISM's
pro-permeability effect. As shown in Figure 4D, inhibiting apoptosis using Z-VAD-fmk suppressed >60% of ISM-induced permeability at 16 h compared with only 30% at 6 h post ISM treatment. Correspondingly, pre-treatment of EC monolayers with Z-VAD-fmk for 30 min partially restored ISM-induced VE-cadherin and β-catenin to cell–cell junctions (see Supplementary material online, Figure S2B).

When Src activation was inhibited by PP1, apoptosis induction by ISM was not affected (Figure 4C). In fact, PP1 alone induced apoptosis partially attenuated ISM-induced permeability. The pan-caspase-inhibitor Z-VAD-fmk was used at various concentrations as indicated (n = 3). (E) ISM-induced permeability can be reversed after ISM withdrawal more readily at 6 h (83%) than at 16 h (58%). HUVEC monolayers were treated with ISM in full growth media for 6 (or) 16 h following which ISM was removed and replaced with fresh media for another 16 h (n = 3). *P < 0.05 by one-way ANOVA; ns, not significant.

Figure 4  ISM induces VP through Src activation and apoptosis. (A) ISM dose-dependently activates Src in vitro and in vivo (n = 4). In vitro, ISM-induced Src activation peaked at 6 h followed by a gradual decline to basal level by 24 h (n = 3). In vivo, Src activation was observed at 15 min after intradermal injection of ISM. (B) Inhibition of Src activation attenuated ISM-induced permeability. EC permeability was measured at 6 and 16 h post ISM treatment in the presence of Src kinase inhibitor PP1 (2 and 20 μM) (n = 3). (C) PP1 does not attenuate ISM-induced apoptosis (n = 3). (D) Inhibition of apoptosis partially attenuated ISM-induced permeability. The pan-caspase-inhibitor Z-VAD-fmk was used at various concentrations as indicated (n = 3). (E) ISM-induced apoptosis in a time-dependent manner (n = 3). (F) Z-VAD-fmk at 20 μM completely inhibited ISM-induced apoptosis. (G) ISM-induced permeability can be reversed after ISM withdrawal more readily at 6 h (83%) than at 16 h (58%). HUVEC monolayers were treated with ISM in full growth media for 6 (or) 16 h following which ISM was removed and replaced with fresh media for another 16 h (n = 3). *P < 0.05 by one-way ANOVA; ns, not significant.
and contributed to the total apoptosis when ISM and PP1 were both present. Since PP1 potently suppressed ISM-induced permeability (Figure 4B), this further proves that ISM triggered VP via Src activation independent of apoptosis induction.

In addition, ISM-induced VP can almost be completely reversed upon its withdrawal after 6 h treatment. In contrast, when ISM withdrawal occurred after 16 h treatment, VP can only be reversed ~50% (Figure 4G). These results suggest that Src-mediated VP can be reversed upon ISM withdrawal, but apoptosis-mediated VP cannot be reversed even after ISM withdrawal.

Altogether, we conclude that ISM induces VP directly through Src activation and indirectly via apoptosis induction.

### 3.5 ISM induces permeability through both cell-surface receptors

Previously, we have shown that antibody neutralization of either integrin αvβ5 or GRP78 inhibited ISM-induced apoptosis.1,2 To decipher which ISM receptor mediates its permeability-inducing activity, we used antibody neutralization to block integrin αvβ5 or GRP78 individually in EC monolayer permeability assays. While blocking αvβ5 did not have a significant effect on the basal permeability, it partially attenuated ISM-induced permeability in an anti-αvβ5 dose-dependent manner (Figure 5A). Interestingly, blockage of GRP78 by neutralizing antibody also dose-dependently attenuated ISM-induced permeability, with high anti-GRP78 doses almost completely blocking the induced permeability (Figure 5B). Thus, ISM induces permeability through signals mediated by both αvβ5 and GRP78 receptors.

