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Thrombin Responses in Human Endothelial Cells

CONTRIBUTIONS FROM RECEPTORS OTHER THAN PAR1 INCLUDE THE TRANSACTIVATION OF PAR2 BY THROMBIN-CLEAVED PAR1*

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Peter J. O'Brien‡§, Nicolas Prevost‡, Marina Molino¶, M. Katherine Hollinger‖, Marilyn J. Woolkalis‖, Donna S. Woulfe‡, and Lawrence F. Brass‡§**

From the Departments of ‡Medicine and §Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the ¶Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, Santa Maria Imbaro 66030, Italy, and the ‖Department of Physiology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The recent identification of two new thrombin receptors, PAR3 and PAR4, led us to re-examine the basis for endothelial cell responses to thrombin. Human umbilical vein endothelial cells (HUVEC) are known to express PAR1 and the trypsin/tryptase receptor, PAR2. Northern blots detected both of those receptors and, to a lesser extent, PAR3, but PAR4 message was undetectable and there was no response to PAR4 agonist peptides. To determine whether PAR3 or any other receptor contributes to thrombin signaling in HUVEC, PAR1 cleavage was blocked with two selective antibodies and PAR1 activation was inhibited with the antagonist, BMS200261. The antibodies completely inhibited HUVEC responses to thrombin, but BMS200261 was only partly effective, even though separate studies established that the antagonist completely inhibits PAR1 signaling at the concentrations used. Since peptides mimicking the PAR1 tethered ligand domain can also activate PAR2, we asked whether the remaining thrombin response in the presence of the antagonist could be due in part to the intermolecular transactivation of PAR2 by cleaved PAR1. Evidence that transactivation can occur was obtained in COS-7 cells co-expressing PAR2 and a variant of PAR1 that can be cleaved, but not signal. There was a substantial response to thrombin only in cells expressing both receptors. Conversely, in HUVEC, complete blockade of the thrombin response by the PAR1 antagonist occurred only when signaling through PAR2 was also blocked. From these observations we conclude that 1) PAR1 is the predominant thrombin receptor expressed in HUVEC and cleavage of PAR1 is required for endothelial cell responses to thrombin; 2) although PAR3 may be expressed, there is still no evidence that it mediates thrombin responses; 3) PAR4 is not expressed on HUVEC; and 4) transactivation of PAR2 by cleaved PAR1 can contribute to endothelial cell responses to thrombin, particularly when signaling through PAR1 is blocked. Such transactivation may limit the effectiveness of PAR1 antagonists, which compete with the tethered ligand domain rather than preventing PAR1 cleavage.

Thrombin and cell surface receptors for thrombin have been shown to play a central role in vascular development and in the response of endothelial cells to vascular injury (reviewed in Ref. 1). Thrombin activates receptors that stimulate phospholipases A_2, C, and D and causes an increase in cytosolic Ca^{2+}. This leads to cell proliferation and the release of a wide range of vasoactive substances (2–5). The only signaling receptors that have been unequivocally identified for thrombin to date are members of the protease-activated receptor family, a group of G protein-coupled receptors that are activated by proteolytic cleavage at a unique site within each receptor's NH_2 terminus. Receptor cleavage exposes a new NH_2 terminus that serves as a tethered ligand by binding to sites within the body of the receptor (reviewed in Ref. 6). Three of the four currently identified PAR[1] family members (PAR1, PAR3, and PAR4) have been shown to be cleaved and activated by thrombin. These receptors are closely related with different, but overlapping expression patterns in human tissues (7). The fourth PAR family member, PAR2, is known to be expressed on human endothelial cells. PAR2 is cleaved and activated by trypsin and tryptase, but not by thrombin (8). It is not yet clear, however, which (if either) of these proteases is the physiological activator of PAR2 in endothelial cells. Stimulation of PAR1 or PAR2 evokes a range of responses in endothelial cells, most of which are common to both receptors (6). In addition to proteolytic activation, three of the PARs can also be activated by synthetic peptides corresponding to their tethered ligand. PAR3, for which no peptide agonist has been reported, is the sole exception.

