Protease-activated Receptor-1 Activation of Endothelial Cells Induces Protein Kinase Cα-dependent Phosphorylation of Syntaxin 4 and Munc18c

ROLE IN SIGNALING P-SELECTIN EXPRESSION*

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Endothelial cells exhibit regulated exocytosis in response to inflammatory mediators such as thrombin and histamine. The exocytosis of Weibel-Palade bodies (WPBs) containing von Willebrand factor, P-selectin, and interleukin-8 within minutes after stimulation is important for vascular homeostasis. SNARE proteins are key components of the exocytic machinery in neurons and some secretory cells, but their role in regulating exocytosis in endothelial cells is not well understood. We examined the function of SNARE proteins in mediating exocytosis of WPBs in endothelial cells. We identified the presence of syntaxin 4, syntaxin 3, and the high affinity syntaxin 4-regulatory protein Munc18c in human lung microvascular endothelial cells. Small interfering RNA-induced knockdown of syntaxin 4 (but not of syntaxin 3) inhibited exocytosis of WPBs as detected by the reduction in thrombin-induced cell surface P-selectin expression. Thrombin ligation of protease-activated receptor-1 activated the phosphorylation of syntaxin 4 and Munc18c, which, in turn, disrupted the interaction between syntaxin 4 and Munc18c. Protein kinase Cα activation was required for the phosphorylation of syntaxin 4 and Munc18c as well as the cell surface expression of P-selectin. We also observed that syntaxin 4 knockdown inhibited the adhesion of neutrophils to thrombin-activated endothelial cells, demonstrating the functional role of syntaxin 4 in promoting endothelial adhesivity. Thus, protease-activated receptor-1-induced protein kinase Cα activation and phosphorylation of syntaxin 4 and Munc18c are required for the cell surface expression of P-selectin and the consequent binding of neutrophils to endothelial cells.

The rapid migration of neutrophils across the vascular endothelial barrier in response to inflammatory stimuli requires the expression of endothelial adhesivity (1, 2). Endothelial adhesiveness can be induced within minutes in response to the release of mediators such as thrombin and histamine. The exocytosis of secretory granules called Weibel-Palade bodies (WPBs) in endothelial cells plays an important role in the initiation of the adhesive response (3, 4). WPBs are storage compartments containing von Willebrand factor, P-selectin, and interleukin-8. von Willebrand factor, a mediator of hemostasis, acts as a chaperone for coagulation factor VIII and thus controls the aggregation of platelets at sites of vascular injury (5). The membrane-inserted glycoprotein P-selectin is involved in inducing the rolling of neutrophils and their recruitment into tissue within minutes (2). Stimulation of endothelial cells by thrombin and histamine causes the translocation of WPBs to the plasma membrane, and WPB fusion with the membrane induces the secretion of von Willebrand factor and the insertion of P-selectin into the membrane (6–8). Because little is known about the signaling events mediating WPB exocytosis in endothelial cells, we addressed the role of the “exocytic machinery” in the mechanism of the translocation of P-selectin to the endothelial membrane.

Matsumot et al. (9) have demonstrated that the N-ethylmaleimide-sensitive factor (NSF), which is known to induce disassembly of the SNARE (soluble NSF attachment protein receptor) complex, was expressed in endothelial cells and that NSF activity was inhibited by nitric oxide through S-nitrosylation. These studies showed that the inhibition of NSF activity by nitric oxide prevented the exocytosis of WPBs and thereby reduced vascular inflammation. SNARE proteins, the central components of the exocytic machinery in neurons and some non-neuronal cells (10–12), regulate exocytosis by promoting vesicle fusion with the plasma membrane. According to the SNARE hypothesis (13), target SNAREs (syntaxin and SNAP-25) present on the target membrane bind to vesicle SNAREs (vesicle-associated membrane protein) on the vesicle membrane to form a ternary SNARE complex. The SNARE complex then brings the two membranes in apposition, a necessary step in overcoming the energy barrier required for membrane fusion. Modulators of intracellular calcium and protein kinases, known to stimulate exocytosis in neurons, have been shown to regulate the SNARE complex formation (14–18). The forma-

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S The on-line version of this article (available at http://www.jbc.org) contains additional data on syntaxin 4 and Munc18c in the form of supplemental Figs. S1 and S2, supplemental experimental procedures, and supplemental references.

