Neutrophil Proteinase 3 Acts on Protease-Activated Receptor-2 to Enhance Vascular Endothelial Cell Barrier Function

Christopher J. Kuckleburg, Peter J. Newman

Objective—The principle role of the vascular endothelium is to present a semi-impermeable barrier to soluble factors and circulating cells, while still permitting the passage of leukocytes from the bloodstream into the tissue. The process of diapedesis involves the selective disruption of endothelial cell junctions, which could compromise vascular integrity. It is therefore somewhat surprising that neutrophil transmigration does not significantly impair endothelial barrier function. We examined whether neutrophils might secrete factors that promote vascular integrity during the latter stages of neutrophil transmigration, in particular, the role of neutrophil serine proteinase 3 (PR3).

Methods and Results—Endothelial cells were treated with PR3 either in its soluble form or in a complex form with cell surface NB1. We observed that PR3 mediated the enhancement of endothelial cell junctional integrity and that this required its proteolytic activity, as well as endothelial cell expression of the protease-activated receptor-2. Importantly, PR3 suppressed the vascular permeability changes and disruption of junctional proteins induced by the action of protease-activated receptor-1 agonists.

Conclusion—These findings establish the potential for neutrophil-derived PR3 to play a role in reestablishing vascular integrity after leukocyte transmigration and in protecting endothelial cells from protease-activated receptor-1-induced permeability changes that occur during thrombotic and inflammatory events. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: calcium signaling ■ NB1 ■ PECAM-1 ■ serine protease

The vascular endothelium plays a critical role in maintaining hemostasis, preserving the integrity of the circulatory system and regulating the inflammatory response. The latter role produces particular challenges for endothelial cells, which must permit the emigration of leukocytes from the bloodstream into the subendothelium, while at the same time preserving vascular integrity. One of the classical hallmarks of inflammation is edema and localized swelling. The contribution of emigrating leukocytes to vascular leakage has long been debated. Previous evidence has indicated that leukocytes can promote an increase in vascular permeability. However, despite the fact that leukocytes are capable of releasing cytokines, proteolytic enzymes, and reactive oxygen intermediates that promote local tissue damage, induce endothelial cell contraction, and recruit additional leukocytes, a number of studies have found that leukocyte transmigration results in only a transient, negligible opening of adherens junctions, with little induction of macromolecule leakage. Further evidence that neutrophil transmigration does not cause increased endothelial cell permeability during the inflammatory response is suggested by both in vivo and in vitro studies showing a general lack of correlation between neutrophil transmigration and increased vascular permeability. For example, in an aseptic model of wound healing, Kim et al. reported that the maximal increase in vascular permeability occurred a full 6 hours before peak neutrophil influx, with the magnitude of vascular leakage unaffected by neutrophil depletion. Burns et al. and Inglis et al. found that neutrophil transmigration across interleukin-1β- and fMLP-stimulated endothelial cell monolayers actually improved vascular cell barrier function. Despite considerable progress in this area, however, it is still not clear how leukocytes, particularly neutrophils, might be able to preserve vascular integrity during the process of transmigration.

Neutrophils contain in their cytoplasmic granules a number of serine proteases, including cathepsin G (CG), neutrophil elastase, and proteinase 3 (PR3). Once released, these proteolytic enzymes can be concentrated in neutrophil extracellular traps or rebound to the cell surface, where they can exert widespread effects, including induction of bactericidal activity, degradation of extracellular matrix proteins, promotion of neutrophil transmigration, and regulation of vascular integrity. In vivo, the activity of CG and neutrophil elastase is inhibited by secretory leukocyte protease inhibitor. CG is also inhibited by antichymotrypsin. Neutrophil elastase and PR3 are inhibited by α1-antitrypsin.
and elafin. The rapid inhibition of these proteases in vivo has the effect of restricting their activity to areas of local neutrophil accumulation.

Of particular interest is PR3, also known as elastin degrading protease, the most abundant serine protease in neutrophils. After neutrophil activation, PR3 is secreted from azurophil granules and rebinds to the neutrophil surface through an association with NB1 (CD177, HNA-2a) — a 60-kDa glycosylphosphatidylinositol (GPI)-linked, cell surface glycoprotein that is expressed on a subpopulation of neutrophils in 97% of healthy individuals. This interaction is unique to PR3, and does not occur for other neutrophil serine proteases. PR3, in association with NB1, is partially protected from proteolytic inactivation — a property that may significantly increase its efficacy. In addition, NB1 has been reported to be a heterophilic binding partner for endothelial cell PECAM-1, and disrupting NB1-PECAM-1 interactions has been shown to significantly inhibit neutrophil transmigration. As PECAM-1 is expressed at endothelial cell junctions where transmigration occurs, it is possible that NB1 directs at least a subpopulation of PR3 molecules to these areas to aid in neutrophil diapedesis, perhaps, through degrading junctional proteins or the extracellular matrix. Another possibility is that PR3 acts, with or without NB1, at the endothelial cell apical surface, where it can interact with endothelial cell receptors proximal to PECAM-1.

