Differential Actions of PAR₂ and PAR₁ in Stimulating Human Endothelial Cell Exocytosis and Permeability

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Abstract—Endothelial cell proteinase activated receptors (PARs) belong to a family of heterotrimeric G protein–coupled receptors that are implicated in leukocyte accumulation and potentiator of reperfusion injury. We characterized the effect and the signal transduction pathways recruited after stimulation of endothelial PAR₂. We used von Willebrand Factor (vWF) release and monolayer permeability to peroxidase to report Weibel-Palade body (WPB) exocytosis and por formation, respectively. Human umbilical vein endothelial cells (HUVECs) were stimulated with the selective PAR₂ agonist peptide SLIGRL-NH₂ or PAR₁ agonist peptide TFLLR-NH₂. PAR₁ stimulation resulted in WPB exocytosis like PAR₂ stimulation but, unlike PAR₁, failed to increase monolayer permeability. BAPTA-AM inhibited PAR₂-induced exocytosis, indicating a PAR₂ calcium–dependent signal in ECs. Moreover, PAR₁-like PAR₂-stimulated exocytosis requires actin cytoskeleton remodeling, because vWF release is inhibited if the cells were pretreated with Jasplakinolide. Rho-GTPase activity is required for PAR-stimulated exocytosis, because inactivation of this family of actin-regulatory proteins with Clostridium difficile toxin B blocked exocytosis. Expression of dominant-negative mutant Cdc42¹⁷N inhibited exocytosis whereas neither dominant-negative Rac¹⁷N expression nor C3 exotoxin treatment affected vWF release. PAR₂ stimulated RhoA-GTP weakly compared with the PAR₁ agonist. We conclude that both PAR₂ and PAR₁ elicit WP body exocytosis in a calcium and Cdc42 GTPase-dependent manner. In contrast, the differential effect of PAR₁ versus PAR₂ activation to increase monolayer permeability correlates with weak RhoA activation by the PAR₂ agonist. (Circ Res. 2003;92:272-278.)

Key Words: vascular endothelium ■ reperfusion injury ■ thrombin receptors ■ Rho-GTP-binding proteins

R eperfusion of tissue after revascularization of an arterial thrombosis or preservation of a solid-organ allograft is accompanied by microvascular endothelial cell injury, local activation of the coagulation cascade, and diminished endothelial anticoagulant properties.¹–³ In addition, platelet and leukocyte adhesion to the endothelium is thought to contribute to inhomogeneous perfusion and persistent ischemic injury of the parenchymal cells of the tissue.⁴–⁶ Activation of specific receptors on the microvascular endothelial cells by the serine proteinases of the coagulation cascade appears to be an important mechanistic link between tissue injury and the early recruitment of leukocytes.²

The proteinase activated receptors (PARs) are a novel family of G protein–coupled receptors that require proteolytic cleavage at the amino terminus to stimulate signaling (see review⁷). Cleavage allows the newly exposed tethered ligand to initiate an intramolecular interaction necessary for signal transduction, which can be mimicked by peptide analogues of the newly exposed N-terminus. The PAR family members, PAR₁–₄, are expressed constitutively on endothelial cells, and PAR₁ and PAR₄ are further upregulated on inflamed endothelium.⁸,⁹ PAR₁, PAR₃, and PAR₄ are substrates for thrombin, whereas PAR₃ can be stimulated by the activity of tryptase, a product of mast cell degranulation, and coagulation Factors VIIa and Xa.¹⁰,¹¹

Stimulation of endothelial cells with thrombin or peptide analogues of the PAR₁-tethered ligand (eg, TRAP or SFLLRN-NH₂), results in reorganization of the cytoskeleton and discharge of endothelial storage granules, or Weibel-Palade bodies (WPBs). Exocytosis of WPBs plays a major role in the recruitment of leukocytes to sites of inflammation.¹² WP body exocytosis releases stored IL-8 and mobilizes the adhesion molecule P-selectin to the luminal surface of the endothelial cell.¹³,¹⁴ In addition, WPB exocytosis promotes platelet adhesion through the release of von Willebrand’s factor (vWF).¹³ Thrombin stimulation elicits capillary leak in vivo and increased endothelial monolayer permeability in vitro.¹⁵,¹⁶ PAR stimulation, then, promotes...
leukocyte adhesion to vascular endothelium and links inflammation and coagulation in a variety of pathological settings.