Prior to the identification of PAR3 (9) and PAR4 (10, 11), most studies of thrombin signaling in endothelial cells focused on PAR1 (12–17). However, the results of those studies leave open the question of whether thrombin responses in endothelial cells are mediated solely by PAR1. Although peptide agonists of PAR1 can clearly produce thrombin-like effects, at least two reports have shown that PAR1 antibodies are only partially effective in blocking thrombin responses (18, 19). In addition, HUVEC have been shown to express mRNA encoding PAR3 and to bind a polyclonal PAR3 antibody (20). The existence of PAR4 in endothelial cells has not been reported.

Therefore, in the present studies, we have revisited the basis for endothelial cell responses to thrombin. Using human umbilical vein endothelial cells (HUVEC) as a model, we have asked the following questions. First, which of the known members of the PAR family are expressed in these cells and what

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** To whom correspondence should be addressed: University of Pennsylvania, Rm. 913 BRB-II, 421 Curie Blvd., Philadelphia, PA 19104. E-mail: brass@mail.med.upenn.edu.

[1] The abbreviations used are: PAR, protease-activated receptor; SDF-1α, stromal derived factor α; PCR, polymerase chain reaction; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.

are their respective contributions to the events that occur when endothelial cells are activated by thrombin? Second, are thrombin responses in endothelial cells mediated in part by additional receptors that have not yet been discovered? These could be unidentified PAR family members or unrelated receptors with different mechanisms of activation. Finally, we also asked whether endothelial cell PAR2, which is not a substrate for thrombin, might indirectly contribute to signaling in response to thrombin by a novel mechanism involving cleaved PAR1. The results that will be described show that PAR1 cleavage is required for endothelial cell responses to thrombin and that subsequent signaling can be mediated by either the direct activation of PAR1 (the predominant mechanism) or by the indirect "transactivation" of PAR2. Thrombin receptors other than PAR1 are either not expressed on endothelial cells or not able to support a thrombin response on their own, but to the extent that transactivation of PAR2 by PAR1 contributes to the thrombin response, it will limit the effectiveness of PAR1 antagonists that compete with the tethered ligand domain rather than preventing PAR1 cleavage.

EXPERIMENTAL PROCEDURES

Reagents—[^{3}H]Myoinositol (specific activity, 22.2 Ci/mmol) was obtained from NEN Life Science Products. Highly purified α-thrombin (~3000 units/mg, 1 unit/ml ≈ 10 nM) was provided by Dr. John Fenton (New York State Department of Health, Albany, NY). Human SDF-1α was provided by Dr. James Hoxie (University of Pennsylvania, Philadelphia, PA). BMS200261 (21) was the generous gift of Dr. Donna Oksenberg (COR Therapeutics, South San Francisco, CA). Histamine (dihydrochloride salt), biotinylated M2 anti-Flag IgG_1 monoclonal antibody, and hirudin were from Sigma. Bovine serum albumin was from ICN, and Fura-2/AM was from Molecular Probes. R-phycoerythrin-conjugated streptavidin was from BIOSOURCE International. The peptide GYPGQV, corresponding to residues 48–53 of human PAR4, was synthesized and high performance liquid chromatography-purified at the University of Pennsylvania Medical Center Protein Chemistry Facility.

Cell Culture—HUVEC were isolated from term umbilical cords and maintained in complete medium (Medium 199, 10 mM HEPES, pH 7.4, 10% fetal calf serum, 1 mM glutamine, 12 units/ml heparin, 100 μg/ml crude endothelial cell growth supplement, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37 °C on fibronectin-coated tissue culture dishes (16). The cells were used at passages 1–3. COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. REH cells were cultured in RPMI 1640 with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Antibodies—WEDE15 and ATAP2 are IgG_1 monoclonal antibodies directed against epitopes within the hirudin-like domain and the tethered ligand domain of human PAR1, respectively (22, 23). Antibody PC143 is an IgG_1 monoclonal antibody that recognizes a peptide corresponding to residues 19–34 of human PAR1 within the fragment that is released when the receptor is cleaved by thrombin. PC143 binds to intact PAR1, but not thrombin-cleaved PAR1 (24). Monoclonal antibody SAM11 is an IgG_{2a} antibody directed against the peptide SLIGKVDGTSHVTG, corresponding to residues 37–50 of human PAR2 (17). Monoclonal antibody 12G5 is an IgG_{2a} antibody directed against the first and second extracellular loops of human CXCR4 (25, 26). Antibody 19 is an IgG_{2a} mouse monoclonal antibody against human CD4 (25). Antibody EH1 is an IgG_1 mouse monoclonal antibody reactive with the human immunodeficiency virus type I nef protein (23). Antibodies were purified from ascites by protein A affinity chromatography using a MAPS II kit (Bio-Rad).