Similar to other serine proteases, PR3 has been reported to interact with protease-activated receptors (PARs). PR3 has been shown to activate platelets, dendritic cells, and endothelial cells through PAR-1 and PAR-2. Because members of the PAR family are associated with regulating vascular permeability, the potential for PR3 to act on these receptors suggests a possible mechanism for neutrophil regulation of barrier function. In the present study, we demonstrate that the serine protease PR3 is able to significantly enhance endothelial cell barrier function through a PAR-2-dependent pathway. In addition, we show that PR3 induces sustained endothelial cell calcium signaling, while at the same time inhibiting the permeability changes and disruption of endothelial cell junctional proteins induced by PAR-1 agonists.

Materials and Methods

Cell Lines

Primary isolated human umbilical vein endothelial cells were maintained in RPMI (Invitrogen) with 10% FBS, 2 mmol/L L-glutamine, and 500 μg/mL gentamycin. Cells were used between passages 3 and 4.

Antibodies

Antibodies against NB1 (MEM166), VE-cadherin (H-72), PR3 (PR9G-2), β-actin, PAR-1, and PAR-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Reagents

Interleukin-1β was purchased from Peprotech (Rocky Hill, NJ). The protease inhibitors elatin and AEBSF were purchased from AnaSpec (San Jose, CA) and Roche (Mannheim, Germany), respectively. Peptide agonists for PAR-1 (SFLLRN) and PAR-2 (SLIGKV) were purchased from Peptides International (Louisville, KY). PR3 was purchased from Enzo LifeScience (Farmingdale, NY) and was isolated from human sputum with a reported purity of >95%. Human neutrophil elastase and CG were purchased from EMD Millipore (Darmstadt, Germany). Soluble recombinant NB1 and NB1-IgG were kindly provided by Dr. Sentot Santoso (Justus Liebig University, Giessen, Germany). Fluorescein and BAPTA-AM were purchased from Invitrogen. Thapsigargin and ionomycin were purchased from Sigma-Aldrich.

Neutrophil Isolation

Neutrophils were isolated as previously described. Briefly, blood from healthy, consenting adult donors was collected in vacutainer tubes (BD Bioscience, Franklin Lakes, NJ) using 2 mmol/L EDTA as an anticoagulant. Donors were later characterized as either NB1-positive or NB1-negative by flow cytometry. Whole blood was layered over a ficoll-histopaque gradient (Sigma) and centrifuged for 30 minutes at 1000 g. Neutrophils were isolated from the buffy coat layer and the volume brought to 10 mL with PBSA (Dulbecco’s PBS without calcium or magnesium with 0.1% BSA). Neutrophils were then washed twice with PBSA at 200g for 10 minutes before being quantified.

Electric Cell-Substrate Impedance Sensing (ECIS) Measurements

ECIS measurements were performed in 8W10E+ electrode arrays on an ECIS Z0 instrument (Applied Biophysics, Troy, NY). Cells were plated on arrays coated with 0.2% collagen (Sigma-Aldrich) and allowed to grow to confluence for 1 to 2 days. On the day of the experiment, the culture media was replaced with media containing 1% FBS. Resistance (in units of Ω) at 4000 Hz was recorded and baseline barrier function was normalized within each experiment to combine results from multiple experiments for analysis. Within each experiment, all treatments were done in duplicate and the mean value was collected. Cells were stimulated with agonists for PAR-1 (SFLLRN, 1–10 μmol/L), PAR-2 (SLIGKV, 1–10 μmol/L), or serine proteases including PR3 (1–0.1 U/mL), CG (1–0.1 U/mL), human neutrophil elastase (1–0.1 U/mL), or thrombin (1 U/mL).

ECIS With Neutrophils

In some ECIS experiments, endothelial cells were stimulated 4 hours with interleukin-1β (1 ng/mL) before the addition of neutrophils. This stimulation alone did not result in a significant change in vascular permeability (data not shown). Stimulated endothelial cells were then incubated with neutrophils (either NB1-positive or NB1-negative) in ECIS arrays. Cells were incubated for 5 minutes with the protease inhibitors AEBSF (10 μmol/L) or elatin (2 μmol/L) before being added to the endothelial cells. Incubation of these inhibitors alone did not significantly affect endothelial cell electric resistance (data not shown).

Transfection of siRNA

Human umbilical vein endothelial cells were transfected with siRNA for PAR-1 (100 nmol/L, sc-36663), PAR-2 (100 nmol/L, sc-36188), or control siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA) using Lipofectamine (Invitrogen, Grand Island, NY) and after the manufacturer’s protocol. After 48 hours, siRNA inhibition of protein expression was determined by western blot analysis. Transfections were done in both 12 well plates as well as in 8W10E+ arrays used for ECIS.