Stimulation of the PAR2 is also proinflammatory in vivo. Injection of selective peptide analogues of the PAR2 receptor tethered ligand is sufficient to initiate inflammation in vivo.17 Moreover, impaired inflammatory responses are observed in the PAR2-deficient mouse.18 In vitro, trypsin, like thrombin or TRAP, treatment elicits WPB exocytosis,19 but fails to induce monolayer permeability.20 However, the proteinases may act on surface proteins other than PARs,21 and the PAR1 agonist peptides modeled on the PAR2 tethered ligand activate both PAR1 and PAR2.22 Hence, characterization of the effects of selective endothelial PAR2 stimulation, and the signaling pathways recruited by PAR2 agonists to stimulate the endothelial cell remain to be explored.

Materials and Methods

Reagents

PAR agonist peptides (AP) TFFLR-NH2 and SLIGRL-NH2 were obtained from the peptide synthesis facility of the Health Sciences Center of the University of Calgary or from the Alberta Peptide Institute and were >95% pure by HPLC. Jasplakinolide was obtained from Molecular Probes. ELISA reagents for vWF quantitation, were obtained from Cederlane Laboratories, and a vWF standard through Precision Biologic. Ionomycin and C3 exotoxin were obtained from Calbiochem. The rho-kinase inhibitor Y-27632 was a gift of Welfide Corporation (Osaka, Japan). The Closstradium difficile toxin B (TcdB) was obtained from List Biochemicals. The rhotekin Rho binding domain (RBD)-GST fusion protein cDNA was a generous gift of Dr M. Schwartz (Scripps Research Institute, San Diego, Calif). Anti-RhoA mAb 26C4, and phospho-specific myosin light chain kinase pAb, were obtained from Santa Cruz Biotechnology. Peroxidase-conjugated secondary pAb were from Jackson Immunoresearch. M199, FBS, and Hanks balanced salt solution were from Invitrogen. Cell dissociation solution, cytochalasin D, H-7, and all other reagents were from Sigma.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from several umbilical cords, pooled, and cultured in complete media (M199 with 20% FBS, penicillin, streptomycin, and glutamine). Cells were serially passed in complete media supplemented with ECGS. HUVECs were cultured on a gelatin matrix and used in the experiments at passage 2 to 5. The passaged endothelial cells were harvested by washing twice with Hanks’, then incubated with a nonenzymatic Cell Dissociation Solution (Sigma) until the ECs were seen to lift off the plate. For use in an experiment, the harvested cells were then replated onto fibronectin (Fn)-coated C6 wells and incubated at 37°C in complete medium for 24 to 48 hours until confluent.

Where indicated, Cytochalasin D (1 μmol/L), jasplakinolide (45 mmol/L), or DMSO vehicle were diluted in supplemented M199 and added to the existing media. After 1 hour of incubation at 37°C, the media was removed and discarded, and fresh complete media with the agonist or vehicle diluted to the desired concentration was applied to the HUVECs. To inhibit Rho-GTPases, HUVECs were pretreated with 50 μmol/L C3 exotoxin for 24 hours and a mobility shift of endothelial Rho on SDS-PAGE was confirmed as described previously.24 To inhibit rho-kinase, endothelial cells were pretreated with H-7 (0.5 μmol/L) or Y-27632 (15 μmol/L) for 120 minutes before stimulation. To stimulate WP body exocytosis, the following agonists were used: phorbol 12-myristate 13-acetate (PMA) at 100 nmol/L, PAR, AP (TFFLR-NH2) at 30 nmol/L, PAR, AP (SLIGRL-NH2) at 30 μmol/L, and Ionomycin at 1 μmol/L, except where otherwise specified.

