Thrombin Receptors Activate $G_o$ Proteins in Endothelial Cells to Regulate Intracellular Calcium and Cell Shape Changes*

Jurgen F. Vanhauwe‡§, Tarita O. Thomas‡§, Richard D. Minshall¶, Chinnaswamy Tiruppathi†, Anli Li‡, Annette Gilchrist‡, Eun-ja Yoon*, Asrar B. Malik‡, and Heidi E. Hamm‡§**

From the ‡Institute for Neuroscience and Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, Illinois 60611, the ‡Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232, and the ¶Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois 60612

Thrombin receptors couple to $G_{i/o}$, $G_{o}$, and $G_{12/13}$ proteins to regulate a variety of signal transduction pathways that underlie the physiological role of endothelial cells in wound healing or inflammation. Whereas the involvement of $G_{i}$, $G_{o}$, $G_{12}$, or $G_{13}$ proteins in thrombin signaling has been investigated extensively, the role of $G_{o}$ proteins has largely been ignored. To determine whether $G_{o}$ proteins could contribute to thrombin-mediated signaling in endothelial cells, we have developed minigenes that encode an 11-amino acid C-terminal peptide of $G_{o}$ proteins. Previously, we have shown that use of the C-terminal minigenes can specifically block receptor activation of G protein families (1). In this study, we demonstrate that $G_{o}$ proteins are present in human microvascular endothelial cells (HMECs). Moreover, we show that thrombin receptors can stimulate $[\text{GTP}]_{i}$-guanosine-5′-O-(3-thio)triphosphate binding to $G_{o}$ proteins when co-expressed in SF9 membranes. The potential coupling of thrombin receptors to $G_{o}$ proteins was substantiated by transfection of the $G_{o}$ minigene into HMECs, which led to a blockade of thrombin-stimulated release of $[\text{Ca}^{2+}]_{i}$ from intracellular stores. Transfection of the $\beta$-adrenergic kinase C terminus blocked the $[\text{Ca}^{2+}]_{i}$ response to the same extent as with $G_{o1}$ minigene peptide, suggesting that this $G_{o}$-mediated $[\text{Ca}^{2+}]_{i}$ transient was caused by $G_{o}$ stimulation of PLC$\beta$. Transfection of a $G_{12/13}$ minigene had no effect on thrombin-stimulated $[\text{Ca}^{2+}]_{i}$ signaling in HMEC, suggesting that $G_{o}$ derived from $G_{o}$ but not $G_{i}$ could activate PLC$\beta$. The involvement of $G_{o}$ proteins on events downstream from calcium signaling was further evidenced by investigating the effect of $G_{o1}$ minigenes on thrombin-stimulated stress fiber formation and endothelial barrier permeability. Both of these effects were sensitive to pertussis toxin treatment and could be blocked by transfection of $G_{o1}$ minigenes but not $G_{12/13}$ minigenes. We conclude that the $G_{o}$ proteins play a role in thrombin signaling distinct from $G_{12/13}$ proteins, which are mediated through their $G_{o}$ subunits and involve coupling to calcium signaling and cytoskeletal rearrangements.

Thrombin is a multifunctional serine protease that catalyzes conversion of fibrinogen to fibrin, a process that is crucial in blood coagulation (1). In addition, thrombin plays a central role in a variety of biological functions such as platelet aggregation, mitogenesis of fibroblasts, monocytic cell chemotaxis, and endothelial cell monolayer permeability (2–4). Many of its actions, including the regulation of biochemical, transcriptional, and functional responses in endothelial cells occur through activation of protease-activated receptors (PARs), which belong to the superfamily of $G$ protein-coupled receptors.

Four PARs have been cloned so far, but only PAR1, PAR3, and PAR4 can be activated by thrombin (5). The activation and signal transduction pathways of PAR1, the prototype of the PAR family, have been studied in great detail. Thrombin cleaves the N-terminal extracellular domain of PAR1 at a specific site, which unmask a new N terminus that then serves as a tethered agonist ligand and activates the receptor by binding intramolecularly to the body of the receptor (6). Cleaved, i.e. irreversibly activated, PAR1 can couple to members of the $G_{i/o}$, $G_{o}$, and $G_{12/13}$ protein families and regulate a variety of intracellular effectors (1).