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tion of the SNARE complex is tightly controlled by the syntaxin-binding protein Munc18 (also named nsec-1 in neurons) (11, 19). Disruption of the interaction with syntaxin with Munc18 is believed to be critical for SNARE complex formation (11, 20). The closed conformation adopted by syntaxin prior to the formation of the SNARE complex is stabilized by Munc18 binding; thus, syntaxin has to be freed from Munc18 inhibition to adopt the open conformation and thereby form the ternary complex (11, 20). Studies also showed that phosphorylation of syntaxin and Munc18 may cause their dissociation, allowing the formation of the SNARE complex (21, 22). Recent studies have suggested that Munc18/sec1 proteins play an important role in vesicle docking (23, 24), but little is known about this process in the regulation of WPB exocytosis in endothelial cells.

In the present study, we investigated the role of SNARE proteins in the mechanism of regulated WPB exocytosis in endothelial cells and the cell surface expression of P-selectin. We had the following objectives: (i) to identify specific syntaxin isoforms involved in the regulated exocytosis of WPBs in human lung microvascular endothelial cells (HLMVECs); (ii) to delineate key signaling pathways regulating the SNARE protein function induced by thrombin activation of endothelial cells; and (iii) to describe the role of SNARE proteins in the neutrophil adhesion response in endothelial cells. Our results demonstrate the following: (i) that syntaxin 4 regulates the exocytosis of WPBs and the subsequent adhesion of neutrophils to HLMVECs induced by thrombin; and (ii) that syntaxin 4 and Munc18c are both the targets of thrombin-induced PKCα activation, which disrupts syntaxin 4-Munc18c interaction and promotes the translocation of P-selectin to the plasma membrane.

MATERIALS AND METHODS

**Antibodies and Reagents**—Rabbit anti-syntaxin 3 antibody was generated against a glutathione S-transferase (GST) fusion protein containing the cytosolic region of syntaxin 3 as described (25). An affinity-purified rabbit anti-syntaxin 4 antibody was purchased from Calbiochem. The isofrom specificity of syntaxin 3 was confirmed using GST-syntaxin fusion proteins (syntaxin 1A, 2, 3, and 4) as described (25). We carried out studies to examine the isofrom specificity of the syntaxin 4 antibody (see supplemental Fig. S1, available in the online version of this article). Anti-PKCα, anti-PKCb2, and anti-Munc18c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-P-selectin antibody was purchased from BD Biosciences. Donkey Alexa-594 secondary antibody was purchased from Life Technologies; and the anti-P-selectin antibody was purchased from BD Biosciences. Rabbit anti-syntaxin 3 antibody was generated against a glutathione S-transferase (GST) fusion protein containing the cytosolic region of syntaxin 3 as described (25). An affinity-purified rabbit anti-syntaxin 4 antibody was purchased from Calbiochem. The isofrom specificity of syntaxin 3 was confirmed using GST-syntaxin fusion proteins (syntaxin 1A, 2, 3, and 4) as described (25).

**Cell Cultures**—Human lung microvascular endothelial cells were purchased from Cambrex (East Rutherford, NJ) and cultured in EGM2-MV medium (Cambrex) supplemented with 10% fetal bovine serum. HLMVEC cultures in a T75 flask pre-coated with 0.2% gelatin were maintained in an incubator at 37 °C in 5% CO2 and 95% air.

**Transfection of Microvascular Endothelial Cells**—Human syntaxin 4-specific small interfering RNA (siRNA) (5′-GAGGAGAGAGCTGTAG- GAGAGC-3′), syntaxin-3-specific siRNA (5′-AACGCAGGACACACT- GAA-3′), and scrambled control siRNA (5′-AAGAGTGACAGAT- GGCAGA-3′) were synthesized using the Silencer siRNA construction kit (Ambion, Austin, TX). Validated PCKα-specific siRNA was obtained directly from Ambion (catalog number 51070). HLMEVCs were plated on 0.2% gelatin-coated plates. At 24 h, cells (∼50% confluence) were transfected with the siRNAs using the GeneEraser siRNA transfection reagent (Stratagene, Kirkland, WA) according to the manufacturer's protocol. For western blotting or surface P-selectin assay 2 days after the transfection procedure was accomplished.

**ELISA for Cell Surface P-selectin Expression**—Cell surface P-selectin expression was assessed by modified ELISA of non-permeabilized cells as described (7). Peroxidase activity was detected using a 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB; Pierce) and quantified by reading the plates on a spectrophotometric microplate reader at 450 nm.

