Thrombin enhances the barrier function of rat microvascular endothelium in a PAR-1-dependent manner

B. Troyanovsky, D. F. Alvarez, J. A. King, and K. L. Schaphorst

University of South Alabama Center for Lung Biology, Mobile, Alabama

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Thrombin enhances the barrier function of rat microvascular endothelium in a PAR-1-dependent manner. Am J Physiol Lung Cell Mol Physiol 294: L266–L275, 2008. First published December 14, 2007; doi:10.1152/ajplung.00107.2007.—Thrombin is a multifunctional coagulation protease with pro- and anti-inflammatory vascular effects. We questioned whether thrombin may have segmentally differentiated effects on pulmonary endothelium. In cultured rat endothelial cells, rat thrombin (10 U/ml) recapitulated the previously reported decrease in transmonolayer electrical resistance (TER), F-actin stress fiber formation, paracellular gap formation, and increased permeability. In contrast, in rat pulmonary microvascular endothelial cells (PMVEC), isolated on the basis of Griffonia simplicifolia lectin recognition, thrombin increased TER, induced fewer stress fibers, and decreased permeability. To assess for differential proteinase-activated receptor (PAR) expression as a basis for the different responses, PAR family expression was analyzed. Both pulmonary artery endothelial cells and PMVEC expressed PAR-1 and PAR-2; however, only PMVEC expressed PAR-3, as shown by both RT-PCR and Western analysis. PAR-1 activating peptides (PAR-APs: SFLLRN-NH₂ and TFLLRN-NH₂) were used to confirm a role for the PAR-1 receptor. PAR-APs (25–250 μM) also increased TER, formed fewer stress fibers, and did not induce paracellular gaps in PMVEC in contrast to that shown in pulmonary artery endothelial cells. These results were confirmed in isolated perfused rat lung preparations. PAR-APs (100 μg/ml) induced a 60% increase in the filtration coefficient over baseline. However, by transmission electron microscopy, perivascular fluid cuffs were seen only along conduit veins and arteries without evidence of intra-alveolar edema. We conclude that thrombin exerts a segmentally differentiated effect on endothelial barrier function in vitro, which corresponds to a pattern of predominant perivascular fluid cuff formation in situ. This may indicate a distinct role for thrombin in the microcirculation.

proteinase-activated receptor; permeability

THROMBIN, a multifunctional serine protease, is the key effector enzyme of coagulation, which activates fibrin cross-linking and subsequent coagulation and hemostasis. Since its initial discovery, many studies have described roles for thrombin that go beyond its original role as an activator of fibrin clot formation (3, 37, 39). Of note, thrombin has been shown to have a role as an important anticoagulant enzyme when it interacts with thrombomodulin, a membrane protein expressed on the apical surface of endothelial cells, which enables thrombin to generate activated protein C (18). In addition, thrombin has been shown to exert prominent proinflammatory effects on vascular cells and is well established as an edemagenic agonist of conduit vessel endothelial cells (14, 15), inducing endothelial cell activation, contraction, paracellular gap formation, and vascular barrier disruption with consequent increases in vascular permeability (8, 15, 41). The panoply of possible roles for thrombin suggest a complex regulation and suggest that its actions depend on the context during which it is generated and on the tissue on which it acts.

The substrates for thrombin’s proteolytic activity, in addition to fibrinogen, include the family of cell surface-expressed, seven transmembrane-spanning domain, heterotrimeric G-protein-coupled receptors collectively known as the protease-activated receptor (PAR) family. The PAR family includes four receptors, PAR-1 through PAR-4, whose activation is unique: these receptors sense protease activity in the extracellular fluid by binding proteases as ligands (3, 32), which then subsequently cleave the NH₂ terminus of the PAR receptor. The newly cleavage-generated NH₂ terminus of the receptor then folds back and interacts with the second extracellular loop and a stretch of amino acids from positions 85 to 89 (the “tethered ligand mechanism”), resulting in receptor activation. Although thrombin was the first cognate ligand described for the PAR family, other coagulation serine proteases have also been shown to have the ability to activate these receptors in vitro and in vivo in either a cofactor-dependent or -independent manner. These coagulation system proteases, such as thrombin, are the major activators of PARs, suggesting that PARs regulate cellular functions associated with the response to vascular injury (37). Whereas thrombin can activate PAR-1, PAR-3, and PAR-4, its primary receptor on endothelial cells is PAR-1 (6, 27). Endothelial PAR-1 activation results in the recruitment and activation of several heterotrimeric G proteins at the cytoplasmic portion of the receptor: Gαi, Gq, and G12/13, the subunits of which mediate subsequent endothelial cell signaling events.