Although the role of $G_{o}$ proteins has been generally believed to be confined to the brain and heart, several reports indicate that $G_{o}$ proteins may serve to regulate various intracellular pathways in non-neuronal cell lines (7, 8). In addition, several new effectors have been identified that are specifically or differentially regulated by $G_{o}$ proteins (versus $G_{i}$ proteins) (9–11). Pertussis toxin (PTX)-mediated ADP-riboseylation of the $C_{y2}^{351}$ residue in the C terminus of $G_{i}$ and $G_{o}$ proteins disables their interaction with receptors and thus prevents receptor-mediated activation of these G proteins. Many effects of thrombin are mediated through pertussis toxin-sensitive $G$ proteins (12–18). This method, however, does not distinguish between $G_{i}$ and $G_{o}$ proteins, and the importance of the latter subtype could be inadequately appreciated (5).

To dissect out the contribution of $G_{12/13}$ and $G_{o1}$ proteins in thrombin-regulated signaling pathways in HMECs, we have designed a dominant negative strategy based on minigene vectors that encode the C-terminal 11-amino acid sequence from Ga. Previously, we have shown that these minigenes are quite specific in such a way that $G_{o}$-based minigenes blocked only thrombin activation of $G_{o}$ protein-mediated pathways (phos-

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‡ These authors contributed equally to this work.

** To whom correspondence should be addressed: Dept. of Pharmacology, Vanderbilt University Medical Center, 442 Robinson Research Bldg., Nashville, TN 37232. E-mail: heidi.hamm@vanderbilt.edu.

1 The abbreviations used are: PAR, protease-activated receptor; GTP$\gamma$S, guanosine-5′-O-(3-thio)triphosphate; $\beta$ARK-ct, $\beta$-adrenergic kinase C terminus; CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GFF, green fluorescent protein; HBSS, Hank’s buffered salt solution; HMEC, human microvascular endothelial cell; NECA, N-ethyl-5′-carbamoyladenosine; PTX, pertussis toxin; SF9, Spodoptera frugiperda 9; TRAP, Thrombin Receptor Activating Peptide.

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phosphatidylinositol bisphosphate hydrolysis and intracellular calcium increase) but not G11a or G12/13 protein-mediated pathways (1, 19). The specificity of Go C-terminal peptides has been shown dramatically by Gilchrist et al. (20), where one or two amino acid substitutions inhibited the ability of peptides to block receptor-mediated activation of signaling pathways.

To delineate specific functions for Gt and Gc proteins in the signaling of thrombin receptors, we have introduced these minigenes into HMECs. Our findings indicate that HMECs contain Gt proteins and that PAR1 has the potential to couple to Gt and Gc proteins when co-expressed in Sf9 cell membranes. In addition, we show that Gt minigenes block thrombin-stimulated release of [Ca2+]i, whereas G11a minigenes do not. The involvement of Gt proteins, but not Gc proteins, was further established in pathways that are known to be downstream of calcium signaling, such as stress fiber formation and endothelial barrier permeability. Together our data demonstrate the importance of Gt proteins in the signaling of thrombin receptors in endothelial cells.

MATERIALS AND METHODS

Reagents—All of the cell culture reagents were purchased from Invitrogen; pEGFP was from CLONTECH, retroviral Tet-inducible vectors pRevTRE2 and pRevTRE2-dEGFP were from CLONTECH (Palo Alto, CA). All of the restriction enzymes were procured from New England Biolabs (Beverly, MA). The highly purified α-thrombin (~2000 units/ng) and PTX were obtained from Calbiochem. Alexa Fluor 568 phalloidin, DAPI, Oregon Green Bapta-1 acetoxymercuril, Pluronic F127, and the Prolong Antifade kit were purchased from Molecular Probes (Eugene, OR). Anti-G11a antibodies were from Dr. D. Manning (University of Pennsylvania, Philadelphia, PA), 3-Isobutyl-1-methylxanthine, forskolin, and isoproterenol were from Sigma. Electrodes for endothelial monolayer resistance measurements were obtained from Applied Biosciences (Troy, NY). Virions producing the rat Goαi1, Goα13, and Gai1 were obtained from Dr. S. Graber (West Virginia University, Morgantown, WV), whereas those for PAR1 were obtained from Dr. C. Chinni (University of Cambridge, Cambridge, UK). [35S]GTPγS was from PerkinElmer Life Sciences.