**Immunofluorescence**—Endothelial cells grown on gelatin-coated coverslips were washed 3× in ice-cold PBS and fixed in freshly prepared 3% paraformaldehyde. Cells were then permeabilized in PBS with 0.1% Fries-K-100 for 10 min at room temperature. After permeabilization, cells were incubated with anti-syntaxin antibodies for 2 h, washed 3× in PBS, and incubated with Alexa-594-labeled secondary antibodies. After three washes in PBS, the coverslips were mounted on objective glasses using Prolong Antifade mounting medium (Molecular Probes). HLMVECs were stained with anti-syntaxin antibodies and viewed on a Zeiss LSM 510 confocal microscope with a 63 × 1.2 numerical aperture objective.

**Phosphoprotein Isolation**—HLMVECs cultured on 100-mm dishes were treated with thrombin (Sigma) or the PAR-1 or PAR-4 peptide agonist in the presence or absence of PKC inhibitors or siRNAs in Hepes-buffered saline solution at 37 °C. Cell lysates were prepared at the indicated time points. Phosphorylated and non-phosphorylated proteins from HLMVECs were separated by an affinity chromatography procedure using a phosphoprotein purification kit from Qiagen (Valencia, CA). Using a phosphoprotein purification kit, 7–15% of total proteins are typically phosphorylated in cells grown under basal conditions. Immunodetection by a phospho-specific antibody showed that the kit yields a complete separation of non-phosphorylated (flowthrough) and phosphorylated proteins (elution fraction). In our studies, after equilibrating the column with phosphoprotein lysis buffer, 1.5 ml of cell lysate (from a 100-mm dish) was loaded onto the phosphoprotein purification column and incubated for 15 min at room temperature. The lysate was then allowed to pass through the column. A 1.5-ml flow-through containing non-phosphorylated proteins was collected. After thorough washes with phosphoprotein lysis buffer, the same volume (1.5 ml) of phosphoprotein elution buffer was applied, and the eluted fraction containing phosphorylated proteins was collected. Equal volumes (20 μl) of samples from either the flow-through or eluted fraction were then loaded onto a gel for immunoblotting studies. The results are presented as the percentage of changes of non-phosphorylated and phosphorylated syntaxin 4 or Munc18c. To confirm the results obtained using the kit, we also determined the phosphorylation of Munc18c in endothelial cells labeled with 32P as described (26). These results were consistent with the data obtained using the kit (see supplemental Fig. S2, available in the on-line version of this article).

**Immunoprecipitation and Immunoblotting**—Cells were lysed in a PBS buffer containing 1% Triton X-100 and 1% protease inhibitor mixture (Sigma). Protein concentrations were determined. For immunoprecipitation experiments, cell lysates were incubated with 5 μl of an appropriately suspended (25%; v/v) protein A/G Plus-agarose (Santa Cruz Biotechnology). Cell lysates were then incubated with 2 μg of Munc18c antibody for 1 h at 4 °C. 20 μl of protein A/G Plus-agarose was added later to the cell lysates, which were incubated at 4 °C on a rotating device overnight. Pellets were collected by centrifugation and washed with PBS. Proteins on the beads were eluted by the addition of sample buffer. Eluted samples or cell lysates were analyzed by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Invitrogen). After transfer, membranes were blocked by incubating with 5% (w/v) nonfat dry milk in PBS solution with 0.05% Tween 20 h at room temperature or overnight at 4 °C. Membranes were then incubated with the indicated antibodies and developed using the enhanced chemiluminescence method (Pierce). Densitometry analysis was carried out using the ImageJ program.

**Neutrophil Adhesion Assay**—HLMVECs were seeded and cultured (50,000 cells/well) in gelatin-coated 24-well plates. siRNA transfection was carried out as described above. Cells were used for the polymorphonuclear neutrophil adhesion assay 2 days after siRNA transfection. Human neutrophils from the whole blood of healthy donors were isolated using Polymorph prep (Accurate, Westbury, NY). Neutrophils were first incubated with 5 μM calcein AM (Molecular Probes) for 30 min at 37 °C. After incubation, neutrophils were washed 2× with prewarmed (37 °C) Hepes-buffered saline solution and resuspended in the same medium. Labeled saline solution. Labeled neutrophils were then added to the prepared endothelial monolayers at 2 × 106 cells/well and were incubated at 37 °C for 30 min. Non-adherent calcein-labeled neutrophils were removed by careful washing. Neutrophil fluorescence readings were obtained using a fluorescein filter set. The readings were normalized to control cells without thrombin stimulation.