Because the endothelium is a central regulator of vascular homeostasis, the effects of endothelial thrombin challenge and PAR-1 activation on endothelial barrier function have been extensively studied. The endothelium is a centrally important tissue that makes up the permeability barrier that restricts solutes and fluid to the intravascular compartment (21). When the vascular endothelium becomes activated in response to inflammatory stimuli, transendothelial permeability is increased by the formation of paracellular gaps in the confluent endothelial lining, enabling both fluid and solutes to extravasate into the surrounding perivascular space (8). Increased vascular permeability is a cardinal feature of acute inflammation and can become a clinically prominent feature in the pathobiology of inflammatory vascular diseases such as acute lung injury and...
the sepsis syndrome. Although studies have supported a role for thrombin in mediating increased vascular permeability in sepsis, antecedent thrombin infusion has provided a significant survival benefit in endotoxin-infused dogs (43) and thrombin inhibition has exacerbated LPS-induced microvascular dysfunction (19). Furthermore, thrombin inhibition in PAR-1 receptor knockout models has failed to lead to an improvement in survival in animal models of sepsis. These findings indicate that the vascular effects of thrombin in sepsis are complex. Although the beneficial effects seen from thrombin in sepsis have been assumed to be due to the thrombomodulin-mediated generation of activated protein C, we questioned whether thrombin may directly exert heterogeneous, segmentally differentiated vascular effects on the barrier function of pulmonary conduit vessel and pulmonary microvascular endothelial cells (PMVEC).

In the present study, we studied a homogenous subpopulation of PMVEC selected on the basis of recognition by the lectin *Griffonia simplicifolia*, which has been shown to specifically recognize rat endothelial cells lining microvessels of less than ~20 μm diameter (23). We compared the barrier function of these primarily septal capillary endothelial cells with rat pulmonary artery endothelial cells (PAEC) after stimulation with thrombin or PAR-1 agonist peptides and found that the PMVEC exhibited a novel increase in barrier function and decrease in permeability. These observations indicate that vascular responses to thrombin have a physiologically relevant segment-specific heterogeneity in the pulmonary circulation.

**MATERIALS AND METHODS**

The research protocol was approved by the University of South Alabama Animal Care and Use Committee.

**Endothelial cell isolation, sorting, and culture.** Main pulmonary arteries were isolated as previously described (4, 23, 40). Briefly, 300- to 400-g Sprague-Dawley rats were euthanized by an intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal; Abbott Laboratories, Chicago, IL). The heart and lungs were excised en bloc and placed in a DMEM (GIBCO BRL, Grand Island, NY) and incubated for 1 h at 37°C to allow the alveolar epithelial lining to be easily removed. Thin strips were removed from the lung periphery adjacent to the pleural surface, finely minced, and transferred with 3–5 ml of complete medium to a 15-ml conical tube containing 3 ml of digestion solution (0.5 g BSA, 10,000 U type 2 collagenase ( Worthington Biochemical, Lakewood, NJ), and calcium- and magnesium-free-PBS (GIBCO BRL) to make 10 ml total volume). The digestion mixture was allowed to incubate at 37°C for 15 min before it was poured through an 80-mesh sieve into a sterile 200-ml beaker. An additional 5 ml of normal medium [10% FBS (Hyclone, Logan, UT) with 30 μg/ml penicillin and streptomycin in DMEM] was used to wash the sieve. The isolation mixture was transferred to a 15-ml conical tube and centrifuged to 300 g for 5 min, the medium was aspirated, and the cells were resuspended with 5 ml of complete medium [1 part microvascular conditioned medium-three parts incomplete medium (80% RPMI 1640, 20% FBS, 12.3 U/ml heparin; Elkins-Sinn, Cherry Hill, NJ) and 6.7 μg/ml Endogro (Vec Technologies, Rensselaer, NY) with 30 μg/ml penicillin and streptomycin]. Centrifugation/aspiration was repeated, and the cells were resuspended in 2–3 ml of complete medium and allowed to incubate at 37°C for 30 min before they were placed drop wise onto 35-mm culture dishes. After 1 h at 37°C with 5% CO₂, 3 ml of complete medium were added. The dishes were checked daily for contaminating cells, which were removed by scraping and aspiration. Endothelial cell colonies were isolated by clonal rings, trypsinized, resuspended in 100 μl of complete medium, and placed as a drop in the center of a T25 flask. The cells were allowed to attach (1 h at 37°C with 5% CO₂) before the addition of 5 ml of complete medium.

The endothelial identity of all cultures was verified by acetylated LDL uptake, factor VIII-R immunocytochemical staining, and the absence of immunostaining with smooth muscle cell α-actin antibodies. Cultures were also characterized with scanning electron microscopy and a lectin-binding panel.