Chemical inhibitors were used at concentrations that maintained HUVEC viability >85% of mock-treated controls as assessed by the XTT assay of mitochondrial activity.25

ELISA Assay of vWF

 Supernatant from mock- or agonist-stimulated HUVECs was removed into separate tubes and kept on ice until analysis. The vWF concentration was determined by ELISA. Calibration was performed with a vWF standard (Human reference plasma, Precision Biologics). Regulated vWF release was calculated by subtracting the vWF concentration in unstimulated wells from the stimulated wells. The effect of the inhibitors was determined by comparing the mean vWF release to its control group. The mean difference of the treatment among several experiments, as noted in the figure legends, was calculated and tested for statistical significance (P<0.05) by ANOVA using SPSS (SPSS).

Endothelial Monolayer Permeability

HUVECs at passage 3 were plated onto fibronectin-coated polycarbonate membranes (5-um pore size Transwell, Costar) at 6×104 cells/cm2 in M199/20% FBS. The monolayers were cultured for 5 days with media changes every 2 days and were not disturbed for 24 hours before the experiment. Horse radish peroxidase (HRP, MW 44kDa, Sigma) was added to the upper chamber at a final concentration of 1 μg/mL, then the specific peptide agonist was added at the concentration indicated in the figure legends. Where indicated, the monolayers were pretreated as described in the figure legend. Medium was harvested from the lower chamber after 20 minutes, and the HRP activity was determined colorimetrically by absorbance at 490 nm of the o-phenyl diamine reaction product. The mean HRP concentration from triplicate experimental wells was compared with the HRP concentration in wells treated with ionomycin 10 μmol/L according to the formula: (experimental−media control)/(ionomycin−media control)×100=% maximum permeability.

The difference of the means between treatment and controls among several experiments was calculated and tested for statistical significance (P<0.05) by ANOVA using SPSS.

Affinity Precipitation of Rho-GTP

The RBD-GST fusion protein was prepared and isolated using glutathione beads (Amersham, Baie d’Urfé, PQ), then used to affinity precipitate GTP-Rho as described previously.26 Rho was detected with anti-Rho mAb 26C4 then the blots were developed using chemiluminescence (Supersignal, Pierce). Images were acquired from film using a CCD camera and the density of the bands were quantitated using the Fluor-S Max software package (BioRad).

Transient Transfection of Endothelial Cells

Dominant-negative myc-tagged mutant Rac127 and Cdc4227 cDNA were generously provided by Dr Gary Bokoch (Scripps) then subcloned into pCSD7 (a gift of John Elliott, University of Alberta, Edmonton, Canada) for transfection of HUVECs. The cDNAs were sequenced to confirm the presence of the mutation before use. Briefly, HUVECs were plated at confluence on Fn matrix in C-6 plates in growth medium 1 day before transfection. The cells were washed with OptiMEM (Life Technologies), then 0.2 μg DNA/well was introduced into the cells using the Effectene kit (Qiagen) according to the instructions of the manufacturer. After 3 to 4 hours, the wells were washed and the media was replaced with M199/10% FBS and ECGS. Pilot experiments indicated that expression of the myc-tagged mutant GTPase was optimal at approximately 24 hours after transfection; therefore, the cells were used in the experiments as indicated at this time. Endothelial cell expression of the myc-tagged protein was confirmed in each experiment.

Results

Weibel-Palade Body Exocytosis Is Stimulated by Activating Peptides Specific for PAR2

First, we sought to examine the effect of selective PAR2 activation compared with PAR1 activation on endothelial cell
PAR 1 or PAR 2 (SLIGRL-NH₂) agonist peptide, then the media was assayed for vWF by ELISA. Regulated vWF release was calculated as described in Materials and Methods and expressed as a fraction of vWF release stimulated by PMA. Mean ± SEM of at least 4 independent experiments is shown.

We next examined if selective PAR 2 stimulation altered endothelial monolayer permeability. Permeability of confluent endothelial monolayers to HRP after stimulation with PAR 1 (TFLLR-NH₂, 50 μmol/L) or PAR 2 (SLIGRL-NH₂, 50 μmol/L) was determined as described in Materials and Methods. Agonist-stimulated monolayer permeability is shown relative to monolayers stimulated with ionomycin (10 μmol/L). Data represent the mean ± SEM of 3 independent experiments.