Receptor Expression Constructs—The cDNA for human PAR3 was provided by Dr. Shaun Coughlin (University of California, San Francisco, CA). The cDNA for human PAR4 in the neomycin resistance vector pBK-CMV was provided by Dr. Andrew Darrow (R. W. Johnson Pharmaceutical Research Institute, Spring House, PA). Human CXCR4 in pCDNA3 was provided by Dr. James Hoxie. Human PAR2 in pRK7 was described previously (27). A PCR-induced CTC to CCC mutation in the PAR1 cDNA that changes leucine 258 to proline was identified and subcloned into the previously described PAR1 cDNA (27) in the mammalian expression vector pRK7 to create PAR1-L258P. P1NT-CXCR4 was generated by the PCR amplification of CXCR4 using the reverse primer TATTTGACCAGCTCCTGGATCTTCCTGCCCACCATCTAC and the T7 universal primer with 30 cycles of PCR with the temperature profile 94 °C for 1 min, 50 °C for 1 min, then 72 °C for 2 min. This reaction yields a product with a *Pfl*MI restriction site at the amino-terminal end of the putative first transmembrane domain of CXCR4. The corresponding domains of PAR1 were removed from the full-length construct in pRK7 using *Pfl*MI and *Xba*I, and the CXCR4 PCR product was subcloned onto the PAR-1 amino terminus, generating a chimeric receptor designated P1NT-CXCR4. The construct contains the amino acid sequence YLTSSW_IFLPT, with the underscore representing the junction between PAR-1 and CXCR4. The amino termini of PAR3 and PAR4 were amplified by PCR from plasmid DNA to insert a *Pfl*MI site at residues analogous to the site in PAR1, and the PCR products were subcloned into P1NT-CXCR4, replacing the PAR1 NH_2-terminal fragment and creating P3NT-CXCR4 and P4NT-CXCR4, respectively.

Northern Analysis—Northern blots of PARs were performed using standard protocols (28). Briefly, total RNA was isolated using the RNAgents system (Promega). RNA (15 μg/sample) was denatured by boiling and electrophoresed on a 1.4% agarose/formaldehyde gel. The gel was washed for 30 min in 20× SSC and then transferred onto nylon membranes by capillary action. After transfer, RNA was UV-cross-linked, then prehybridized for 4–5 h at 63 °C and hybridized with 10^6 cpm/ml denatured probe. The membrane was washed in 0.2% SSC, 0.1% SDS at 63 °C for 30 min, then for 2 h at room temperature in 0.1% SSC, 0.1% SDS. Probes for the PARs were derived from the following restriction fragments of their cDNAs: PAR1 *Pst*I-*Eco*RI (nucleotides 764–2123), PAR2 *Bam*HI (nucleotides 1–1266), and PAR3 *Hin*dIII-*Kpn*I (nucleotides 1–405). Membranes were analyzed, stripped in 0.1% SDS at 90 °C, and rehybridized with a probe to RPL32 to assay RNA loading (29).

Reverse Transcription and PCR Amplification of RNA—Total RNA from Dami cells or HUVEC (5 μg) was treated with RQ1 DNase (Promega) according to manufacturer's protocols. cDNA synthesis was carried out using 100 units of avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.) and 20 pmol of random primers for 30 min at 37 °C. Positive control PCR for PAR1 was performed with the forward primer CACCGGAGTGTTTGTAGTCA and the reverse primer TAACTGCTGGGATCGGAACT. Conditions were as follows: 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min for 30 cycles. PAR4 amplification used the forward primer CTTGGCAAGCTTCGGCACGAGCAGAAGCTG and the reverse primer GAGTGTCACCAGGAGCTGGCCGGGAGCTCCAGGGTGTC with the temperature profile: 94 °C for 1 min, 72 °C for 2 min for 30 cycles. Negative control reactions were carried out as above with no reverse transcription.