**Measurement of endothelial barrier function.** Electrical resistance across endothelial cell monolayers was measured by using an electrical cell substrate impedance-sensing (ECIS) system (Applied Biophysics, Troy, NY) as previously described (13). Briefly, cells were grown (25–50 × 10⁴/well for 24–36 h) on gold microelectrodes (model 5301A; EG&G Instruments, Princeton, NJ) with a built-in differential preamplifier (model 5316A, EG&G Instruments). The in-phase and out-of-phase voltages between the electrodes were monitored in real time with the lock-in amplifier and converted to scalar measurements of transendothelial impedance of which resistance was the primary focus. Transmonolayer electrical resistance (TER) was monitored for 30 min to establish a baseline resistance. Wells with baselines that exceeded two standard deviations from the pooled mean were rejected from analyses. All electrical resistance data are presented as values normalized by the basal resistance for each well.

Endothelial permeability was also assayed by measuring the transmonolayer flux of a tracer molecule, FITC-inulin (7 kDa; Mr ~15,000). FITC-inulin (Sigma) was dissolved in PBS by heating in warm water. Cell monolayers were grown on porous polycarbonate filters separating two chambers (0.4 μm-pore-size Transwell membranes; Costar) in 24-well plates. Before treatment, cells were washed in serum-free DMEM and equilibrated for 3–5 h. FITC-inulin was added to the luminal chamber to a final concentration of 20 μM and incubated for 1 h before treatment. After thrombin or PAR-1 agonist peptide addition to the luminal chamber, FITC-inulin transfer to the abluminal chamber was monitored by measuring absorbance at 1-h intervals with a spectrophotometric plate reader ( Molecular Devices, Sunnyvale, CA).

**Fluorescence microscopy for F-acetin.** After treatment, endothelial cells were washed with PBS and fixed with 3.7% formaldehyde for 15 min. Cells were then rinsed twice with PBS, permeabilized with 0.25% Triton X-100 for 5 min at room temperature, and rinsed twice with Tris-buffered saline that contained 0.1% Tween 20. Coverslips were incubated in Texas red phalloidin for 60 min at room temperature and rinsed four times with Tris-buffered saline-0.1% Tween 20 before they were mounted on glass slides. A Nikon Eclipse TE3000 microscope and Sony DKC-5000 digital camera connected to a personal computer were used to visualize and analyze filamentous F-acetin.

**Detection of PAR family member receptor expression.** For RT-PCR, total RNA was isolated with Trizol LS reagent (Invitrogen) according to the manufacturer’s instructions. To exclude any genomic DNA contamination, all RNA samples were pretreated with DNA-free...
DNase treatment and removal reagents (Ambion). One microgram of total RNA and the Qiagen OneStep RT-PCR kit were used for the RT-PCR reactions with specific primers. The primers used for these reactions are shown in Table 1 (38). PAR-1 and PAR-3 were amplified with 30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 60 s), PAR-2 was amplified with 30 cycles (94°C for 30 s, 54°C for 30 s, 72°C for 60 s), and PAR-4 was amplified with 30 cycles (94°C for 30 s, 59°C for 30 s, 72°C for 30 s). GAPDH was used as a housekeeping gene for control and amplified with 30 cycles (94°C for 30 s, 49°C for 30 s, and 72°C for 60 s). The identity of PCR products was confirmed by cloning into pDrive cloning vector (Qiagen) and by sequencing.

PCR data were also confirmed at the protein level by Western immunoblotting. Homogenates were prepared by scraping 60-mm² dishes of confluent endothelium into 0.5 ml of Tris-buffered saline, pH 7.4, containing 1% SDS. DNA was sheared by passing homogenates through a tuberculin syringe. The homogenates were then boiled for 5 min at 100°C; resolved by SDS-PAGE, transferred electrophoretically to nitrocellulose membranes, and then probed with specific antibodies for either PAR-1 or PAR-3 (Santa Cruz). Final detection was performed using an enhanced chemiluminescence detection protocol according to the manufacturer’s instructions (Amersham).