Preparation of Sf9 Membranes—Sf9 cells were grown at 27 °C and at an ambient atmosphere in suspension in a shaking incubator and transfected as described before (21). Harvested Sf9 cells were washed with ice-cold 50 mM Tris-HCl buffer, pH 7.4, resuspended in hypotonic 10 mM Tris-HCl buffer, pH 7.4, and homogenized with 10 strokes of a Bio-Homogenizer (BioSpec Products, Inc.) at high speed. The homogenate was centrifuged at 30,000 × g for 30 min at room temperature in a volume of 125 μl with ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 5 mM MgCl2, and 1 mM EDTA. The DNA was brought up in sterile double distilled H2O (stock concentration, 100 μM). Complementary DNA was annealed in 1× NE Buffer (3) (50 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, 1 mM dithiotreitol; New England Biolabs) at 85 °C for 10 min and then allowed to cool slowly to room temperature. The annealed cDNA were ligated for 1 h at room temperature into a Tet-inducible retroviral vector pRevTRE2. As a control, we used a retroviral minigene virus (pRevTRE2-dEGFP; CLONTECH). Retroviral minigenes were generated by infecting the packaging cells GP-293 with pRevTRE2 minigenes and vesicular stomatitis virus glycoprotein using Effectene (Qiagen). For our studies we used a human dermal microvascular endothelial cell line that was transformed using SV-40 (HMEC-1; obtained from Dr. E. Ades (Centers for Disease Control, Atlanta, GA). The cells were maintained in MCDB 131 medium supplemented with 5% fetal bovine serum, penicillin/streptomycin (5000 units/ml, 5000 μg/ml), hydrocortisone, growth factor (0.01 μg/ml), and L-glutamine (2 mM) in an atmosphere of 95% air, 5% CO2 at 37 °C. The cells were seeded at 1 × 105 cells/ml and subcultured after detachment with 0.05% trypsin/0.5 mM EDTA. All of the studies utilized cell passages 18–26.

Plasmid Constructs—cDNA minigene constructs were designed as described previously (19). The C terminus of the G protein-coupled receptor kinase 2 (βARK-ct) has been shown to be a potent and specific Gβγ inhibitor (23). The βARK-ct construct codes for residues 548–671 of the rat homolog βARK.

Retroviral minigenes were constructed as follows. The cDNA encoding the last 11 amino acids of human Go subunits (Goα13 and Goαi1) or rat Gαi1 C terminus (36 amino acids) was isolated from pRevTRE2 (Great American Gene Company) with newly engineered 5′- and 3′-ends. The 5′-end contained a BamHI site followed by the ribosome-binding consensus sequence [5′-GCGGCAACCC-3′], a methionine (ATG) for translation initiation, and a glycine (GGA) to protect the ribosome-binding site during translation and the nascent peptide against proteolytic degradation. A HindIII site was synthesized at the 3′-end immediately following the translation stop codon (TGA). The DNA was brought up in sterile double distilled H2O (stock concentration, 100 μM). Complementary DNA was annealed in 1× NE Buffer (3) (50 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, 1 mM dithiotreitol; New England Biolabs) at 85 °C for 10 min and then allowed to cool slowly to room temperature. The annealed cDNA were ligated for 1 h at room temperature into a Tet-inducible retroviral vector pRevTRE2. As a control, we used a retroviral minigene virus (pRevTRE2-dEGFP; CLONTECH). Retroviral minigenes were generated by infecting the packaging cells GP-293 with pRevTRE2 minigenes and vesicular stomatitis virus glycoprotein using Effectene (Qiagen). For optimal results, the retroviral vectors were transfected using the pAnon GP-293 cell line (CLONTECH) with vesicular stomatitis virus glycoprotein, an envelope glycoprotein from the vesicular stomatitis virus. As a control, we used the enhanced GFP inserted into the parental vector (pRevTRE2-dEGFP, CLONTECH). Retroviral minigenes were generated by infecting the packaging cells GP-293 with pRevTRE2 minigenes and retroviral minigene virus glycoprotein using Effectene (Qiagen). Use of retroviral minigenes led to 100% transfection efficiency. This was confirmed using infection of HMECs with BamHI and HindIII. Following ligation, the samples were heated to 65 °C for 5 min to deactivate the T4 DNA ligase. The ligation reaction (1 μl) was electroverted into 50 μl of competent AR1814 cells (Bio-Rad Escherichia coli Pulsar), and the cells were immediately placed into 1 ml of superoptimal catabolite medium (Invitrogen). After 1 h at 37 °C, 100 μl was spread on LB/ampicillin plates and incubated at 37 °C for 16 h. Transformants were selected on 1× LB medium with 100 μg/ml sodium acetaminophen, and the transformants were grown overnight in LB/ampicillin, and their plasmid DNA was purified (Qiagen SpinKit). The plasmid DNA was digested with NcoI (New England Biolabs, Inc.) for 1 h at 37 °C and run on a 1.5% (3:1) agarose gel. Vector alone produced one band (6.5 kb), whereas vector with insert resulted in two bands (5.1 and 1.4 kb). DNA with the correct pattern was sequenced (Northwestern University Biotechnology Center) to confirm the appropriate sequence.