**Statistics**—Data are presented as means ± S.E. Statistical analysis was carried out by student's t test. Differences were considered significant at p < 0.05.
RESULTS

Cellular Distribution of Syntaxin 3 and Syntaxin 4 in Endothelial Cells—A typical HLMVEC contained hundreds of WPBs in the perinuclear region before thrombin stimulation that disappeared after thrombin challenge. The rapid exocytosis of WPBs observed in HLMVECs after thrombin stimulation is similar to what was found in other types of human endothelial cells; e.g., human umbilical vein endothelial cells and human intestinal microvascular endothelial cells (6, 8). To determine the molecular basis of thrombin-stimulated exocytosis of WPBs in endothelial cells, we examined whether specific isoforms of syntaxin were expressed in HLMVECs. Syntaxins are critical components of SNARE machinery that regulate membrane trafficking (11). Syntaxin 3 and syntaxin 4 have been shown to regulate exocytosis in non-neuronal cells (27–29). Thus, we examined the expression and cellular distributions of syntaxin 3 and syntaxin 4 in HLMVECs by immunofluorescence. Syntaxin 3 staining was shown to be different from syntaxin 4. In HLMVECs, syntaxin 3 exhibited intense stainings at cell-cell contacts, and there was some intracellular punctate staining (Fig. 1, left). We observed both cell surface and intracellular syntaxin 4 staining (Fig. 1, right).

Role of Syntaxin Isoforms in Mechanism of Thrombin-induced Surface P-selectin Expression in Endothelial Cells—To address the functional role of syntaxin 3 and syntaxin 4 in the regulated exocytosis of WPBs after thrombin stimulation, we used siRNAs against syntaxin 3 and syntaxin 4 to knock down their expression. The effects of siRNAs on the exocytosis of WPBs were examined by P-selectin surface expression in HLMVECs. Using syntaxin 3-specific siRNA, we reduced the expression of syntaxin 3 in HLMVECs without affecting the expression of syntaxin 4 (Fig. 2A). Syntaxin 4 siRNA transfection specifically decreased syntaxin 4 expression (Fig. 2B) and had no effect on syntaxin 3 or P-selectin expression. Syntaxin 4 siRNA caused a significant reduction in the thrombin-induced P-selectin surface expression over the 60-min time course compared with that in control cells, whereas syntaxin 3 siRNA had no significant effect (Fig. 2C). As syntaxin 4 expression was not completely inhibited by the siRNA (Fig. 2B), it is possible that this residual syntaxin 4 expression may have been sufficient to support some degree of the WPB exocytosis seen in Fig. 2C.

Thrombin Stimulation Induces Phosphorylation of Syntaxin 4 and Munc18c in Endothelial Cells—We next addressed the possibility that the phosphorylation of SNARE proteins was involved in the mechanism of WPB exocytosis in endothelial cells. Thus, we examined the phosphorylation of syntaxin 4 and its regulatory protein, Munc18c, after thrombin challenge. Thrombin induced the marked phosphorylation of both syntaxin 4 and Munc18c (Fig. 3). The non-phosphorylated syntaxin 4 and Munc18c were significantly reduced, whereas phosphorylated syntaxin 4 and Munc18c were markedly increased within 5 min after thrombin exposure (Fig. 3, A and B). The phosphorylated syntaxin 4 and Munc18c remained elevated during the 30-min thrombin exposure period.

PAR-1 Signaling Induces Phosphorylation of Syntaxin 4 and Munc18c—Because endothelial cells express two thrombin receptors, PAR-1 and PAR-4 (30–32), we determined the contributions of these receptor in signaling syntaxin 4 and Munc18c phosphorylation. HLMVECs were stimulated with a selective PAR-1 or PAR-4 peptide agonist (described under “Materials and Methods”). The PAR-1 peptide had a similar effect as that of thrombin on the phosphorylation of syntaxin 4 and Munc18c (Fig. 4A). Moreover, only the PAR-1 peptide was capable of stimulating the P-selectin surface (Fig. 4B).