Determination of the filtration coefficient in the isolated perfused rat lung. Adult male CD40 rats (250–300 g) were anesthetized with pentobarbital sodium (50 mg/kg ip), and a tracheotomy catheter was inserted. The lungs were ventilated at inspiratory and expiratory pressures of 6.0 and 2.0 cmH₂O, respectively, with room air until the heart was cannulated; animals were then ventilated with 21% O₂-5% CO₂-74% N₂. After a subdiaphragmatic thoracotomy, the mediastinal pressure equilibrium was disturbed by increasing hydrostatic forces equally opposed by oncotic forces. The weight equilibrium was disturbed by increasing pressure (Pc) was estimated by double occlusion. When lung weight and colloid oncotic pressures are constant. Edema formation (net volume) was measured by gravimetric method and was expressed as milliliters per minute per millimeter of mercury per 1 g of dry lung tissue. Left lung dry weight was measured after the lung was dried in a desiccator for 48 h. In all experiments, isolated lungs were allowed to equilibrate and attain isogravimetric conditions for 20 min, after which baseline Pc was determined.

Transmission electron microscopy. To identify segment-specific leak sites, after the final Pc measurements, the lungs were perfused with 3% glutaraldehyde in cacodylate buffer at increased (Pv ~10 cmH₂O) until the fixative was recovered from the venous outflow. The lungs were then immersed in the same fixative. Specimens were postfixed in 1% osmium tetroxide, dehydrated with graded alcohol series, and embedded in PolyBed 812 (Polysciences, Warrington, PA) resin. Thick sections (1 µm) were cut with a glass knife, stained with 1% toluidine blue, and examined by light microscopy. Representative images of the extravascular and the septal compartment were then selected and processed for transmission electron microscopy (TEM). Thin sections (80 nm) were cut with a diamond knife, stained with uranyl acetate, and counterstained with Reynolds’s lead citrate. The sections were examined by TEM (Philips CM 100; FEI, Hillsboro, OR), and representative images were selected for publication.

Data analysis. Numerical data are reported as means ± SE. One-way ANOVA was used to evaluate differences between experimental groups, with Student-Newman-Keuls post hoc test as appropriate. Significance was considered at P < 0.05.

RESULTS

Effect of rat thrombin on the barrier function of cultured rat PAEC and PMVEC. Rat PAEC and rat PMVEC were isolated based on differential lectin binding as previously reported (23). Isolation of endothelial cells from rat pulmonary vessel segments is based on differential lectin recognition. Rat PAEC is specifically recognized by the Helix pomatia lectin, whereas endothelial cells from pulmonary microvessels (<20 µm diameter) are specifically recognized by the lectin Griffonia simplicifolia both in vitro and in vivo (23). Endothelial cell lineage was confirmed by immunostaining for endothelial cell-specific markers as described in MATERIALS AND METHODS. To begin characterizing the vascular segment-specific responses of thrombin on pulmonary endothelial cells, we first compared the effect of rat thrombin (Sigma, St. Louis, MO) on the barrier function of rat PMVEC and PAEC in vitro (Fig.)

| Table 1. Primers used for RT-PCR analysis of PAR family receptor expression in endothelium |
|---|---|---|---|
| Receptor | Primer Sequence (5’ to 3’) | Position | Accession Code | PCR Product |
| PAR-1 | Sense: CCTATGAGACAGGAGCAGATTTC | 146 | M81642 | 355 bp |
| | Antisense: GCCCTCTGGACCTCATCCGTTC | 500 | | |
| PAR-2 | Sense: GCCGGCTGCTGGAGTAGATACT | 19 | U61373 | 742 bp |
| | Antisense: GGAACAGAAGACACGTCGAGAATG | 760 | | |
| PAR-3 | Sense: GTGCTCTGCGACGCACCTATTGTG | 18 | AF310076 | 581 bp |
| | Antisense: ATACGGACATACGATGTCGGC | 598 | | |
| PAR-4 | Sense: GAAATGCCGACAGCAGGAGACATC | 54 | AF310216 | 559 bp |
| | Antisense: GCTAGAGGCGGTGACGCGCA | 612 | | |

The primers listed are as described by Rohatgi et al. (38). The run conditions are provided in MATERIALS AND METHODS. PAR, protease-activated receptor.
1A). In rat PAEC, thrombin addition provoked a rapid drop in resistance, similar to that reported previously in PAEC from other species. In contrast, thrombin challenge in PMVEC produced a significantly different TER response. After thrombin addition, TER rapidly increased to a level ~30–50% above baseline and remained stable.

We next compared the dose responsiveness of the two types of cultured endothelium. Whereas PAEC were unresponsive to doses of thrombin < 1 U/ml (not shown), higher doses induced a decrease in TER (Fig. 1B). In contrast, PMVEC were responsive to lower doses of thrombin and exhibited a dose-dependent increase in peak resistance after challenge with thrombin doses of 0.5–10 U/ml (Fig. 1C).