Cytosolic Ca^{2+}—Confluent HUVEC were loaded with 5 μM FURA-2/AM in complete medium for 1 h and then released from the tissue culture dishes by incubating for 15 min with phosphate-buffered saline containing 1 mM EDTA. The detached cells were washed, resuspended in medium 199, incubated for 15 min, washed again, and resuspended in RPMI 1640 without phenol red at 1×10^6 cells/ml. Cytosolic Ca^{2+} in REH cells was measured exactly as described previously for HEL cells (30). In all cases, changes in Fura-2 fluorescence were measured with an SLM/Aminco model AB2 fluorescence spectrophotometer within 30 min (16). In experiments where cells were treated with multiple agonists and/or antagonists, reagents were added to the cuvette sequentially. In desensitization experiments, cells were stimulated with agonist, then cytosolic Ca^{2+} stores were allowed to refill for 5 min, during which time data collection was suspended (31).

Antibody Blockade of Thrombin Cleavage—The ability of antibody mixtures to prevent thrombin cleavage of PAR1 was first determined using flow cytometry. Cells were transiently transfected by the calcium phosphate method with a total of 20 μg of plasmid DNA for 16 h, shocked with 10% Me_2SO in Dulbecco's modified Eagle's medium, detached with trypsin, and then replated. The next day, cells were detached with PBS/EDTA, washed once in PBS, and then resuspended in binding buffer (Dulbecco's modified Eagle's medium with 1% bovine serum albumin). Before thrombin addition, samples were preincubated for 10 min at 37 °C with either PAR1-directed or control antibodies (25 μg/ml each of antibodies WEDE15 and ATAP2, or 50 μg/ml antibody EH1). The cells were then treated with 20 nM thrombin for 45 min at 37 °C. The subsequent steps were performed on ice. Cells were initially resuspended in acid wash buffer (RPMI 1640 with MES, pH 2.5, and 2% bovine serum albumin) for 15 min to remove surface bound antibodies (24) and then washed twice in PBS before being resuspended to 1×10^6/ml in staining buffer (17) supplemented with 10 units/ml hirudin. After 5 min, either biotinylated PC143 or biotinylated M2 antibody was added to a final concentration of 10 μg/ml and incubated for 30 min. The cells were then washed in PBS, resuspended in staining buffer, stained

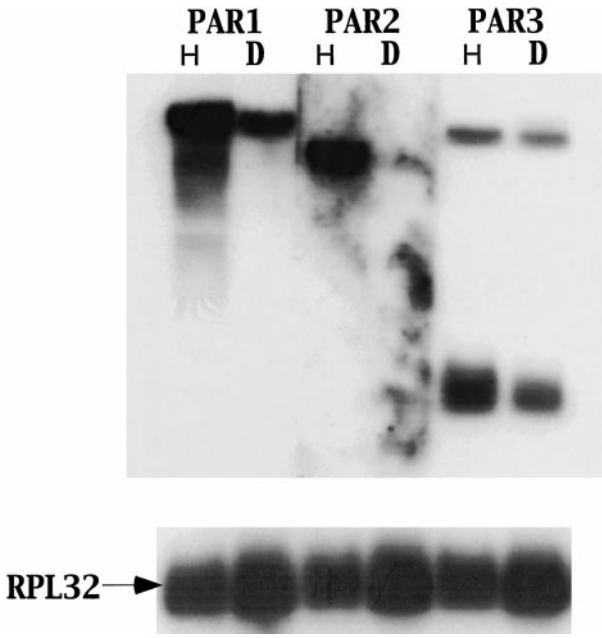

FIG. 1. **Expression of PAR1, PAR2, and PAR3 mRNA in HUVEC.** Northern analysis of total RNA from confluent cultures of HUVEC (*H*) or DAMI cells (*D*) was performed as described under "Experimental Procedures." Membranes were hybridized with specific PAR oligonucleotides as indicated. Blots were then stripped and rehybridized with a probe for RPL32 to assess abundance. Transcript sizes agree with previous reports (9, 34, 53).

with a 1:800 dilution of phycoerythrin-streptavidin for 30 min, washed twice with PBS, and fixed in 2% paraformaldehyde in PBS for analysis by flow cytometry.