For optimal results, the retroviral vectors were transfected using the pAnon GP-293 cell line (CLONTECH) with vesicular stomatitis virus glycoprotein using Effectene (Qiagen). Use of retroviral minigenes led to 100% transfection efficiency. This was confirmed using infection of the pRevTRE2-dEGFP vector, which exhibited expression of GFP in virtually every cell (data not shown).

Western Blot Analysis—Endothelial cell lysates were resolved by SDS-PAGE on a 10–20% separating gel under reducing conditions. The blot was probed with an ABT-101–coupled anti-human βARK and horseradish peroxidase conjugated secondary antibodies and detected using chemiluminescent substrate.
chemiluminescent detection system (LumiGLO, Kirkegaard and Perry Laboratories, Gaithersburg, MD). cAMP Assay—HMECs were seeded onto 6-well plates at 1 × 10^5 cells/well 24 h before transfection. The cells were transfected with retroviral minigene constructs, and 24 h before the assay, the cells were seeded onto 24-well plate. Thereafter, the cells were washed once with serum-free medium containing 1 mM 3-isobutyryl-1-methylxanthine, a phosphodiesterase inhibitor, and further incubated for 20 min in 500 μl of serum-free medium containing 1 mM 3-isobutyryl-1-methylxanthine. After the preincubation, 50 μl of preincubation medium supplemented with forskolin (final concentration, 10 μM) was added to each well. To detect the inhibitory effect, 100 nM thrombin or 10 μM NBCC was added along with forskolin. Basal cAMP accumulation was measured in the absence of forskolin and compounds. The reactions were terminated by the addition of 100 μl of 1 N HClO4. The samples were frozen and thawed, and 200 μl of KOH/K2PO4 (0.5 M, pH 13.5) was added to neutralize the samples (final pH 7.4). After formation of the KClO4 precipitate (30 min at 4 °C), the plates were centrifuged (10 min at 650 × g, 4 °C). The amount of cAMP in each well was determined with a commercial [3H]-labeled cAMP radioimmunoassay kit (Biomedical Technologies Inc., Stoughton, MA).

([Ca2+]i) Response—In single cell fluorescence measurements, the DaRED (CLONTECH) fluorescence reporter gene was used to confirm the transfected cells. HMECs were transfected with pcDNA-Giα1, pcDNA-Giα2, pcDNA-Giα3, or pDNA-Giα4 minigene DNA and with or without pARK-expressing DNA along with DaRED. After 48 h, the cells were transferred to coverslips at a low confluency in a 24-well plate and allowed to adhere for at least 2 h. The medium was aspirated, and each coverslip was incubated at 37 °C for 30 min in 0.5 ml of loading buffer (20 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 0.83 mM Na2HPO4, 0.17 mM NaH2PO4, 1 mg/ml bovine serum albumin, 25 mM mannone) containing 0.1% (v/v) Phorunuc P127 and 10 μM Oregon Green Bapta-1 acetoxymethyl ester. The cells were washed twice with and incubated in Ca2+ buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.5 mM CaCl2, 0.55 mM MgCl2). The coverslips were placed in the chamber that was mounted on the stage of an upright microscope. The experiment was performed at room temperature. The transfected cells were identified using a green filter by observing DaRED fluorescence. The basal conditions were established for 40 s before addition of thrombin (~70 μM). Recordings (exposure time) were made every 10 s and continued for 170 s after stimulation with thrombin. The images were quantified using the NIH Image Program.