### 3.6 ISM induces Src activation through GRP78

Since Src activation by ISM is required for ISM's permeability induction activity, we studied which ISM receptor mediated Src activation. As shown in Figure 5C, blocking cell-surface GRP78 attenuated ISM-induced Src activation. On the other hand, no effect on

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**Figure 5** Blocking GRP78 or αvβ5 each attenuates ISM-induced permeability. (A) Anti-αvβ5 antibody partially blocks ISM-induced VP. Post-confluent HUVEC monolayers were co-incubated with ISM and increasing doses of αvβ5 antibody for 6 h following which permeability was measured (n = 3). (B) Anti-GRP78 antibody significantly blocks ISM-induced VP (n = 3). (C) Anti-GRP78 suppressed ISM-induced Src activation (n = 3), ratios correspond to representative blot. (D) Anti-αvβ5 had no effect on ISM-induced Src activation (n = 3), ratios correspond to representative blot. (E and F) Inhibition of clathrin-dependent ISM internalization using chlorpromazine led to attenuation of ISM-induced VP and Src activation. (G) Inhibition of Src activation had no effect on ISM internalization. *P < 0.05 compared with control by one-way ANOVA.
ISM-induced Src activation was observed when αvβ5 integrin was blocked by antibody (Figure 5D). In comparison, antibody blocking of αvβ5 integrin generated a partial inhibition of ISM-induced permeability (Figure 5A). Thus, ISM activates Src through cell-surface GRP78 but not αvβ5 integrin.

ISM binding with cell-surface GRP78 is known to trigger ISM-GRP78 complex internalization via clathrin-dependent endocytosis. Inhibition of clathrin-dependent endocytosis with chlorpromazine also attenuated ISM-induced permeability and Src activation in a dose-dependent manner (Figure 5E and F). However, inhibiting Src activation using PP1 had no effect on ISM internalization (Figure 5G). Thus, ISM-induced Src activation and permeability require the ligation of ISM to GRP78 and its subsequent internalization, but Src activation is not required for ISM-GRP78 endocytosis.

3.7 ISM exposure induces/enhances direct GRP78 interaction with Src

Although GRP78 has been reported to function as a receptor kinase, the interaction of cell surface GRP78 with intracellular partners has not been reported. Since ISM-induced Src activation is dependent on ISM binding to GRP78, a GRP78 receptor-mediated intracellular signal transduction is likely to ensue. To clarify whether GRP78 interacts with Src, we performed co-IP experiment with recombinant GRP78 and Src in vitro. No interaction between the two proteins was observed in the experimental conditions tested (data not shown). However, in the presence of ISM, GRP78-Src interaction was observed (Figure 6A). When anti-Src or anti-GRP78 antibody bound protein A/G beads were incubated with whole cell lysate from ISM-treated or control HUVECs, binding between GRP78 and Src was observed in both control and ISM-treated cell lysate. Notably, there was an obvious increase in GRP78-Src interaction in ISM-treated cell lysate (Figure 6A).

We then analysed whether GRP78 and Src co-localize and interact intracellularly. Upon overexpression of GRP78-mCherry and Src-GFP in both HUVECs and HEK293Ts, a significant increase in GRP78 and Src co-localization was observed after 24 h exposure to ISM (Figure 6B and C). To further prove that GRP78 and Src indeed interact directly, PLA, a method allowing visualization and quantification of specific protein interaction events in situ, was performed in both HUVECs and HEK293Ts co-expressing GRP78-mCherry and Src-GFP. PLA signal represents direct interaction between two proteins in their native state and location. Notably, PLA signals were observed in the cytosol only in ISM-treated cells but not in control cells, suggesting direct interaction between GRP78 and Src upon ISM treatment (Figure 6D and E). The interaction between ISM and GRP78 in the same experimental setting was used as a positive control. No interaction between ISM and Src was observed.

Based on these results, we conclude that ISM binding to cell-surface GRP78 triggers ISM-GRP78 complex internalization and elicits GRP78-Src interaction in the cytosol. This interaction is likely responsible for ISM-induced Src activation.

3.8 ISM is up-regulated and contributes to LPS-induced ALI and pulmonary hyperpermeability in mice

ALI is a condition triggered by systemic or local lung inflammation that involves pulmonary vascular hyperpermeability and oedema. The cellular and molecular mechanisms of ALI-associated pulmonary hyperpermeability and oedema are not fully understood. Since ISM is expressed in the lung, and systemic infusion of rISM induced VP most potently in mouse lung (Figure 2), we investigated whether ISM is involved in pathological pulmonary hyperpermeability using a mouse model of ALI induced by LPS. Mice were challenged with 50 μg LPS and a substantial increase in pulmonary permeability was observed within 24 h following LPS challenge (Figure 7A). Both lung tissue lysates as well as BAL fluid showed significant up-regulations of ISM in LPS-challenged mice compared with saline control (Figure 7B and C). ISM up-regulation was observed in both bronchial and alveolar epithelial cells (Figure 7D). Furthermore, systemic infusion of an anti-GRP78 antibody targeted to block ISM’s high-affinity receptor led to significant attenuation of LPS-induced pulmonary hyperpermeability (Figure 7E). The same anti-GRP78 antibody also effectively attenuated ISM-induced endothelial monolayer permeability in vitro (Figure 5B).