Effect of Cytochalasin D and Jasplakinolide on PAR 2 Agonist Peptide-Stimulated Exocytosis

Both endothelial cell exocytosis and monolayer permeability changes are thought to require rearrangement of the f-actin cytoskeleton, hence we sought to determine if PAR 2 stimulation induced f-actin reorganization. Cytochalasin D and jasplakinolide have been previously shown to depolymerize or stabilize the f-actin cytoskeleton, respectively.²⁷,²⁸ To determine if the endothelial cytoskeleton was reorganized in PAR 2 -stimulated exocytosis, HUVECs were pretreated with either agent, then treated or not with peptide agonist. Neither agent changed constitutive vWF release. However, cytochalasin D pretreatment led to a modest augmentation of both PAR 2 - and PAR 1 -regulated WP body exocytosis (Figure 3). These data indicate that an intact cytoskeleton is not required for PAR 2 -mediated exocytosis.

Jasplakinolide was used to test the requirements of each agonist for remodeling of the intact cytoskeleton to elicit vWF release (Figure 3). HUVECs pretreated with jasplakinolide then stimulated with PAR 1 agonist peptide, TFLLR-NH₂, released 54 ±15% of vWF compared with mock-pretreated cells (P=0.03, n=6 experiments). Similarly, jasplakinolide inhibited vWF release on stimulation with PAR 2 agonist peptide, SLIGRL-NH₂, to 43 ±16% of mock-pretreatment release (P=0.05, n=5 experiments). These data indicate that PAR 2 -mediated exocytosis requires active remodeling of the endothelial f-actin cytoskeleton.
PAR2 Exocytosis Requires Intracellular Calcium

The initiation of a calcium flux has previously been identified to play a critical role in thrombin-stimulated WP body exocytosis and monolayer permeability.29,30 To determine if PAR2-stimulated exocytosis was similarly dependent on calcium-dependent signaling, HUVEC monolayers were pretreated with BAPTA-AM, then stimulated with the PAR agonist peptides. As noted in Figure 4A, PAR2-stimulated vWF release was markedly inhibited under these conditions. This indicates that WP body exocytosis, stimulated by either PAR1 or PAR2, is dependent on elevated intracellular calcium concentration.

Our data suggest that both cytoskeletal reorganization and elevated intracellular calcium are required for efficient PAR-stimulated exocytosis. However, high concentrations of jasplakinolide may inhibit sustained intracellular calcium levels after stimulation via G protein-coupled receptors.31 Because a calcium flux is sufficient to induce WP body exocytosis,29 we sought to determine if calcium ionophore-induced vWF release was affected by jasplakinolide. First, HUVECs were stimulated with ionomycin to define a concentration-effect curve (data not shown). Ionomycin (1 μmol/L) induced vWF release comparable to that of the PAR-activating peptides at 30 to 50 μmol/L. Jasplakinolide pretreatment diminished ionomycin-induced vWF release to 52±13% (Figure 4B; P=0.02, n=5 experiments) of mock-pretreated controls. This observation indicates that the effect of jasplakinolide lies downstream of the generation of a calcium flux and suggests that PAR2 stimulation results in a calcium-dependent reorganization of the endothelial cytoskeleton.

Involvement of Rho-GTPase Signaling After PAR2 Stimulation

The Rho family of small GTP-binding proteins regulate the f-actin cytoskeleton and have been implicated in thrombin-stimulated increased endothelial monolayer permeability,16,32 and in exocytosis in a variety of other cell types.33,34 Rho kinase, a Rho-GTP–dependent serine/threonine kinase, acts to phosphorylate and inactivate myosin light chain phosphorylation. Myosin light chain phosphorylation is also implicated in both thrombin-stimulated permeability and exocytosis.16,29 We therefore sought to determine if a Rho-GTPase-dependent signaling pathway was recruited after PAR2 stimulation.

We pretreated confluent HUVEC monolayers with C3 exotoxin to selectively inhibit the small GTPases, Rho, Rac, and Cdc42. PAR2- and PAR1-mediated exocytosis were markedly inhibited (Figure 5A). This strongly suggests that Rho-GTPases serve a critically important function to effect PAR-stimulated exocytosis in endothelial cells.