Immunofluorescent Microscopy—As a marker for transfected cells, the pEGFP plasmid containing the gene for enhanced green fluorescent protein was transiently co-transfected together with minigene constructs as described above. HMECs were grown on gelatin-coated coverslips, serum-starved for 24 h, washed with HBSS, and fixed with 4% paraformaldehyde. The coverslips were washed three times for 5 min in 100 mM glycine in HBSS to quench and remove the fixative between receptors and all members of the Gi subfamily (except Gαo) through ADP-ribosylation of the Cys351 residue in the Cα subunit. The effect of PTX has often been shown to be sensitive to PTX, e.g., intracellular calcium release, activation of Na+/H+ exchanger, arachidonic acid release, and endothelial relaxation (12–18). PTX abolishes the interaction between receptors and all members of the Gα subfamily (except Gαo) through ADP-ribosylation of the Cys351 residue in the Cα terminus of the Gα subunit. The effect of PTX has often been attributed to Gαi proteins, because Gαi proteins are generally believed to play a role in the brain, where it constitutes about 1% of the total protein content (26).

To determine whether PAR1, the most characterized thrombin receptor, can potentially couple to Gαi proteins, we expressed this receptor in SF9 cells along with Gαi1, Gβ1, and Gγ2, and measured TRAP-stimulated [35S]GTPγS binding to membranes prepared from these cells. As a negative control, we co-expressed PAR1, Gβ1, and Gγ2 (but not Gαi) in SF9 cells. Fig. 1 shows that TRAP-stimulated [35S]GTPγS binding up to 700% above the basal level in a concentration-dependent manner in SF9 membranes co-expressing PAR1 and Gαi1β1γ2 proteins. In SF9 membranes co-expressing only PAR1 and Gβ1γ2, TRAP-stimulated [35S]GTPγS binding to a significantly lower level (230% ± 10% above basal level, p < 0.05). In addition, we found that TRAP could stimulate [35S]GTPγS binding to membranes prepared from SF9 cells co-expressing PAR1 and Gαa1β1γ2, Gαa2β1γ2, or Gαaβ1γ2 heterotrimers. The level of stimulation in the latter membranes was apparently lower than in membranes co-expressing the Gαaβ1γ2 heterotrimer. Because we did not determine the level of PAR1 expression (because of the lack of commercially available radioligands), we...
could not conclude whether the lower stimulation level by Go,β1γ2 reflected a lower coupling efficiency or a lower expression of receptor or G protein.

Presence of Go Proteins in HMECs—Because we demonstrated coupling of PAR1 to Go, proteins when co-expressed in SF9 cells, we next investigated the presence of Go, proteins in HMECs. HMEC lysates were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The presence of Go, proteins was detected with an antibody that recognizes both Go,1 and Go,2 proteins, but not Go,i proteins (8). Fig. 2 shows that HMECs contain Go,i proteins. This unexpected finding led us to further investigate the coupling of Go,i proteins to thrombin receptors in HMECs.

Inhibition of Adenyl Cyclase—To determine whether Go, or G, proteins are involved in thrombin-mediated inhibition of adenyl cyclase, HMECs were infected with pRevTRE2-G,1/2 or pRevTRE2-Go, minigenes. We could not demonstrate thrombin-mediated inhibition of adenyl cyclase after either forskolin or isoproterenol stimulation of HMEC. This might be attributable to a different source of HMECs than used previously (27). We tested several different conditions including preincubation with thrombin before challenging the cells with forskolin and thrombin: leaving out thrombin in either the preincubation or incubation step (to eliminate receptor desensitization), elimination of 3-isobutyl-1-methylxanthine (to reduce protein kinase A effects), different concentrations of forskolin, or variation of the incubation or preincubation times. Under none of these experimental conditions did we demonstrate inhibition of forskolin- or isoproterenol-stimulated cAMP formation by thrombin (data not shown). Indeed, thrombin-mediated inhibition of adenyl cyclase has only been reported in specific endothelial cell lines (28–31).