**Effect of thrombin on FITC-inulin permeability on rat PAEC and PMVEC in vitro.** We next sought to confirm the findings from our ECIS experiments with studies of the transmonolayer molecular transfer of the tracer molecular FITC-inulin (Mr ~15,000) in a dual-chamber system (Fig. 2). The absorbance of FITC in the abluminal chamber was measured spectrophotometrically at 1-h intervals after addition of rat thrombin (Fig. 2). Thrombin increased the transmonolayer flux of FITC-inulin in the PAEC monolayers, manifested as a faster rate of increasing FITC absorbance over time compared with control (Fig. 2A). In contrast, when thrombin was added to monolayers of PMVEC, the rate of absorbance increase over time was less than that for untreated controls (Fig. 2B). In aggregate for these conditions, thrombin produced a 30.6 ± 3.8% increase in FITC-albumin flux across PAEC monolayers but produced a significantly different 16.2 ± 7.2% decrease in flux across PMVEC monolayers (Fig. 2C). These data agree with our transmonolayer resistance studies that showed thrombin-disrupting barrier function (decreasing resistance) in PAEC but thrombin-enhancing barrier function (increasing resistance) in PMVEC (Fig. 1).

**Effect of rat thrombin on F-actin organization in cultured rat pulmonary endothelia.** Because changes in endothelial barrier function have been linked to alterations in the endothelial actin cytoskeleton (8, 15, 41), we examined actin organization in thrombin-challenged pulmonary endothelium (Fig. 3). At basal conditions in both cell types, actin was arranged in a fine reticular pattern with some actin localized at the cell periphery (Fig. 3, A and C). In PAEC, thrombin challenge (5 U/ml for 5 min) increased F-actin fluorescence, organized actin into axially oriented stress fibers, and formed paracellular gaps (Fig. 3B, solid arrow). These findings are consistent with previous reports of thrombin-induced F-actin reorganization, stress fiber formation, and gap formation in other conduit vessel endothelia. In contrast, when PMVEC were challenged with thrombin, a different actin morphology resulted (Fig. 3D). Actin did not arrange into stress fibers, and peripheral actin remained intact in many cells (Fig. 3D, solid arrow). In addition, in contrast to thrombin-challenged PAEC, paracellular gaps were...
not observed. The segment-specific responses of thrombin on pulmonary endothelial barrier function correlate with segmentally differentiated patterns of thrombin-induced actin organization.

**RT-PCR and Western immunoblot identification of PAR subtypes in cultured rat pulmonary endothelia.** Although PAR-1 was the first receptor described for which thrombin is a ligand, thrombin can cleave and activate other members of the PAR family. Because PAR expression in pulmonary microvessels has not been characterized, we tested using RT-PCR analysis whether the segmentally differentiated effects of thrombin on rat pulmonary endothelial cells could be explained by different patterns of receptor message expression. Interestingly, only PMVEC expressed message for PAR-3, whereas neither endothelium expressed detectable levels of PAR-4 (Fig. 4A). We confirmed these results at the protein level by performing Western immunoblotting analysis for PAR-1 (Fig. 4B) and PAR-3 (Fig. 4C) on rat pulmonary endothelial homogenates. Thus, although the PAR-1 receptor is expressed at the mRNA and protein levels in both types of pulmonary endothelium, PAR-3 receptor was detected only in the PMVEC.

**Effect of PAR agonist peptides on the barrier function of cultured rat pulmonary endothelia.** Because thrombin can proteolytically cleave and activate several receptors in the PAR family, we next tested whether thrombin-induced endothelial barrier enhancement was due to ligation of PAR-1 by using specific peptide agonists of the PAR-1 receptor. PAR activating peptides were used for a number of reasons: because the peptides are not proteolytically active, they should not replicate behaviors elicited by thrombin from the cleavage of non-PAR targets. Also, because they do not possess the domains necessary for binding to thrombomodulin or for activating protein C, they should simplify the system and directly probe PAR-1 activation. Finally, because the peptides can be made to resemble the activating neo-NH2 terminus of a particular PAR, they can be highly specific for a particular PAR subtype. We used two peptides for these experiments: S6 (SFLLRN-NH2) and the PAR-1-specific T6 (TFLLRN-NH2). The effects of these PAR activating peptides on TER were examined (Fig. 5). When S6 (100 μg/ml) was added to wells on which PAEC were grown, an immediate decline in TER was observed. In contrast, when S6 was added to wells containing PMVEC monolayers, a rapid and sustained increase in TER was observed (Fig. 5A). These responses in TER were highly similar to those produced by thrombin challenge of PAEC and PMVEC (Fig. 1). The barrier-enhancing effects of the PAR activating peptides S6 (Fig. 5B) and T6 (Fig. 5C) on
PMVEC was manifest by doses of peptide ranging from 25 to 250 μM.