To determine if RhoA activity elicited by PAR2 stimulation was involved in exocytosis, we tested the effect of specific inhibitors of the RhoA signaling pathway. First, we inhibited Rho activity with C3 exotoxin.34 C3 pretreatment did not inhibit vWF release using the PAR2 agonist peptide to stimulate endothelial cell exocytosis (Figure 5A). We similarly observed that inhibition of Rho kinase with either Y-27632 or H-7 (data not shown) failed to inhibit, and tended to augment, PAR-stimulated vWF release. In contrast, PAR2 stimulation of monolayer permeability was markedly attenuated by inhibition of Rho kinase with Y-27632 (Figure 2). These data indicate that RhoA-GTPase signaling is not required for PAR2-stimulated exocytosis and suggest that other Rho-GTPase family members account for the TcdB-mediated inhibition.
negative mutant Rac\textsubscript{17N} or Cdc42\textsubscript{17N} in the endothelial cells. Both PAR\textsubscript{2}- and PAR\textsubscript{1}-stimulated exocytosis was inhibited in HUVEC-expressing Cdc42\textsubscript{17N} but not Rac\textsubscript{17N} (Figure 5B). Taken together these observations indicate that stimulation of either endothelial PAR receptor recruits a signal that is dependent on Cdc42 activity to elicit exocytosis.

PAR\textsubscript{2} Stimulation Elicits Weak RhoA Activity

PAR\textsubscript{2}-stimulated monolayer permeability depends on Rho kinase activity (Figure 2), hence we directly assessed RhoA activation after PAR\textsubscript{2} stimulation to determine if the failure of PAR\textsubscript{2} to elicit pore formation correlated with RhoA-GTP. As shown in Figure 6, Rho-A-GTP was robustly activated by PAR\textsubscript{1} stimulation, but only weakly by PAR\textsubscript{2} stimulation at receptor-selective concentrations of the agonist peptides. To determine if the weak RhoA-GTP stimulation by PAR\textsubscript{2} could account for the failure of pore formation through failing to increase cell contractility, we examined myosin light chain phosphorylation. Both PAR\textsubscript{2} and PAR\textsubscript{1} agonist peptides induced phosphorylation of myosin light chain, with a much greater effect of PAR\textsubscript{1} (Figure 7). Interestingly, stimulation of MLC phosphorylation by either agonist peptide was inhibited by pretreatment of the HUVECs with Y27632 (data not shown). These observations indicate that the failure of PAR\textsubscript{2} stimulation to elicit monolayer pore formation correlates with weak signaling through RhoA.

Discussion

The major observations of this article are as follows: first, PAR\textsubscript{2}-stimulated exocytosis is dependent on both remodeling...
the actin cytoskeleton and eliciting an intracellular calcium flux. PAR₂-stimulated exocytosis does not require either myosin light chain phosphorylation, or RhoA-GTPase-dependent Rho kinase activity. However, both PAR₁ and PAR₂-stimulated exocytosis is sensitive to TcdB inhibition of the Rho family of GTPases. Cdc42 signaling appears to be the principal Rho-GTPase required for endothelial Weibel-Palade body exocytosis. Interestingly, unlike PAR₁, PAR₂ failed to elicit increased endothelial monolayer permeability, despite generating stress fibers and myosin light chain phosphorylation to enable cell contractility. Activity of RhoA-GTP and myosin light chain phosphorylation are required for PAR₂-mediated monolayer permeability.

We studied selective agonists of both the PAR₂ and PAR₁ G protein–coupled receptors and observed that each stimulated similar amounts of vWF release from the endothelial cell storage pool. The use of a selective activator of PAR₂ indicates that the PAR system can have as great an impact on Weibel-Palade body exocytosis and hence luminal display of P-selectin as the thrombin-regulated PAR₁ system. Interestingly, like PAR₁, PAR₂-mediated exocytosis required f-actin reorganization, and involves a calcium-dependent rather than a cAMP-dependent second messenger. A similar requirement for f-actin reorganization was observed using ionomycin to mimic the calcium flux initiated by the PAR receptors, indicating that the requirement for f-actin remodeling likely occurred downstream of the PAR-stimulated calcium signal. These observations are in agreement with previous work that inferred activation of the PAR₂ receptor using trypsin to stimulate Weibel-Palade body exocytosis and that detected a calcium flux in endothelial cells after trypsin stimulation.