Calcium Measurements

Calcium signaling in endothelial cells was detected using the calcium sensitive dye Fluo4 (Invitrogen). Endothelial cells cultured in 2-well chamber slides were incubated 15 minutes with Fluo4 (10 μmol/L). After Fluo4 loading endothelial cells were washed once, fresh media were added and the cells were transferred to a heated incubation chamber and observed using an Olympus FV1000 MPE confocal microscope (Center Valley, PA). After a baseline was established, cells were treated with ionomycin, PAR-1 or PAR-2 peptide agonists, or PR3. In some experiments, endothelial cells were preloaded with...
BAPTA-AM (10 μmol/L) for 30 minutes. In other experiments cells were pretreated with thapsigargin (1–10 μmol/L) immediately before agonist stimulation. Calcium fluxes were recorded for 10 minutes and later analyzed offline using Olympus Fluoview software. For every experiment, 10 cells were randomly selected from each field of view for line scan analysis.

Confocal Microscopy

Confocal microscopy was used to identify morphological changes in endothelial cell VE-cadherin localization and disruption of cell–cell junctions. Endothelial cells were cultured on 8 chamber slides (1×10^5 cell/mL) until confluent. Monolayers were first pretreated with PR3 (0.1 U/mL) or a PAR-2 peptide agonist (SLIGKV, 1 μmol/L) for 15 minutes, then treated with a PAR-1 peptide agonist (SFLLRN, 1 μmol/L). After 30 minutes, cells were fixed with 2% paraformaldehyde for 2 hours at 4°C. The cells were then washed in PBS and permeabilized with ice-cold 0.5% Triton X-100 in PBS for 3 minutes and then washed with PBS. The cells were next blocked 1 hour at 37°C with 5% FBS in PBS. Cells were then stained with antibodies against VE-cadherin overnight at 4°C in PBS containing 0.3% bovine albumin (PBSA). After being washed with PBS, the cells were stained 1 hour with Texas Red conjugated antirabbit antibodies at room temperature. The slides were then stained with DAPI (10 μmol/L) for 10 minutes at room temperature. Slides were coverslipped using Prolong Gold antifade (Invitrogen; Eugene, OR) and analyzed for VE-cadherin localization using an Olympus FV1000 MPE confocal microscope.

Statistical Analysis

Results, where applicable, are expressed as mean±SEM. Statistical analysis was performed on GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA), and significance was determined using ANOVA and the Bonferroni post hoc test. Results were considered statistically significant above the α<0.05 level.

Results

Neutrophils and PR3 Enhance Endothelial Cell Barrier Function

Neutrophils in contact with endothelial cells have been reported to enhance endothelial cell barrier function.18,44 To confirm these findings, we cultured endothelial cells on gold-coated chamber slides, added neutrophils, and measured neutrophil-induced changes in barrier function using Electric-Cell substrate Impedance Sensing (ECIS). As shown in Figure 1A and B, addition of neutrophils induced an immediate enhancement in endothelial cell barrier function, as expected. In contrast, neutrophils added to endothelial cells in the presence of serine protease inhibitors (AEBSF, elafin) failed to augment junctional integrity. To explore directly the ability of neutrophil-derived serine proteases to mediate these effects, we incubated endothelial cells with CG, neutrophil elastase (NE), PR3, or a positive control PAR-2 peptide agonist (SLIGKV) previously shown to enhance barrier function.45 As shown in Figure 1C and 1D, both PR3 and the PAR-2 peptide agonist significantly increased endothelial cell monolayer integrity, whereas CG and NE had little effect. To determine whether PR3 activity was required for the change in endothelial cell barrier function, PR3 was either heat-inactivated by boiling for 30 minutes or pretreated with elafin (2 μmol/L) for 15 minutes. In these conditions PR3 was unable to produce a change in barrier function compared with untreated cells (data not shown). These data demonstrate that both neutrophils and neutrophil-derived PR3 are able to enhance endothelial cell barrier function.

Role of Neutrophil NB1 (CD177) in Endothelial Cell Barrier Function

Only 40% to 60% of the neutrophils in healthy individuals express NB1 on their surface, whereas 3% to 5% of the population (henceforth termed NB1-null individuals) do not express NB1 at all because of a frame-shift mutation leading to early termination of the NB1 transcript.46 Activated neutrophils secrete PR3, some of which rebinds to the cell surface in an NB1-dependent manner.