To confirm whether PAR activating peptides produced the same effects on F-actin organization as those that were observed with rat thrombin challenge, rat PAEC and PMVEC were grown to confluence on glass coverslips and challenged with 100 μM S6 and T6 (Fig. 6). The control endothelial cells again exhibited a fine central reticular actin pattern, with some actin present at the cell periphery (Fig. 6, A and D). Both S6 and T6 challenge resulted in increased F-actin fluorescence in both PMVEC and PAEC, although the organization of actin fibers was distinct between the cell types. After 5 min of S6 and T6 challenge in PAEC, the F-actin was prominently organized into axially oriented stress fibers in many of the observed cells (Fig. 6, B and C; solid white arrows indicate regions of stress fibers). These findings are typical of the responses previously observed after PAR agonist peptide challenge in other conduit vessel endothelial cells. In contrast, when PMVEC were challenged with PAR agonist peptides, a different F-actin organization was seen (Fig. 6, E and F). Compared with peptide-challenged PAEC, the F-actin in PMVEC was cleared from the center of the cells and more prominently localized at the cell periphery (Fig. 6, E and F, yellow arrowheads). This actin organization is similar to that observed in endothelial cells challenged with other barrier-enhancing agents such as sphingosine 1-phosphate (11) and hepatocyte growth factor (25) and is similar to the actin organization produced in PMVEC in response to thrombin (Fig. 3).

**Effect of PAR-1 agonist peptides on vascular permeability in the isolated-perfused rat lung.** Based on our in vitro data demonstrating segment-specific responses to rat thrombin, we hypothesized that rat pulmonary capillaries would not increase permeability after exposure to PAR-1 agonist peptides (no increase in capillary $K_f$). However, we also hypothesized from our in vitro data that total $K_f$ would increase after challenge with PAR-1 agonist peptides, as previously reported in mice (44), but that this would result from edema fluid accumulation in perivascular cuffs rather than in alveolar septae or alveolar spaces (an increase in extracapillary $K_f$). The results of exposing isolated-perfused rat lungs to the PAR-1-specific T6 peptide are shown in Fig. 7. This produced a change of ~60% in $K_f$ from baseline (Fig. 7A). As positive controls, measurements of $K_f$ were performed for agonists that had previously been determined to increase permeability in rat PAEC in a segment-specific manner (Fig. 7B). 14,15-Epoxyeicosatrienoic acid (14,15-EET) is a physiologically relevant arachadonic acid metabolite that increases pulmonary microvascular permeability but does not increase permeability in extracapillary segments (1). Thapsigargin activates store-operated calcium...
channels and potently induces gap formation and increases permeability in rat PAEC but not in rat PMVEC (48). Both thapsigargin (150 nM) and 14,15-EET (3 μM) produced significant increases in total $K_f$ (300%) over baseline, whereas the PAR-1 agonist T6 had a smaller (60%) overall effect on total $K_f$ (Fig. 7B).

Although PAR agonist peptide did increase total $K_f$, this measurement cannot distinguish between capillary permeabil-

**DISCUSSION**

The effects of thrombin on vascular endothelial permeability have been extensively studied. These studies have mainly

**Fig. 7. Effect of PAR-1 agonist peptides on fluid filtration in the isolated-perfused rat lung.** A: filtration coefficient ($K_f$) at baseline (BL) and at the end of the experiment after the addition of T6 (TFLLRN-NH$_2$) to the perfusate ($n = 4$ experiments). $K_f$ was significantly increased (~60%) by T6 addition *P < 0.05. B: $K_f$ measurements compared with 100 μM T6 and the segment-specific permeability-inducing agonists 3 μM 14,15-epoxyeicosatrienoic acid (14,15-EET) and 150 nM thapsigargin (Thap) ($n = 4$–7 experiments). S6 was much less potent in inducing fluid filtration in the isolated lung than either of these other agents. *Significant change from corresponding baseline, $P < 0.05$.  