Coupling of PAR₂ to endothelial cytoskeletal-associated proteins has not been examined previously. However, regulated exocytosis by mast cells or pancreatic acinar cells can be facilitated by dissolution of the cortical actin barrier and is thought to be a prerequisite for vesicle fusion to the plasma membrane. The endothelial PAR₂-stimulated calcium signal is required for exocytosis and may regulate disassembly of cortical f-actin, for example by augmenting the activity of actin-severing proteins. The sensitivity of PAR₂-stimulated exocytosis to the actin-stabilizing effect of jasplakinolide is consistent with this model.

Regulation of f-actin reorganization occurs under the influence of the Rho family of monomeric GTP-binding proteins. For example, Rac1 is linked to PAR signaling pathways that regulate stress fiber induction and cortical actin remodeling. In addition, GTP-γS–induced exocytosis in permeabilized mast cells is inhibited by C3 exotoxin, which specifically ADP-ribosylates and inactivates Rho. Rac and Cdc42 activity are also implicated in regulated exocytosis in basophilic leukemia cells and pancreatic β-cells. Our observation that pretreatment of the HUVECs with TcdB, to inactivate Rho, Rac, and Cdc42, markedly inhibits PAR₂-stimulated vWF release, clearly implicating Rho-GTPases in both the PAR₁ and PAR₂ signaling pathways that direct Weibel-Palade body exocytosis.

Rho-GTP rapidly increases after stimulation of HUVECs with either the PAR₁ or PAR₂ agonist. To determine the participation of Rho directly in PAR-stimulated WP body exocytosis, we used the C3 exotoxin to selectively modify and abrogate interaction of Rho with downstream effector molecules. We found no consistent inhibitory effect of C3 exotoxin when used at concentrations known to markedly ADP-ribosylate endothelial Rho proteins. Moreover, inhibition of Rho kinase with Y27632 also fails to block PAR-stimulated exocytosis. This result indicates that neither RhoA nor Rho kinase activity is required for WP body exocytosis after stimulation of either PAR₁ and confirm recent data examining the role of RhoA in thrombin-stimulated exocytosis. In contrast, we observed that expression of the Cdc42 mutant significantly decreased PAR-stimulated Weibel-Palade body exocytosis. Because the transfection efficiency in these experiments was approximately 30% to 50%, diminished Cdc42 activity likely accounts for much of the inhibition observed with TcdB treatment. Taken together these experiments implicate an obligatory role for Cdc42 activity in the cascade of events leading to endothelial exocytosis downstream of either protease activated receptor. However, we cannot exclude that Rho-GTPase family members function partially redundantly in this system.

Interestingly, despite the ability to mobilize intracellular calcium and elicit f-actin remodeling and myosin light chain phosphorylation, PAR₂ signaling, in contrast with PAR₁, fails to increase endothelial monolayer permeability. Our observations confirm and extend those of Compton et al who observed no endothelial monolayer permeability change after trypsin stimulation. We observe that PAR₂-mediated monolayer permeability required Rho-GTP and Rho kinase activity for this event and demonstrate that PAR₂ recruited Rho-GTP, albeit only weakly. These results indicate that unlike PAR₁, PAR₂ fails to sufficiently activate the RhoA, and possibly other, signal transduction pathways necessary for monolayer pore formation.

In summary, we describe a role for cytoskeleton remodeling and the Rho family of GTP-binding proteins in both PAR₁ and PAR₂ signaling to endothelial Weibel-Palade body exocytosis. The signal involves Cdc42 GTP-binding protein acting independently of Rho kinase or Rac1. Our data are consistent with a model of Cdc42 GTP-binding protein-dependent cortical cytoskeletal remodeling. However, in comparison with PAR₁, PAR₂ elicits less RhoA-GTP and MLC phosphorylation and does not elicit increased monolayer permeability.

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Differential Actions of PAR$_2$ and PAR$_1$ in Stimulating Human Endothelial Cell Exocytosis and Permeability: The Role of Rho-GTPases

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