Phosphoinositide Hydrolysis—Early passage HUVEC were loaded overnight with 4 μCi/ml [^{3}H]myoinositol in complete growth medium. Cells were washed once, then serum-starved for 2 h in Medium 199. 20 mM $LiCl_2$ was added 15 min before the addition of either buffer or thrombin (2 units/ml). Before agonist addition, some samples were preincubated for 10 min at 37 °C with either the anti-PAR1 antibody mix or EH1 control antibodies. Cells were then incubated for 45 min at 37 °C, extracted in perchloric acid/EDTA, and then neutralized. Total inositol phosphates were measured by ion exchange chromatography on Dowex columns and scintillation counting (32). For transactivation experiments, COS cells were transiently transfected with 10 μg of each receptor construct and assayed approximately 48 h after transfection for surface expression of transfected PAR1 and PAR2, using flow cytometry with antibodies WEDE15 and SAM11, respectively. Cells with comparable transfection efficiencies and surface expression of transfected receptors were assayed for thrombin-induced phosphoinositide turnover in response to 20 nM thrombin, as described previously (27).

RESULTS

Previous reports show that human umbilical vein endothelial cells (HUVEC) express PAR1 and PAR2 (15–17, 33) and that these receptors are capable of being activated by thrombin and trypsin, respectively (8, 34). A recent report by Schmidt and co-workers (20) suggests that human endothelial cells also express PAR3 but, in the absence of a selective agonist or antagonist, the ability of the receptor to signal in HUVEC could not be tested directly. In the studies shown in Fig. 1, mRNA from early passage HUVEC was probed for PAR3 using PAR1 and PAR2 as positive controls. As reported (20), PAR3 mRNA was detectable, but with less intensity than PAR1 or PAR2. Evidence for PAR4 expression in HUVEC was sought by reverse transcription-PCR and by stimulating the cells with a PAR4 peptide agonist. Using primers based upon the PAR4 NH_2 terminus (see "Experimental Procedures") and PAR1 as a positive control, we were able to detect PAR4 in the megakaryoblastic Dami cell line, but not in HUVEC (data not shown). Furthermore, the PAR4 peptide agonist, GYPGQV, failed to produce an increase in cytosolic Ca^{2+} when added to HUVEC loaded with Fura-2 (Fig. 2). The same peptide agonist caused platelet aggregation (Ref. 10 and data not shown).

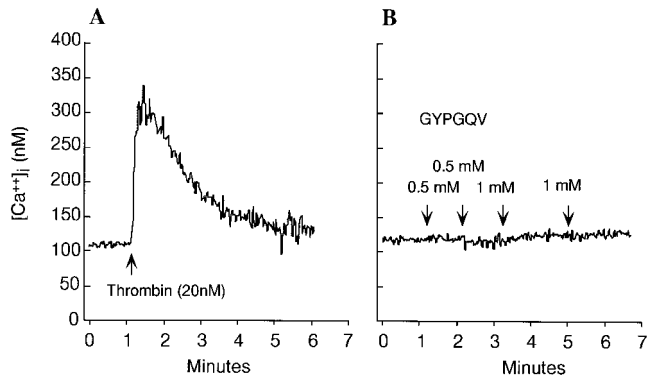

FIG. 2. **Activation of HUVEC by thrombin and the PAR4 peptide GYPGQV.** To measure changes in the cytosolic Ca^{2+} concentration, HUVEC were loaded with Fura-2 and stimulated with GYPGQV and thrombin as indicated. Results are representative of those obtained in three such experiments.