To verify whether our experimental conditions were sufficient to measure inhibition of adenyl cyclase activity, we measured it in a CHO cell line that stably expressed A3 adenosine receptors, which couple preferentially to G, proteins. Our results show that stimulation of A3 adenosine receptors inhibited adenyl cyclase in CHO cells (Fig. 3). When these CHO cells were infected with retroviral minigene virus and treated with PTX, the cAMP levels were set at 100% and are represented by a dashed line (Fig. 3A). As expected, the addition of the Go,i minigene virus (Fig. 3B) and forskolin-stimulated cAMP formation was blocked almost completely (Fig. 3A). The inhibitory action of A3 adenosine receptors on cAMP formation was blocked almost completely (Fig. 3A). The infecton of a control virus (pRevTRE2-G,iR) (paired Student’s t test; p < 0.05). B shows the cAMP levels of cells treated with NECA and thrombin as a percentage of the forskolin level. forskolin levels were set at 100% and are represented by a dashed line. The asterisks indicate that the cAMP levels were significantly higher than in cells infected with control virus (pRevTRE2-G,iR) (paired Student’s t test; p < 0.05).

Stimulation of Intracellular Calcium Release—Previously, we have shown that thrombin-mediated stimulation of [Ca2+]i was blocked in HMECs transfected with pcDNA-G,i, but not pcDNA-G,1/2 minigene vectors, which eliminated a role for G,i proteins in this signaling event of thrombin receptors (1). In this study, we loaded HMECs transfected with pcDNA-G,1/2, pcDNA-G,i, or pcDNA-G,iR minigene vectors with Oregon Green Bapta-1 for 30 min and measured the thrombin-induced [Ca2+]i increase.-green Bapta-1, in single cells. Fig 4 shows that HMECs transfected with pcDNA-G,i minigene vector showed a marked reduction in their calcium response to thrombin, but HMECs transfected with pcDNA-G,1/2 or pcDNA-G,iR minigene vectors were unaffected. This means that G,i, proteins, but not G,i, proteins, play a role in the thrombin-stimulated [Ca2+]i increase.

To further delineate the mechanism whereby G,i proteins participate in the thrombin-mediated calcium signaling pathways, we used a scavenger of Gβγ, the β-ar-GPCR kinase C-terminal vector (23), which would allow us to differentiate the role of the Go versus Gβγ subunits of G,i. We co-transfected βARK ct DNA along with minigene DNA and measured [Ca2+]i. (Fig. 5). βARK ct expression in G,i minigene transfected cells led to the same amount of inhibition of [Ca2+]i, as in G,i minigene transfected cells, suggesting that G,i,βγ is involved in activation of PLCβ. As expected, the addition of the βARK ct
with the Gq minigene together had no further effect on [Ca2+]i, which is through the Gq subunit of Gq. Thus, Gq, but not Gi, activation by PAR1 in HMEC cells leads to Gβγ activation of PLCβ. Because Gi can couple to PAR1 when overexpressed in SF9 cells, these data provide evidence of a cell type-specific coupling of PAR1 to PLCβ.

Stimulation of Stress Fiber Formation—The unexpected finding that Giq proteins are involved in the calcium response to thrombin in HMECs led us to further investigate the importance of Giq proteins in downstream signaling events. It has already been shown that thrombin-mediated F-actin stress fiber formation can in part be regulated by [Ca2+]i (32). Therefore, HMECs were co-transfected with pcDNA-GFP and pcDNA-Gi1/2 or pcDNA-Gi1 minigene vectors, and thrombin-induced stress fiber formation was monitored 48 h after transfection. The cells were serum-starved for 24 h prior to thrombin stimulation, permeabilized, and stained for F-actin with Alexa 568-phalloidin, and their nuclei were stained with DAPI. Transfected cells were distinguished from untransfected cells by detecting GFP fluorescence. Thrombin-stimulated stress fiber formation is blocked almost completely in Gi1 minigene transfected cells. In contrast, the presence of the Gi1/2 minigene did not change the level of thrombin-stimulated stress fiber formation as compared with cells transfected with the GiR minigene (Fig. 6, section II).