Figure 1. Neutrophil PR3 induces an increase in endothelial cell barrier function. Human umbilical vein endothelial cells (HUVEC, 1×10^5) cultured on BW10E+ arrays and barrier function was measured by electric cell-substrate impedance sensing. The arrow indicates the time of endothelial cell treatment. A, HUVEC were stimulated 4 hours with interleukin (IL)-1β (1 ng/mL) before the addition of neutrophils (1×10^5) in the presence or absence of serine protease inhibitors AEBSF (10 μmol/L) or elafin (2 μmol/L). Results represent the mean±SEM of 2 wells from 1 of 4 representative experiments. B, Neutrophils caused a significant increase in endothelial cell barrier function compared with untreated endothelial cells. Inhibition of neutrophil serine proteases with elafin or AEBSF significantly reduced the increase in endothelial cell barrier function induced by neutrophils (mean±SEM from 4 separate experiments, **P<0.01). C, Endothelial cells treated with a positive control protease-activated receptor (PAR)-2 activating peptide (SLIGKV, 10 μmol/L), proteinase 3 (PR3, 0.1 U/mL), neutrophil elastase (NE, 0.1 U/mL), or cathepsin G (CG, 0.1 U/mL). Results represent the mean±SEM of 2 wells from 1 of 4 representative experiments. D, PAR-2 and PR3 induced a significant increase in endothelial cell barrier function 60 minutes after treatment (mean±SEM from 4 separate experiments, **P<0.01).
PR3/NB1 complex formation has also been shown to significantly increase on the neutrophil surface during the process of transendothelial migration. Although NB1-null individuals are unable to present PR3 on the neutrophil surface, they still express normal levels of PR3 in their intracellular granules. To determine whether NB1 plays a role in the ability of PR3 to promote an increase in endothelial cell barrier function, we incubated neutrophils derived from NB1-positive and NB1-null individuals increased endothelial cell barrier function. The increase was significantly greater with NB1-positive neutrophils (Figure 2B). To determine whether NB1 itself might be responsible for this enhancement of monolayer integrity, we incubated endothelial cells with soluble monovalent NB1 or bivalent NB1-IgG. As shown in Figure 2C, although PR3 was able to increase barrier function, NB1 alone in either form was without an effect. These data demonstrate that NB1-bound PR3 can function as an effective positive modulator of vascular integrity.

**PR3 Promotes Barrier Function via its Action on Endothelial Cell PAR-2**

Endothelial cells express 4 PARs, but of these, only PAR-1 and PAR-2 have been shown to play a role in regulating vascular barrier function in response to serine proteases. Activation of PAR-1 by thrombin or thrombin agonist peptides induces rapid endothelial cell contraction and vascular permeability, however thrombin does not activate PAR-2. In contrast, PAR-2 can be activated by coagulation factor Xa and promotes endothelial cell barrier protection. Factor VIIa has also been reported to enhance endothelial cell barrier function but acts on PAR-1, rather than PAR-2, to exert its effects. To examine whether PR3 augments endothelial cell barrier function by acting on either PAR-1 or PAR-2, we examined its effects on endothelial cells in which protein expression of PAR-1 and PAR-2 had been knocked down using PAR-specific siRNAs (Figure 3A). As shown in Figure 3B, a PAR-1 agonist peptide was effective in disrupting endothelial cell monolayer integrity in wild-type endothelial cells and in endothelial cells in which PAR-2 had been knocked down, but had no effect in PAR-1-deficient endothelial cells, as expected. Similarly, PAR-2-induced barrier protection was abrogated only in endothelial cells lacking PAR-2 expression, demonstrating the specificity of the assay system. Importantly, although PR3 was effective in augmenting barrier function in wild-type and PAR-1-deficient endothelial cell monolayers, it failed to have an effect on endothelial cells in which PAR-2 had been knocked down (Figure 3C). Although these experiments demonstrate the importance of endothelial PAR-2 expression for PR3-mediated barrier function, it was not clear whether PR3 could cleave PAR-2 on the cell surface, a process required for physiological PAR-2 activation. To investigate this, 293 human embryonic kidney cells were transfected with a PAR-2 alkaline phosphatase reporter construct (Figure I in the online-only Data Supplement). In cells incubated with PR3, we found significant PAR-2 cleavage, which was completely abrogated in the presence of the inhibitor elafin. Therefore, cell surface expressed PAR-2 is cleaved by PR3. These data demonstrate that the ability of PR3 to exert barrier protective effects is dependent on endothelial cell expression of PAR-2 and not PAR-1.

**PR3 Induces Calcium Signaling in Endothelial Cells**

Many signaling events, including those downstream of PAR-1 and PAR-2 activation, require intracellular calcium signaling. To determine whether PR3 stimulation of endothelial cell

![Figure 2. Neutrophil-expressed NB1 alone does not increase endothelial cell barrier function. A, Human umbilical vein endothelial cells (HUVEC, 1×10⁵) cultured on 8W10E+ arrays and barrier function was measured by electric cell-substrate impedance sensing. The arrow indicates the time of endothelial cell treatment. HUVEC were stimulated 4 hours with interleukin (IL)-1β (1 ng/mL) before the addition of neutrophils (1×10⁶) from either NB1-positive or NB1-null individuals. Results represent the mean±SEM of 2 wells from 1 of 4 representative experiments. **P<0.01. B, NB1-positive neutrophils promoted a significant increase in endothelial cell barrier function compared with NB1-negative neutrophils after 60 minutes of incubation (mean±SEM from 4 separate experiments, **P<0.01). C, Monomeric NB1 (20 μg/mL) or bivalent NB1-IgG (20 μg/mL) did not promote an increase in endothelial cell barrier function. Results represent the mean±SEM of 2 wells from 1 of 3 representative experiments. PR3 indicates proteinase 3.](image-url)
Sustained calcium response.