PKC-dependent Phosphorylation of Syntaxin 4 and Munc18c—Because PKC may regulate SNARE protein function (16, 22, 29), we examined the possible role of PKC in inducing the phosphorylation of syntaxin 4 and Munc18c in endothelial cells. G06976 (an antagonist of calcium-dependent

FIG. 1. Expression of syntaxin 3 and syntaxin 4 in HLMVECs. Immunofluorescence images of HLMVECs incubated with specific anti-syntaxin 3 (left) or anti-syntaxin 4 antibodies (right). Cells were stained with an Alexa-594-conjugated secondary antibody. Results are representative of three independent experiments.

FIG. 2. siRNA-induced knockdown of syntaxin 4 expression inhibits thrombin-induced P-selectin expression on the endothelial cell surface. A, HLMVECs were transfected with scrambled control (Sc) or syntaxin 3-specific siRNAs (S3 siRNA). Cell lysates collected 2 days after transfection were resolved by SDS-PAGE and immunoblotted with anti-syntaxin 3 or anti-syntaxin 4 antibodies. B, HLMVECs were transfected with scrambled control or syntaxin 4-specific siRNAs (S4 siRNA) at the concentrations indicated. Cell lysates collected 2 days after transfection were separated on a SDS-PAGE and immunoblotted with syntaxin 4, syntaxin 3, or P-selectin antibodies. The blots are representative of three separate experiments. C, cell surface P-selectin expression was quantified by modified ELISA. P-selectin surface expression induced by thrombin (1 unit/ml) was determined as a function of the time (0–60 min). HLMVECs were transfected with 10 nM siRNAs of scrambled control (Con), syntaxin 3 (Syn 3), and syntaxin 4 (Syn 4). Thrombin-induced P-selectin cell surface expression was assessed 2 days after transfection. Data are presented as mean ± S.E. (n ≥ 3). Asterisk indicates a value significantly different from that of cells transfected with scrambled control and challenged with thrombin; p < 0.05.
PKC isoforms), the PKC\(_i\) inhibitory peptide, and rottlerin (an inhibitor of PKC\(b\)) were used. We observed that only G0\(\delta\)976 significantly interfered with thrombin-induced phosphorylation of both syntaxin 4 and Munc18c (Fig. 5A).

**PKC-dependent Phosphorylation Disrupts Syntaxin 4 and Munc18c Interaction**—The binding of syntaxin to Munc18, a high affinity syntaxin-binding protein, may limit the availability of syntaxin to form the SNARE complex (11). Three munc18 isoforms have been identified; munc18a and 18b interact with syntaxins 1A, 2, and 3, but not with 4 (33), and munc18c is a high affinity syntaxin 4-binding protein (34). We carried out co-immunoprecipitation studies to examine whether PKC-dependent phosphorylation of syntaxin 4 and Munc18c affected their interaction. Syntaxin 4 and Munc18c co-immunoprecipitated in resting endothelial cells, whereas thrombin stimulation resulted in the disassociation of syntaxin 4 and Munc18c (Fig. 5B). G0\(\delta\)976, which significantly blocked phosphorylation of both syntaxin 4 and Munc18c, prevented the thrombin-induced syntaxin 4/Munc18c disassociation (Fig. 5B). In contrast, the PKC\(_i\) inhibitory peptide and rottlerin had no significant effect (Fig. 5B). The inhibitory effects of G0\(\delta\)976 on phosphorylation and the syntaxin 4/Munc18c interaction were coupled to the inhibition on thrombin-induced P-selectin surface expression (Fig. 5C).

**Phosphorylation of Syntaxin 4 and Munc18c in Endothelial Cells Requires PKC\(a\) Activation**—The inhibitory effects of G0\(\delta\)976 shown above suggest that conventional calcium-dependent PKC isoforms are involved in the phosphorylation of syntaxin 4 and Munc18c. The conventional PKC\(a\) isoform, highly expressed in endothelial cells, regulates endothelial cell barrier function (26, 35). To address whether PKC\(a\) also mediates thrombin-induced phosphorylation of syntaxin 4 and Munc18c, HLMVECs were transfected with a siRNA to knockdown PKC\(a\) expression in HLMVECs. Transfection of a scrambled control siRNA had no effect on PKC\(a\) expression, whereas the PKC\(a\) siRNA reduced PKC\(a\) expression without affecting the expression of PKC\(b\)2 or P-selectin (Fig. 6A). PKC\(a\) siRNA inhibited the thrombin-induced syntaxin 4 and Munc18c phosphorylation, whereas scrambled control siRNA had no effect (Fig. 6B). Knockdown of PKC\(a\) expression also prevented thrombin-induced P-selectin cell surface expression (Fig. 6C).