These results suggest that PAR3 may be present in HUVEC, but PAR4 is not. In order to distinguish the contributions of PAR1 from those of PAR3 (or any other as yet unidentified thrombin receptor), we used two previously developed monoclonal antibodies to block PAR1 cleavage by thrombin. Antibody ATAP2 binds to an epitope within the PAR1 tethered ligand domain (23). Antibody WEDE15 binds to the hirudin-like domain that interacts with thrombin's anion-binding exosite (22). Cleavage of PAR1 was detected with a third monoclonal antibody, PC143, which binds to residues within the NH_2-terminal fragment of PAR1 that is released by thrombin (24). In the studies shown in Fig. 3, COS-7 cells transiently transfected with human PAR1 were exposed to thrombin in the presence of either ATAP2 and WEDE15 or an equivalent amount of an isotype-matched control antibody, EH1. Approximately 30% of the cells expressed the transfected receptor. In the histograms shown in Fig. 3 (*A* and *B*), the transfected cells bound more biotinylated PC143, and appear as a shoulder to the right of the weakly fluorescent, mock-transfected cells (*dotted line*). Thrombin caused a complete loss of PC143 binding and the disappearance of the shoulder in the cells preincubated with the control antibody, EH1 (Fig. 3*A*), but cleavage was completely inhibited by the ATAP2/WEDE15 antibody mixture (Fig. 3*B*).

Based upon the sequence of the peptides that were originally used to prepare them, antibodies ATAP2 and WEDE15 would not be expected to bind to human PAR3 or PAR4. To confirm this specificity, COS-7 cells were transfected with chimeric constructs consisting of the NH_2 terminus of either PAR1, PAR3, or PAR4 fused to the G protein-coupled chemokine receptor, CXCR4 (25). Cells transfected with each of these constructs were recognized by anti-CXCR4 antibody 12G5 (data not shown). Fig. 3*C* shows that in cells expressing individual chimeras, WEDE15 and ATAP2 bind to cells expressing the PAR1-CXCR4 chimera, but not to cells expressing the PAR3- or PAR4-CXCR4 chimeras.

PAR1 Cleavage Is Required for Thrombin Responses in HUVEC—Since antibodies ATAP2 and WEDE15 inhibit PAR1 cleavage by thrombin, but do not bind to PAR3 or PAR4, we used them to determine whether PAR1 cleavage is required for HUVEC to respond to thrombin. In the study shown in Fig. 4, changes in the cytosolic Ca^{2+} concentration in HUVEC were measured in response to thrombin and the PAR2 agonist peptide, SLIGRL. In the presence of isotype-matched control, thrombin produced a robust increase in cytosolic Ca^{2+}, but in