PR3 signaling requires both intracellular and extracellular calcium stores to induce a flux (Figure 4H). Therefore, PR3 signaling requires both inhibition of RhoA and Rac1 activity downstream of PAR-2 is linked to increased barrier function, whereas RhoA activity after PAR-1 stimulation promotes vascular permeability. Using an assay specific for the active, GTP-bound forms of Rac1 and RhoA (Cytoskeleton Inc, Denver, CO) we observed active Rac1-GTP in endothelial cells treated with a PAR-2 agonist peptide or PR3, but no increase in RhoA-GTP (Figure II in the online-only Data Supplement). In contrast, a PAR-1 agonist peptide was able to significantly increase RhoA-GTP, but it did not increase Rac1-GTP binding. Therefore, the activation of Rac1 and inhibition of RhoA may be 1 pathway that PR3 incorporates to inhibit PAR-1–mediated vascular permeability.

PR3 Inhibits PAR-1 Cytoskeletal Changes in Endothelial Cells

Vascular permeability involves both cytoskeletal changes and reorganization of intracellular junctions, both of which contribute to morphological changes, including formation of gaps and interdigitating membrane protrusions at cell–cell borders that can be easily visualized using fluorescent microscopy. To further explore the ability of PR3 to inhibit PAR-1 signaling, we examined the localization of VE-cadherin in resting and agonist-treated endothelial cell monolayers. As shown in Figure 6A, VE-cadherin is present at cell junctions as a tight, representative experiments.

PAR-2 results in release of calcium from intracellular stores, we loaded endothelial cell monolayers with Fluo4 and examined their responses to a series of agonists using confocal microscopy. As shown in Figure 4A, addition of the calcium ionophore ionomycin produced an immediate, robust, calcium flux, whereas stimulation with a PAR-1 agonist produced a characteristic, short-lived response (Figure 4B). In contrast, stimulation with a PAR-2 agonist (Figure 4C) promoted a sustained calcium flux consistent with previous reports.51,52 As shown in Figure 4D, PR3 produced both a rapid primary calcium signal as well as a sustained calcium response, consistent with its action on PAR-2 and not PAR-1. Chelating intracellular calcium with BAPTA-AM blocked both the primary and sustained responses (Figure 4G), whereas depletion of extracellular calcium with extracellular EGTA blocked only the sustained calcium signal induced by PR3 (Figure 4F), suggesting that the sustained signal required influx of extracellular stores. To determine whether the proteolytic activity of PR3 was required for its ability to induce sustained calcium signaling, we inactivated PR3 by heat denaturing or by preincubating it with the serine protease inhibitor, elafin. As shown in Figure 4E and 4F, although PR3 inactivated by either method was able to induce a primary calcium flux, both the amplitude and duration of the response were markedly blunted. However, inhibiting intracellular calcium uptake with thapsigargin before addition of PR3 allowed cells to retain a sustained, although blunted, calcium flux (Figure 4H). Therefore, PR3 signaling requires both intracellular and extracellular calcium stores to induce a sustained calcium response.