**Elevated Intracellular Calcium Concentration Is Necessary for Thrombin-induced Phosphorylation of Syntaxin 4 and Munc18c**—Because PKC\(a\) activation is calcium-dependent, intracellular calcium chelation with membrane permeable BAPTA-AM prevents PKC\(a\) translocation and activation (36–39). Thus, we examined the role of intracellular calcium in signaling the thrombin-induced phosphorylation of syntaxin 4 and Munc18c. BAPTA-AM treatment interfered with thrombin-induced phosphorylation of syntaxin 4 and Munc18c (Fig. 7A) and with cell surface P-selectin expression (Fig. 7B).

**Syntaxin 4 Regulates Neutrophil Adhesion to Endothelial Cells**—We next addressed whether the regulation of P-selectin surface expression by syntaxin 4 is a mechanism for controlling neutrophil adhesion to endothelial cells. Thus, we examined the role of syntaxin 4 in thrombin-stimulated neutrophil adhesion to HLMVECs using the isoform-specific syntaxin siRNAs...
Fig. 5. PKC-dependent phosphorylation of syntaxin 4 and Munc18c disrupts their interaction and increases endothelial cell surface P-selectin expression. HLMVECs were pre-treated with or without (Con) the PKC inhibitors G66976 (G) (200 nM), inhibitory PKCζ peptide (Z) (20 μM), or rottlerin (R) (5 μM) for 30 min, and cells were challenged with thrombin (Th) (1 unit/ml); treatment times were 5 min for phosphorylation and immunoprecipitation studies and 15 min for surface P-selectin assays. Non-phosphorylated (N) and phosphorylated (P) proteins were separated and detected as described under “Materials and Methods.” A, representative blots and bar graphs show percentage changes of non-phosphorylated and phosphorylated syntaxin 4 and Munc18c. B, cell lysates were immunoprecipitated (IP) with anti-Munc18c antibody and blotted with anti-syntaxin 4 antibody. The same membrane was stripped and re-probed with anti-syntaxin 4 or Munc18c antibody. Results shown here are representative of three independent experiments. C, cell surface P-selectin expression was assessed by modified ELISA. Data are presented as mean ± S.E. (n ≥ 3). Asterisk indicates a value significantly different from that of cells challenged with thrombin alone; p < 0.05.

Described above we observed that syntaxin 4 siRNA, but not syntaxin 3 siRNA, significantly inhibited neutrophil adhesion to thrombin-activated HLMVECs (Fig. 8).

Discussion

Studies were carried out to address constituents of the SNARE machinery regulating the exocytosis of WPBs in endothelial cells by thrombin. We demonstrated that SNARE proteins expressed in endothelial cells are early targets of thrombin-activated signaling and thereby play pivotal role in WPB exocytosis. It is known that the activation of G protein-coupled PAR-1 in endothelial cells by thrombin results in PKCα activation (26, 35). On the basis of our findings, we propose that activation of PKCα is important in inducing the phosphorylation of syntaxin 4 and Munc18c and thereby facilitating the formation of the SNARE complex that mediates the fusion of WPBs with the apical endothelial membrane. The resultant increased surface expression of P-selectin and the secretion of von Willebrand factor and interleukin-8 may contribute to the host defense response to inflammatory stimuli and infection.

In the present study, we have focused on syntaxin 4, a constituent of the target SNARE apparatus. We showed that syntaxin 4 plays an essential role in the activation of WPB exocytosis induced by thrombin stimulation of endothelial cells. We observed that a siRNA-induced reduction in syntaxin 4 expression prevented P-selectin surface expression, whereas the inhibition of syntaxin 3 isoform (another syntaxin isoform expressed in endothelial cells) had no significant effect. We set out to address the response to thrombin, because it is a key mediator of vascular inflammation (40) that has been shown to induce the rapid translocation of P-selectin to the plasma membrane of endothelial cells (7, 41). Thrombin activates its receptor PAR-1, a member of a G protein-coupled protease-activated receptor family (31), on endothelial cells. Studies using peptide agonists specific for PAR-1 and PAR-1 knock-out mice showed that thrombin induced vascular endothelial activation through the PAR-1 isoform (30, 32). A principal signal transduction pathway following thrombin cleavage of PAR-1 is the activation of phospholipase C through Gq followed by inositol 1,4,5-trisphosphate and diacylglycerol production, resulting in a rise in intracellular calcium and the activation of PKCα (31). We found that the phosphorylation of syntaxin 4 and Munc18c was increased after stimulation with the PAR-1 agonist peptide, indicating that SNARE proteins are important targets of PAR-1 signaling in the endothelium.