If Gq is required for thrombin-induced stress fiber formation, then pertussis toxin, which ADP-ribosylates Gi proteins as well as Gq α subunit, should inhibit stress fiber formation. Treatment of HMECs with pertussis toxin (100 ng/ml) resulted in a strong blockade of thrombin-induced stress fiber formation (Fig. 6, section III). Together, these data clearly show the involvement of Gq proteins, but not Giq proteins, in F-actin stress fiber formation.

Transendothelial Electrical Resistance Assay—Thrombin-induced stress fiber formation may lead to cellular contraction and disruption of the endothelial monolayer. This leads to an increase in permeability of the endothelial monolayer and may have physiological significance in inflammation and wound healing. To check the involvement of Gq proteins in endothelial permeability, we measured transendothelial electrical resistance on microelectrodes. Confluent HMEC monolayers were seeded onto electrodes and challenged with thrombin. When thrombin induces cell rounding through modification of the cellular cytoskeleton, a transient decrease in resistance can be measured. Fig. 7 shows the thrombin-induced decrease in electrical resistance on electrodes covered with cells infected with pRevTRE2 or pRevTRE2-GiR virus expressing the GiR random sequence in all of the cells. Similarly a decrease in permeability was observed when HMECs were infected with pRevTRE2-Gi1/2 minigene virus. However, thrombin treatment in HMECs infected with pRevTRE2-Gi1 miogene virus or pretreated with PTX (100 ng/ml) resulted in a dramatic blockade of the thrombin-induced transendothelial electrical resistance change compared with either GiR or Gi1/2 minigene transfected HMECs. Hence, Giq proteins are the major Gq family proteins involved in the thrombin-induced endothelial barrier dysfunction.

DISCUSSION

The role of Giq proteins has been primarily established in the brain, where it represents about 1% of the total protein content.
Although the role of $G_o$ proteins had been somewhat obscure, several reports now indicate distinct roles and effectors for these $G_o$ subunits, which share about 80% amino acid identity with $G_i$ proteins. Novel effectors or regulators such as Rap1GAP, VMAT2, RGS14, or Pcp2 have been described that $G_o$ specifically or differentially targets compared with $G_i$ proteins (9–11, 33, 34).

PTX-mediated ADP-ribosylation of $G_i$ or $G_o$ proteins inhibits their interaction with G protein-coupled receptors, and several thrombin-mediated effects have been shown to be sensitive to PTX (12, 13, 17), such as stimulation of PAR1 gene expression (18), NO release (15), endothelial relaxation (14), $Ca^{2+}$ influx, and release of tissue plasminogen activator and Von Willebrand factor (16). To determine whether $G_o$ proteins serve a specific role in the signaling pathways of thrombin receptors in HMECs, we investigated whether PAR1 has the potential to activate $G_o$ proteins in vitro. TRAP stimulation of PAR1 induced a 7-fold increase in $[^{35}S]GTP^\gamma S$ binding to SF9 membranes co-expressing PAR1 and the $G_o^1/G_o^2$ heterotrimer. This stimulation of $[^{35}S]GTP^\gamma S$ binding was significantly higher than in membranes expressing PAR1 along with the $G_i^1/G_i^2$ dimer. We also found in similar experiments that TRAP stimulates $[^{35}S]GTP^\gamma S$ to SF9 membranes co-expressing PAR1 and the $G_o^1/G_o^2$ heterotrimer. As an overlay of actin stress fiber and nuclear staining and GFP fluorescence, Section III, thrombin-induced stress fiber formation in untreated (A and B) and PTX treated (C and D) HMECs. A shows actin stress fiber staining with Alexa Fluor 568-phalloidin; B is an overlay of actin stress fiber and nuclear staining and GFP fluorescence. Section III, HMEC transfected with pRevTRE2-$G_o$ minigene viruses block thrombin-induced endothelial barrier dysfunction. The cells were infected with minigene viruses and plated on gold electrodes. The production of the C-terminal peptides was induced using 2 $\mu$g/ml doxycyclin. After serum deprivation, the cells were stimulated with 25 nM thrombin, and changes in transendothelial electrical resistance were monitored in real time. A representative from at least three experiments is shown. Statistical analysis was performed with the peak values. The peak values from PTX-treated HMECs were significantly smaller than peak values from cells infected with pRevTRE2-$G_i$ minigene virus. A similar reduction was observed in cells infected with pRevTRE2-$G_o$, virus. The cells infected with pRevTRE2-$G_o$ showed a smaller peak value than control, but our results indicate that $G_o$ proteins may play a prominent role in disruption of the endothelial monolayer (paired Student’s $t$ test; $p < 0.05$).
tion of either pRevTRE2-G12 or pRevTRE2-G13 minigene viruses. Taken together, these findings suggest that thrombin receptors do not use G1 or G2 proteins to inhibit adenylyl cyclase in HMECs. Interestingly, PAR1 does couple to G2 to regulate PAR-1 gene expression in HMEC cells (18), endothelial albumin endocytosis (35), or endothelial proliferation.2