PR3 Signaling Partially Abrogates Vascular Permeability Changes Induced by PAR-1

To determine whether PR3-induced activation of PAR-2 might be able to reverse or moderate the junctional disruption effects induced by PAR-1 agonists, we pretreated endothelial cell monolayers with PR3 or a PAR-2 peptide agonist 15 minutes before challenge with a PAR-1 agonist. As shown in Figure 5, PR3 or a PAR-2 agonist significantly inhibited PAR-1–induced permeability, blunting both the immediate increase in (Figure 5A and 5B), as well as markedly improving the rate at which vascular integrity was restored (Figure 5C). Therefore, neutrophil-derived PR3 not only enhances baseline endothelial cell barrier function (Figures 1–3), but also can ameliorate the loss of junctional integrity induced by agonists that act on PAR-1. One mechanism by which PAR-2 activation could inhibit PAR-1–induced permeability is by regulating the small GTPases Rac1 and RhoA. Rac1 activation downstream of PAR-2 is linked to increased barrier function, whereas RhoA activity after PAR-1 stimulation promotes vascular permeability. Using an assay specific for the active, GTP-bound forms of Rac1 and RhoA (Cytoskeleton Inc, Denver, CO) we observed active Rac1-GTP in endothelial cells treated with a PAR-2 agonist peptide or PR3, but no increase in RhoA-GTP (Figure II in the online-only Data Supplement). In contrast, a PAR-1 agonist peptide was able to significantly increase RhoA-GTP, but it did not increase Rac1-GTP binding. Therefore, the activation of Rac1 and inhibition of RhoA may be 1 pathway that PR3 incorporates to inhibit PAR-1–mediated vascular permeability.
uninterrupted band in untreated endothelial cell monolayers. After stimulation with a PAR-1 agonist, VE-cadherin localizes to membrane ruffles and cellular protrusions, indicative of morphological changes that result in increased vascular permeability (Figure 6B). When endothelial cells were pretreated with PR3 or a PAR-2 agonist before PAR-1 stimulation, however, we observed a dramatic inhibition of gap formation and membrane ruffling (Figure 6C and 6D). These data suggest that the improvement in barrier function conferred by PR3 or PAR-2 agonist peptide treatment is attributable to stabilization of VE-cadherin-containing endothelial cell–cell junctions.

**Discussion**

Although transmigrating leukocytes disrupt endothelial cell junctions and degrade extracellular matrix proteins during the process of diapedesis, endothelial cell barrier function has been reported to actually increase during the process. The mechanisms by which neutrophils maintain, and later augment, vascular integrity during transmigration are not well understood. In the present study, we demonstrate that the neutrophil serine protease PR3 acts on PAR-2 to enhance endothelial cell barrier function, revealing a novel pathway for the maintenance of vascular integrity. Furthermore, PR3 stimulation of endothelial cells can counter vascular permeability induced by stimulating the thrombin receptor, PAR-1. These data suggest a heretofore unappreciated mechanism that neutrophils use to maintain the integrity of the vascular endothelium during diapedesis during the inflammatory response.

Activation of endothelial cells by PR3 was found to require endothelial cell expression of PAR-2, a protease-activated receptor capable of enhancing barrier function. Endothelial cells have been reported to express all 4 protease-activated receptors, with PAR-2 functioning to promote endothelial cell barrier function, whereas PAR-1 either increasing or decreasing endothelial cell barrier function, depending on the stimulus. The role of endothelial cell PAR-3 and PAR-4...
in regulating endothelial cell barrier integrity are less clear. PAR-2 can be activated by factor Xa, tissue factor/VIIa, or tertiary complexes of these molecules as well as the serine proteases trypsin and trypcase. Neutrophil serine proteases have been reported to interact with PAR-1 receptors; NE and CG have been shown to cleave and inactivate PAR-1. NE and CG have been shown previously to promote vascular permeability, but this may be through PAR-1–independent pathways. For example, CG has been reported to activate PAR-4, but whether this contributes to vascular permeability is unknown. Similar to CG and NE, PR3 has been reported to inactivate the Endothelial Cell Protein C Receptor (EPCR). This is interesting because activated Protein C has been shown to promote endothelial cell barrier function through the EPCR/PAR-1 axis. It is unlikely that PR3 modulates EPCR function, because loss of PAR-1 had little effect on the ability of PR3 to modulate endothelial cell

Figure 5. Proteinase (PR)3 inhibits protease-activated receptor (PAR)1-mediated vascular permeability. Human umbilical vein endothelial cells (1x10^5) were cultured on BW10E+ arrays and barrier function was measured by electric cell-substrate impedance sensing. The arrows indicate the time of endothelial cell treatment. A, Endothelial cells pretreated with PAR-2 agonist peptide (AP) (SLIGKV, 1 μmol/L) or PR3 (0.1 U/mL) for 15 minutes before being treated with PAR-1 agonist peptide (AP) (SFLLRN, 1 μmol/L). Endothelial cell pretreatment with PAR-2 AP or PR3 significantly inhibited PAR-1 AP–induced barrier function after 1 hour (B) and promoted barrier function as long as 3 hours (C) after treatment. Results represent the means±SEM of 2 wells from 1 of 4 representative experiments. (mean±SEM from 4 separate experiments; **P<0.01).