**Fig. 8. Effect of PAR-1 agonist peptides on segmental vascular permeability in the isolated-perfused rat lung.** A: T6 (100 μM)-perfused lung section showing a large conduit vessel (Lu: vessel lumen) with a perivascular fluid (F) cuff within the adventitia of the vessel (Ad), confirming that fluid accumulation most likely did not arise from extravascular accumulation. Perivascular fluid cuffs are not seen within the adventitia of vessels from normal controls (not shown). Bar = 10 μm. B: another conduit vessel with adjacent septal capillaries demonstrates normal septal capillaries (Ca) and perivascular fluid cuffing (F). Alveolar spaces, free of fluid, are adjacent, as evidenced by the more electron-dense type II pneumocyte. Bar = 10 μm. C: conduit vessel section from a thapsigargin-perfused lung. Perivascular fluid cuffing (F), similar to that seen with T6, is seen. Inset: an intercellular junction is seen that has opened after thapsigargin (*). D: septal capillary with surrounding alveolar spaces (Alv) from 14,15-EET-perfused lung. Fluid-filled membrane blebs within the septal capillary are evident (§), which are absent in T6-perfused lungs.
focused on conduit vessel endothelium (10, 12, 14, 36, 46) and have not resolved the site of increased permeability to a specific vascular segment (5, 30, 44, 47). Moreover, whereas animal studies have generally shown that thrombin increases permeability (Kf) (47), decreases the reflection coefficient, and increases pulmonary lymph flow (30), these studies have not attempted to distinguish between arteriolar, venular, and capillary permeabilities. Whether thrombin or other biologically relevant PAR-1 agonists exert physiologically significant, segmentally differentiated vascular effects remains largely unexplored. In contrast to its barrier-disruptive effects on conduit vessel endothelial monolayers, we have found that both thrombin and PAR-1 agonist peptides enhance the barrier function and decrease the permeability of rat microvascular cells in vitro. A recent report documented that thrombin in very low doses (40 pM) was able to augment the transmonolayer resistance of human conduit vessel endothelial cells (9). To our knowledge, this is the first report of thrombin exerting a barrier-enhancing, rather than barrier-disruptive, effect on PMVEC. This effect was manifest at both lower and higher doses of thrombin. These results suggest that the biological actions of PAR-1 agonism exhibit vascular segment specificity and, moreover, that PAR-1 agonism may have a distinct role in pulmonary microvessels.

We also found that, although PAR-1 agonist peptide increased total Kf in the isolated perfused rat lung, this was predominantly due to edema fluid accumulation in perivascular cuffs around venules and arterioles rather than in the septal interstitium or intra-alveolar spaces. Although these results challenge the prevalent understanding of thrombin as a widespread edemagenic proinflammatory agent, they are consistent with the evolving understanding of thrombin as a complex multifunctional molecule with both pro- and antiangiogenic and pro- and anti-inflammatory roles (42, 43). A potential vasoprotective role for thrombin in the microcirculation was suggested in a previous study of thrombin inhibition with hirudin in a septic hamster model (19). In that study, sepsis diminished the functional capillary density of a skeletal muscle preparation from septic hamsters, whereas the addition of hirudin treatment produced a dramatic further diminution in functional capillary density over that observed in the septic animals alone. Although it may be posited that the protective effects of thrombin observed in that study were due to the thrombin/thrombomodulin-mediated generation of activated protein C, our study suggests that microvascular endothelial PAR-1 agonism can exert vasoprotective responses in a system where activated protein C generation is not possible. This observation suggests that PAR-1-mediated endothelial segmental response heterogeneity is not only ligand mediated but that endothelial functional segmental heterogeneity may also occur at the level of the PAR-1 receptor.

Our results differ from previous in vitro and in situ reports of thrombin-induced increases in microvascular permeability (16, 24, 29, 31, 44, 47). The explanation of these variances arises, at least in part, from distinct methodological differences, depending on whether endothelial barrier function is assayed in vitro or in situ. With respect to previously reported in vitro studies, our own results agree with the wide body of literature that either thrombin or PAR-1 agonist peptides decrease the barrier function and increase the transmonolayer flux of tracer molecules across endothelial monolayers derived from conduit vessels such as pulmonary artery and umbilical vein (14). A number of reports have also examined the effects of PAR-1 agonists on the barrier function and transmonolayer molecular flux of PMVEC in vitro and have shown responses similar to those observed in conduit endothelial monolayers (16, 24, 29, 31). In contrast, here we report the novel finding that PAR-1 agonists increase barrier function and decrease the permeability of rat PMVEC monolayers. A key difference between our findings and those of prior reports is the population of microvascular cells studied. Typical pulmonary microvascular cell isolation methods use cells obtained from a peripheral wedge of lung tissue. The distal pulmonary vasculature of the peripheral lung is mainly microvascular, and the tissue digests from which endothelial cells are isolated in these preparations will contain cells derived from vessels that are both larger and smaller than 20 μm diameter. The isolation method that we used relies on specific lectin recognition (G. simplicifolia), which histochemically specifically decorates rat endothelial cells of vessels <20 μm in diameter, vessels that predominantly comprise the septal capillary networks. Thus our results do not contradict the earlier reports; instead, our results report on the behavior of a distinct subpopulation of G. simplicifolia-positive rat PMVEC derived mainly from pulmonary microvessels of <20 μm diameter.