In this work, we report a novel function of the angiogenesis inhibitor ISM as a VP inducer. We further showed that ISM is up-regulated upon intratracheal LPS challenge in mouse lung and provided evidence that it contributes to the pulmonary hyperpermeability and oedema of LPS-induced ALI. Furthermore, we revealed that ISM directly induces endothelial permeability through its receptor GRP78-mediated Src activation. Thus, ISM is a novel regulator of endothelial barrier function.

Different angiogenesis inhibitors regulate VP in different manners. Thrombospondin-1 (TSP-1) induces tyrosine phosphorylation of Aβ protein complexes such as VE-cadherin, γ-catenin, and p120 to induce paracellular permeability of the endothelial barrier. Similarly, semaphorin 3A was found to induce VP by tyrosine phosphorylation of Aβ proteins. However, other angiogenesis inhibitors such as endostatin and angiostatin are known to reduce permeability stimulator-induced VP. ISM seems to be similar to TSP-1 and semaphorin 3A to both inhibit angiogenesis and promote VE-cadherin tyrosine phosphorylation including VE-cadherin, β-catenin, and p120 catenin (Figure 3). VE-cadherin, β-catenin, and p120 catenin are partners in a complex which hold VE-cadherin on the intercellular junctions. Increased tyrosine phosphorylation of Aβ proteins uncouple the catenins from the cadherins, leading to the disruption of endothelial barrier and opening of paracellular permeability. ISM triggers junctional protein phosphorylation via Src activation. Based on the time course of events upon ISM exposure to endothelial monolayers, it appears that ISM first triggers Src activation, followed by Src-mediated phosphorylation of Aβ proteins and subsequent dissociation of the Aβ, resulting in barrier disruption. In parallel, ISM binding to GRP78 and αvβ5 on the EC cell surface both induces apoptosis through two independent pathways and contribute to enhancing VP. Whether the two functions of ISM (antiangiogenesis and pro-permeability) can be segregated physiology or pathology remains to be fully explored.

The role of Src in endothelial permeability has been well documented including its role in VEGF-induced permeability. Using Src kinase inhibitor PP1, we demonstrated that Src activation is the dominant trigger of ISM-induced endothelial permeability at early time point (6 h post ISM treatment). At later time point (16 h post...
Figure 6  ISM induces direct GRP78-Src interaction. (A) Co-IP of rSrc and rGRP78 in the absence and presence of rISM. HUVEC whole cell lysates were pulled down in the presence or absence of ISM with either anti-GRP78 or anti-Src and probed with the other protein, respectively. NIH3T3 was used as a control as it has undetectable cell-surface GRP78 level. \( n = 3 \). (B) ISM induces co-localization of Src-GFP and GRP78-mCherry in both HEK293T cells and HUVECs. Forty-eight hours post expression plasmids transfection, cells were treated with 1 \( \mu M \) ISM for 24 h. Rp between red and green fluorescent signals is indicated in the far-right panels. (C) Rp value in analysed cells. (D) Determination of intracellular GRP78 and Src interaction using PLA. GRP78 and Src interact only in the presence of ISM. ISM interacts with GRP78 as previously reported but not with Src. (E) Quantification of PLA signals. PLA signal (red) from three microscopic fields from each sample group were calculated by ImageJ and then normalized by cell number. \* \( P < 0.05 \) by one-way ANOVA.
ISM is up-regulated and contributes to LPS-induced ALI and pulmonary hyperpermeability in mice. (A) Intratracheal LPS challenge generated significant lung VP at 24 h post LPS delivery as measured by Evans blue dye leakage. *Significantly changed comparing with control at \( P < 0.05 \) by Student’s t-test. (B) ISM is highly up-regulated in LPS-induced ALI. Western blotting of lung tissue lysates for ISM was normalized with β-actin. ISM level in individual mouse is presented in the distribution dot plot. *Significantly changed comparing with control at \( P < 0.05 \) by Student’s t-test. (C) ISM is up-regulated in BAL-Fluid of LPS-treated lung. S1–S3: saline-treated lung; L1–L4: LPS-treated lung. (D) Up-regulation of ISM in both bronchial and alveolar epithelia shown by IHC. (E) Neutralization of ISM’s receptor GRP78 using 5 mg/kg anti-GRP78 antibody potently attenuated LPS-induced VP. Isotype control antibody and saline were used as control. \( ^* P < 0.05 \) by one-way ANOVA. Number of animals used is indicated in each group.
ISM-induced apoptosis also indirectly contributed significantly to the enhanced permeability (Figure 4). Inhibiting apoptosis by the pan-caspase inhibitor Z-VAD-fmk reduced ISM-induced permeability much more extensively at 16 h compared with at 6 h post ISM treatment. This correlates with the time required for apoptosis to manifest as substantially more apoptosis was observed at 16 h than 6 h post ISM treatment (Figure 4F).