Figure 6. Proteinase (PR)3 inhibits protease-activated receptor (PAR)1 changes in VE-cadherin localization. Confluent monolayers of human umbilical vein endothelial cells (HUVEC) cultured on 8-chamber glass slides were stimulated with a PAR-1 peptide agonist (SFLLR, 1 μmol/L) for 30 minutes. In some experiments, HUVEC were pretreated 15 minutes with PR3 (0.1 U/mL) before PAR-1 stimulation. After 30 minutes, cells were fixed and stained with antibodies against the junction protein VE-cadherin (red) and nuclei were stained with DAPI (blue). PAR-1–stimulated HUVEC demonstrated intercellular gap formation and cellular interdigitations characteristic of decreased vascular barrier function (indicated by arrows). However, endothelial cells pretreated with PR3 or PAR-2 peptide agonist before PAR-1 stimulation showed an absence of VE-cadherin disruption. Results are representative of 5 separate experiments. Scale bar, 10 μm.
barrier integrity (Figure 3). Should PR3 cleave EPCR in vivo, however, it would produce the interesting scenario in which it both increases endothelial barrier function by activating PAR-2 while simultaneously inhibiting the ability of APC to activate PAR-1 activity. Whether these events occur in vivo may depend on temporal and spatially dependant conditions. We also observed that PR3 promotes sustained calcium signaling in endothelial cells (Figure 4). The observation that protease inhibitors or heat inactivation blocks the ability of PR3 to induce sustained cytosolic calcium flux, with only a moderate effect on immediate PR3-induced calcium transients (Figure 4E and 4F), suggests that PR3 might also be interacting with endothelial cells in a manner independent of its proteolytic activity. PR3 has been reported to bind to integrins and the EPCR. Precisely how PR3 interacts with endothelial cells to exert enzyme activity-independent responses may be an interesting topic of future investigation.

Thrombin generation occurs in many inflammatory conditions such as sepsis where increased vascular permeability is associated with disease severity. In some of these inflammatory conditions, there is a large influx of neutrophils, therefore neutrophils recruited to these areas may play a role in helping block or mitigate endothelial cell permeability induced by thrombin signaling through PAR-1. There is also a significant increase in endothelial cell expression of PAR-2 during sepsis, whereas PAR-1 levels are not affected. Likewise, NB1 expression on circulating neutrophils has been reported to significantly increase during sepsis. Because NB1 is required for expression of PR3 on the neutrophil cell surface, this suggests that during sepsis there may be increased PAR3 surface expression on neutrophils. Coupled with increased endothelial cell PAR-2 expression, this could play a role in helping neutrophils promote vascular integrity during inflammatory responses or sepsis. Whether PR3 and NB1 play a role in this regard is an area for future investigation.

Both soluble and NB1-bound PR3 can enhance barrier function (Figure 1). The significance of PR3 interacting with NB1 is 2-fold. First, PR3 interacting with NB1 is protected from proteolytic inactivation by inhibitors such as α1-antitrypsin. PR3 is therefore able to have a sustained affect, under conditions where other serine proteases (eg, NE and CG) become inactivated. Second, NB1 is a receptor for endothelial cell PECAM-1, suggesting that PR3 may be presented to the endothelium at cell–cell junctions where PECAM-1 is localized. In this regard, PR3 might play a role in neutrophil transmigration by degrading proteins at cell borders. Alternatively NB1, through its interaction with PECAM-1, may present PAR2 to endothelial receptors in the vicinity of PECAM-1. For example, PECAM-1 and PAR-1 have been shown to localize to lipid rafts where PR3 may be able to interact with and inactivate PAR-1. Likewise, if PAR-2 is also found in lipid rafts it may be in close proximity to PECAM-1 and be activated by the NB1–PR3 complex. In future studies, we hope to explore the role of NB1–PECAM-1 interactions in promoting PAR3 activation of PAR-2.

Approximately 3% to 5% of the population do not express NB1 on their neutrophils. We found that although NB1-null neutrophils were able to enhance barrier function (Figure 1), the degree to which it had an effect was significantly diminished compared with the effects of neutrophils from NB1+ individuals. The observation that NB1 is required for PR3 surface expression suggests either that PR3 secreted from NB1-negative neutrophils becomes sufficiently concentrated at the endothelial cell surface to retain some of its barrier-promoting activities even in the absence of NB1, or NB1-positive neutrophils have the capacity to use an alternative, PAR3-independent, mechanism to reseal endothelial cell junctions during diapedesis. Because no other serine proteases have been shown to promote barrier function, PR3 is likely to be the major contributor to vascular integrity during neutrophil transmigration. However, we cannot discount the possibility that a compensatory mechanism exists in NB1-null individuals.

One of the most intriguing results from the current study is the observation that PR3 reduced the extent to which PAR-1 agonists disrupt the vascular permeability barrier. Thus, pre-treatment of endothelial cells with PR3 or a PAR-2 agonist not only suppressed the initial permeability change after PAR-1 stimulation, but also restored barrier function significantly faster than cells stimulated with PAR-1 agonists (Figure 5). The mechanism by which PR3 exerted these effects is suggested by the observation that PR3 inhibited PAR-1–mediated cytoskeletal reorganization and endothelial cell gap formation (Figure 6). The signaling pathway from PR3 to the cytoskeleton remains to be investigated.

In summary, we have found that PR3 plays a significant role in promoting vascular integrity by signaling through endothelial cell PAR-2. Our study describes a unique mechanism in which neutrophils promote endothelial cell barrier function during transmigration, as well as inhibiting permeability induced by thrombin receptor signaling. These findings suggest that neutrophils may play a much more significant role in regulating hemostasis and vascular function that is currently believed.