Next, we showed that Gq proteins are expressed in HMECs. This finding further indicates that Gq proteins may play a role in the signaling of thrombin receptors in HMECs. Because calcium signaling has been shown to be sensitive to PTX in several cell lines, we wanted to verify whether transfection of minigene vectors, but not the G12/13 minigene vectors can block thrombin-stimulated intracellular calcium mobilization. Previously, we have shown that the Gq minigenes, but not the G12/13 minigene vectors can block thrombin-stimulated phosphatidylinositol hydrolysis and intracellular calcium mobilization (1). In addition, we demonstrated that a minigene vector encoding the Gq C-terminal peptide in which the last two residues were mutated could not block these effects. These findings demonstrated that Ga terminally based minigenes can specifically block G protein families. We showed in HMEC that Gq minigenes caused a significant reduction in [Ca2+]i response to thrombin compared with G12/13 or GqR minigenes. These results indicate that Gq, but not G1, serves to couple thrombin receptors to calcium signaling. This Ca2+ transient is mediated by Gβγ activation of PLCβ, because a similar inhibition was induced by transfection with a scavenger of Gβγ signaling, the βARK-c peptide, and their effects were not additive. Thus, there is a cell-specific lack of coupling of Gβγ to PLCβ.

To further demonstrate the importance of Gq proteins in signal transduction pathways downstream from calcium events, we measured thrombin-stimulated stress fiber formation in HMECs transfected with minigene vectors or pre-treated with PTX. Stress fiber formation has been shown to depend on a number of signaling events, including intracellular calcium release and/or activation of Ca2+-dependent protein kinase C (32, 36). Thrombin-induced stress fiber formation was blocked in PTX-pre-treated cells as well as in cells transfected with pcDNA-Gq1 minigene vector but not in cells transfected with pcDNA-G12/13 minigene vector, indicating the involvement of Gq proteins, but not G1 proteins, in this signaling event.

Formation of stress fibers often leads to cell rounding, which, in the case of endothelial cells, disrupts the endothelial monolayer. Hence, we measured endothelial monolayer permeability, a physiological response downstream of stress fiber formation. Thrombin induced a decrease in transendothelial electrical resistance, and this effect was blocked in cells pre-treated with PTX. The cells infected with the pRevTRE2-Gq1 minigene virus also exhibited a muted thrombin response. The cells infected with the control virus pRevTRE2-G12 had a normal response to thrombin, whereas the cells infected with pRevTRE2-G12/13 demonstrated a slightly reduced response. Hence, we showed that Gq proteins play an important role in the signaling of thrombin receptors distinct from G1 proteins, and we confirmed its involvement in different levels of the signaling cascade from calcium mobilization to stress fiber formation and endothelial barrier dysfunction.

In conclusion, we have demonstrated a novel role for Gq proteins in the signal transduction of thrombin receptors in HMECs in which they regulate calcium signaling and cytoskeletal rearrangements. In addition, we have shown that the minigene approach can be used to dissect out the effects of pertussis toxin and determine whether they are mediated by G1 or Gq proteins.

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Thrombin Receptors Activate $G\alpha$ Proteins in Endothelial Cells to Regulate Intracellular Calcium and Cell Shape Changes
Jurgen F. Vanhauwe, Tarita O. Thomas, Richard D. Minshall, Chinnaswamy Tiruppathi, Anli Li, Annette Gilchrist, Eun-ja Yoon, Asrar B. Malik and Heidi E. Hamm

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