Since ISM induces apoptosis through both of its cell-surface receptors, neutralizing antibody to either GRP78 or integrin \( \alpha v \beta 5 \) leads to attenuation of ISM-induced permeability. However, only anti-GRP78 antibody suppresses ISM-induced Src activation (Figure 5). Using multiple techniques such as co-IP, fluorescent co-localization by confocal microscopy imaging and PLA, we demonstrated that ISM stimulates a direct interaction between GRP78 and Src, leading to Src activation (Figures 5 and 6). This is the first report revealing that GRP78 activates Src via direct interaction with Src. A schematic illustration of how ISM induces VP is presented in Figure 8.

The low-affinity ISM receptor integrin \( \alpha v \beta 5 \) has been shown to play an important role in VEGF-mediated VP by activating Src and opening up the VE-cadherin-catenin barrier. \(^{11,24}\) However, integrin ligation triggered Src activation requires the Src substrate FAK to form a FAK-\( \alpha v \beta 5 \) signaling complex which lead to VP. \(^{25}\) Since ISM activates Src without activating FAK (data not shown), ISM seems to induce permeability not through the Src-FAK-\( \alpha v \beta 5 \) pathway. The partial attenuation of ISM-induced permeability by neutralizing \( \alpha v \beta 5 \) is likely due to the attenuation of apoptosis induced through ISM-\( \alpha v \beta 5 \) integrin interaction as previously reported.\(^ {1,10}\)

How cell-surface GRP78 transmits signal intracellularly remains to be elucidated. Evidences of GRP78 autophosphorylation have been reported both in vitro and in vivo.\(^ {28,39}\) Upon binding to its ligand \( \alpha 5 \beta 1 \), cell-surface GRP78 was shown to undergo tyrosine phosphorylation.\(^ {28}\) Binding of another ligand Cripto to cell-surface GRP78 is known to cause Src activation.\(^ {29}\) Lead exposure was shown to disrupt tight junctions at the blood–brain barrier via GRP78-mediated Src activation.\(^ {40}\) Recently, cell-surface GRP78 was identified as a signal receptor for oxidized phospholipids (OxPAPC) in human pulmonary artery ECs and human lung microvascular ECs, mediating endothelial barrier enhancement.\(^ {41}\) Binding of OxPAPC to GRP78 also leads to Src activation in ECs. Furthermore, ADAM15 promotes endothelial hyperpermeability in LPS-induced ALI likely via Src activation.\(^ {42,43}\) Although these findings all suggested that ligation of cell-surface GRP78 may trigger a GRP78-Src intracellular signaling pathway, in this work we demonstrated for the first time that ISM (ligand) binding to GRP78 leads to a direct interaction of GRP78 with cytoplasmic Src, triggering Src activation (Figure 6).

ALI is a significant source of morbidity and mortality in critically ill patients. Owing to the complexity of the disease and our incomplete understanding of its pathophysiology and molecular mechanisms, effective treatment and prevention of ALI remain as unmet medical needs. Our work here suggest that ISM may be a new player in vascular hyperpermeability and oedema in ALI. Inhibiting ISM overexpression or function may be a useful therapeutic approach that is worth further investigation.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.
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