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Disclosures
None.

References
22. yIELDS proteolytic fragments with selective migratory properties.


Neutrophil Proteinase 3 Acts on Protease-Activated Receptor-2 to Enhance Vascular Endothelial Cell Barrier Function

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SUPPLEMENT MATERIAL

Materials and Methods

PAR-2 Cleavage Assay: HEK 293 cells were cultured in 24 well tissue culture plates to approximately 70% confluency. The cells were then transiently transfected with a PAR2-ALP reporter construct (1 μg/well) in antibiotic-free Opti-MEM medium using Lipofectamine (Invitrogen, Carlsbad, CA) following the protocol previously described 1. After 6 hours the media was changed and the cells were incubated an additional 48 hours. The cells were then incubated with varying concentration of PR3 or thrombin (0.01 to 1 U/ml) for 4 hours. In some treatments, the PR3 was first incubated 15 minutes with 2 μM elafin before being added to the cells. Conditioned media was collected, centrifuged to remove cell debris and analyzed for ALP activity using the SensoLyte luminescent ALP reporter assay kit (AnaSpec, San Jose, CA) following the manufacturer’s instructions. Results are expressed as the mean ±SEM of four separate experiments.

Rac1-GTP and RhoA-GTP detection: HUVECs cultured on 12 well plates were serum-starved overnight at 37°C in 5% CO₂. The cells were then treated with the PAR1 agonist peptide (AP) (SFLLRN, 10 μM), PAR2 agonist peptide (SLIGKV, 10 μM) or PR3 (1 U/ml) for 5-15 minutes. The cells were then washed with ice-cold PBS, followed by lysis buffer to remove the cells and then flash frozen in liquid nitrogen. Quantification of Rac1-GTP and RhoA-GTP was done using the G-LISA kit (Cytoskeleton, Inc; Denver, CO) following the manufacturer’s instructions. The relative quantity of RhoA-GTP and Rac1-GTP in the cell lysates was determined using a fluorescent plate reader at 490 nM. This was then expressed as a percentage (% of max) of the
RhoA and Rac1 positive control proteins. Results are expressed as the mean ±SEM of four separate experiments.

**Results**

*PR3 cleaves PAR2 of the cell surface*

In cells transiently transfected with PAR2-ALP we found that PR3 could efficiently cleave PAR2, promoting the release of ALP that was detected by our assay (supplemental Figure I). However, if PR3 was first inactivated by elafin we did not detect any significant increase in PAR2 cleavage compared to untreated cells. Thrombin, another serine protease, was unable to significantly cleave PAR-2 from the surface of HEK 293 cells. Therefore, PR3 can cleave cell surface expressed PAR2 and this is inhibited by elafin.

*PR3 promotes Rac1 activation in endothelial cells*

In endothelial cells treated with a PAR2 agonist peptide or PR3 we observed a significant increase in Rac1-GTP activity compared to untreated cells (supplemental Figure II), but no significant increase in RhoA-GTP. In contrast, we found that endothelial cells treated with a PAR1 agonist peptide had a significant increase in RhoA-GTP activity; however, PR3 nor PAR2 agonist peptide were able to significantly increase RhoA-GTP at the time points we used. These results demonstrate that PR3 can promote Rac1-GTP activity while RhoA-GTP activation is not significantly increased.

**Reference**

Supplemental Figure 1. PR3 cleaves PAR-2 expressed on the cell surface. HEK 293 cells were transiently transfected with a PAR-2 alkaline phosphatase (ALP) containing reporter construct. These cells were then incubated with PR3, PR3 treated with elafin (2 μM) or thrombin for 4 hours. Supernatants were then collected and analyzed for ALP activity. Conditioned media from cells treated with PR3 had significantly increased ALP activity compared to media from untreated cells. PR3 inactivated by elafin was not able to cleave PAR-2. (* = p<0.01 compared to untreated cells) Results represent the mean ± SEM of four separate experiments.
Supplemental Figure II. PR3 promotes Rac1-GTP activity. Serum starved endothelial cells in 12 well tissue culture plates were incubated with PR3 (1 U/ml), PAR-1 agonist peptide (SFLRN, 10 μM) or PAR-2 agonist peptide (SLIGKV, 10 μM). Cell lysates were then collected and analyzed for Rac1- and RhoA-GTP binding compared to a positive control protein. Results are expressed as the percent of maximum compared to the positive control protein. Both PR3 and PAR-2 agonist peptide were able to promote a significant increase in Rac1-GTP activity compared to untreated cells. In contrast, only the PAR-1 agonist peptide was able to significantly increase RhoA-GTP activity at the time points we observed. (*) = p<0.01 compared to untreated cells) Results represent the mean ±SEM of four separate experiments.