To address the basis of syntaxin 4 and Munc18c phosphorylation, we initially used PKC inhibitors, because multiple PKC isoforms, PKCα, δ, and ζ, are involved in signaling endothelial inflammatory responses (42–44). We also targeted PKCα specifically by using siRNA to address its role in SNARE protein phosphorylation. Our finding that thrombin-induced phosphorylation of syntaxin 4 and Munc18c was significantly reduced by the inhibitor of calcium-dependent conventional PKC isoforms, G66976, and by PKCα siRNA suggests that PKCα is crucial in regulating SNARE protein phosphorylation. We observed that the inhibition of PKCα-induced phosphorylation of syntaxin 4 and Munc18c prevented their dissociation and that this was coupled to decreased WPB exocytosis in endothelial cells. Thus, our data are consistent with a model in which PKCα regulates exocytosis in endothelial cells by modifying the functions of syntaxin 4 and Munc18c through their phosphorylation. These data point to the important role of PKCα-dependent phosphorylation in disrupting the syntaxin 4 and Munc18c interaction, thus facilitating SNARE complex formation and stimulating WPB exocytosis.
Transfected with scrambled control siRNA and challenged with thrombin (Th) 2 days later (5 min for phosphorylation studies or 15 min for surface P-selectin assays). A, cell lysates were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and immunoblotted with anti-PKCα antibodies. The same membrane was stripped and blotted with anti-PKCβ2 antibody. B, non-phosphorylated (N) and phosphorylated (P) proteins were separated and detected as described under “Materials and Methods.” Representative blots and bar graphs show the percentage changes of non-phosphorylated and phosphorylated syntaxin 4 or Munc18c over their total detection. Results shown are representative of three independent experiments. C, cell surface P-selectin expression. Data are presented as mean ± S.E. (n = 3). Asterisk indicates a value significantly different from that of cells transfected with scrambled control siRNA and challenged with thrombin (Th); p < 0.05.

It is well established that nsec-1 (a homolog of Munc18c) binds to syntaxin 1A with high affinity in biochemical studies (19, 45); however, in vivo evidence of their association is controversial. Some studies failed to show their co-localization (46), but other studies have demonstrated their association in plasma membrane and granule membrane by chemical cross-linking (19, 47). Even though our data support the interaction of Munc18c with syntaxin 4 in endothelial cells and show that their interaction can be regulated by PKCα-dependent phosphorylation, we cannot exclude the possibility that Munc18c may have other functions that contribute to vesicle docking (23, 24).

The elevation of intracellular calcium concentration has diverse regulatory functions in membrane trafficking, including the translocation and fusion of intracellular vesicles to the plasma membrane (15). A localized rise of intracellular calcium may allow tight control of vesicle trafficking in a temporal and spatial manner. We observed that chelating intracellular calcium with BAPTA-AM in endothelial cells inhibited the PAR1-activated phosphorylation of syntaxin 4 and Munc18c and blocked the exocytosis of WPBs, as was the case with the inhibition of PKCα. The role of intracellular calcium in regulating WPB exocytosis may be the result of PKCα activation (36, 39); that is, calcium may regulate exocytosis of WPBs in endothelial cells secondary to calcium-dependent PKCα activation and the subsequent phosphorylation of syntaxin 4 and Munc18c.

Both intracellular calcium rise and PKCα activation are
early signals regulating the vascular inflammatory response (43, 48) and have a variety of downstream targets, including the activation of transcription factors (42, 44). However, syntaxin 4 and Munc18c, on the basis of inducing the release of WPB constituents such as P-selectin, may function to increase endothelial adhesiveness to leukocytes. We observed that the reduction in syntaxin 4 expression inhibited neutrophil adhesion to thrombin-activated endothelial cells, suggesting that inhibition of syntaxin 4-regulated P-selectin exocytosis may prevent the early component of the inflammatory response. Thus, the present study provides insights into the core molecular apparatus of syntaxin 4 and Munc18c and the essential role of PKCα-dependent phosphorylation of these proteins in signaling P-selectin translocation to the endothelial plasma membrane.

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