Our results also agree with previous reports of PAR-1 agonist-mediated increased permeability in situ, as assessed in the isolated perfused lung preparation by measuring Kf. In our studies, PAR-1 agonist peptide produced an ∼60% increase in Kf compared with baseline, which is similar to the degree of Kf augmentation induced by thrombin in other studies (47). Because the surface area of the pulmonary capillary networks is much larger than the surface area of pulmonary arteries and veins, it is often assumed that total Kf is approximately equal to Kf for the pulmonary capillary bed. However, Parker and Yoshikawa (33, 35) have concluded that the basal resistance to fluid transudation in pulmonary capillaries is much higher than that of pulmonary veins and arteries, allowing for a higher contribution to basal fluid permeability from arteries and veins than could be predicted by vascular surface area alone. Furthermore, there are experimental situations in which Kf has been shown not to be equal to capillary Kf (2, 35). In the isolated perfused rat lung preparation, the endosomal Ca2+ ATPase inhibitor thapsigargin produced conduit vessel endothelial permeability with perivascular fluid cuff formation in the absence of intra-alveolar fluid accumulation (2). In this circumstance, Kf bears little resemblance to capillary Kf. In contrast, despite inducing a comparable degree of augmentation of Kf, the arachidonic acid metabolite 14,15-EEt produced the opposite pattern: whereas there were no perivascular fluid cuffs, the septal capillaries demonstrated intraluminal endothelial blebs and the accumulation of intra-alveolar edema (2). In this circumstance, Kf contains a significant component of capillary Kf. Together with the analysis by Parker and Townsley (33, 34), these data clearly establish that caution must be applied when interpreting Kf measurements in the absence of data that can inform about vascular segmental effects of the applied intervention (2). In our own data, PAR-1 agonist peptide-treated rat lungs produced a smaller degree of augmentation of total Kf than had been observed for either thapsigargin or 14,15-EEt (Fig. 7) and produced perivascular fluid cuff formation in the absence of septal capillary endothe-
thelial intraluminal bleb formation or intra-alveolar edema, similar to the histological appearance induced by thapsigargin treatment (Fig. 8). These in situ data agree with our in vitro data, which demonstrated a PAR-1-mediated decrease in barrier function in the PAEC but not in the PMVEC (Figs. 1, 2, and 6). We conclude that, in the in situ preparation, the PAR-1 agonist peptide increases $K_t$ by increasing the permeability of extracapillary endothelial cells but does not augment endothelial permeability in rat pulmonary septal capillaries.

The molecular mechanisms that underlie the observed functional response heterogeneity to PAR-1 agonist stimulation between pulmonary macrovascular and microvascular cells are unknown but presumably involve the differential activation of signals downstream from PAR-1. Differential signaling by PAR-1 in a ligand-dependent manner has been previously described and has been called “ligand-induced differential signaling” (45) or “functional selectivity” (29, 45). Differential signaling by PAR-1 in a cofactor-dependent manner has also been described for other coagulation proteases and activated protein C (9). Although these studies reveal the signaling versatility of the PAR-1 receptor, the same ligand for PAR-1 was used in our studies to elicit heterogeneous barrier responses from endothelium arising from different pulmonary vascular segments. Therefore, the ligand itself cannot be responsible for eliciting differential PAR-1 signaling between these cell types, implicating a possible cofactor that is responsible for initiating differential signaling. Our studies have identified a candidate protein: only the rat PMVEC expressed the PAR-3 receptor as determined by both RT-PCR and Western immunoblotting. Presently, the best described role for PAR-3 is in murine platelets where it acts as a cofactor for the thrombin-mediated activation of PAR-4, which lacks an extracellular thrombin-binding site (20, 22). PAR-3 also appears to be necessary for the activated protein C-induced abrogation of agonist-mediated apoptosis in neurons (17). Because PAR-3 has demonstrated cofactor behavior in another system, the unique agonist expression of PAR-3 in rat PMVEC is potentially meaningful. Recently, an interaction between PAR-1 and PAR-3 has been demonstrated that has the ability to modify PAR-1-mediated responses (28).

We have shown that thrombin and PAR-1 agonist peptides exert a segmentally differentiated effect on rat PAEC in vitro and that PAR-1 activating peptides produce perivascular edema fluid cuffs in situ without evidence of intra-alveolar edema. These data fit into a paradigm of compartmentalized lung edema formation, wherein barrier dysfunction in the pulmonary circulation may have segment-specific physiological consequences (26). The role of the PAR-3 receptor in participating in segment specificity is a topic that is in need of further investigation.

REFERENCES