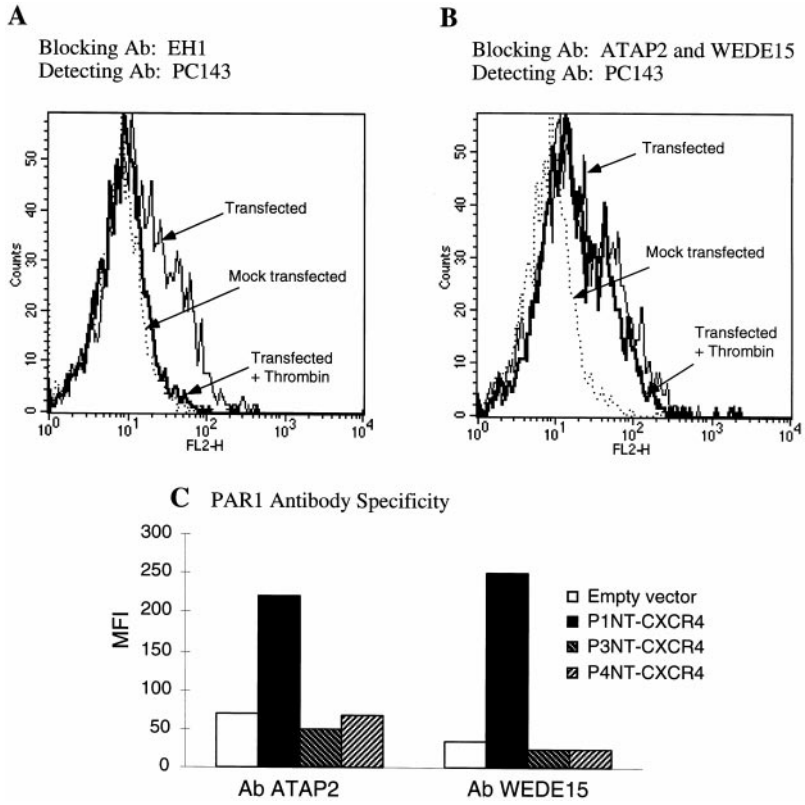

FIG. 3. **Selected monoclonal antibodies can specifically inhibit thrombin cleavage of PAR1.** *A* and *B*, COS-7 cells were analyzed by flow cytometry 36–48 h after transfection with human PAR1 or empty vector. The cells were preincubated with either the control antibody EH1 (*A*) or the anti-PAR1 monoclonal antibodies ATAP2 and WEDE15 (*B*), and stimulated with thrombin. Receptor cleavage was detected as a loss in binding for biotinylated antibody PC143, a cleavage-sensitive PAR1 monoclonal antibody, as described under "Experimental Procedures." *C*, COS-7 cells were transfected with chimeras containing the NH_2 terminus of PAR1, PAR3, or PAR4 fused to CXCR4 as described under "Experimental Procedures." Surface expression of each chimera was confirmed using the monoclonal antibody 12G5 directed against CXCR4 (data not shown). The results are expressed as mean fluorescence intensity (*MFI*) of the population of cells for each transfection, and show the binding of antibody ATAP2 and WEDE15 to cells expressing P1NT-CXCR4, but not the other chimeras. The results shown are representative of two separate experiments.

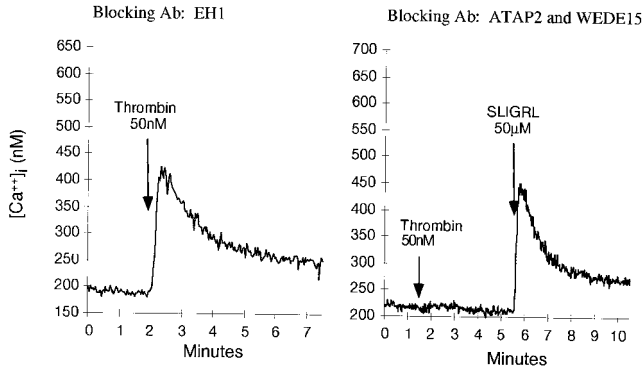

FIG. 4. **PAR1 blocking antibodies inhibit HUVEC thrombin responses.** Fura-2-loaded HUVEC were pretreated with the control antibody EH1 (50 μg/ml) or the anti-PAR1 antibodies ATAP2 and WEDE15 (25 μg/ml of each), then stimulated with thrombin as indicated. Cell responsiveness was confirmed using the SLIGRL peptide as indicated. Results are representative of three separate experiments.

the presence of the PAR1-blocking antibodies there was no response, even at 50 nM thrombin. The response to SLIGRL was the same in either case. Blockade of PAR1 cleavage also inhibited thrombin-induced phosphorylation of the mitogen-activated protein kinase, ERK2 (data not shown), which lies downstream of a number of different effector pathways in HUVEC (35–37).

These results suggest that the rapid thrombin-induced calcium response in HUVEC requires PAR1 cleavage and that PAR3 (despite the Northern analysis) is either not present or not able to signal on its own in any of the assays that were performed. The same conclusion might apply to any other, as yet unidentified, thrombin receptors on HUVEC. Since there is evidence (20) that PAR3 is expressed on HUVEC, we considered the possibility that PAR3 is present, but is cleaved by thrombin at too slow a rate to stimulate mitogen-activated protein kinase phosphorylation or to contribute to the rapid increase in inositol 1,4,5-trisphosphate needed to stimulate the release of stored calcium (38, 39). This possibility was tested by analyzing thrombin-induced inositol phosphate accumulation in the presence of control or PAR1-blocking antibodies while inhibiting inositol phosphatase activity with Li^+. The results in Fig. 5 show that thrombin-induced inositol accumulation in HUVEC was reduced by 93% in the presence of the PAR1 blocking antibodies, making a substantial contribution from PAR3 seem unlikely.

Transactivation of PAR2 by PAR1—Since PAR1 cleavage is necessary for HUVEC thrombin responses, we sought to determine if it is sufficient to account for the myriad responses induced by thrombin in these cells. In other words, could some part of the HUVEC thrombin response result from the activation of another receptor by a mechanism that depends upon PAR1 cleavage? One likely candidate for such a role is PAR2, which is also expressed on endothelial cells. Although PAR2 has been shown to be activated by trypsin (8) and, less efficiently, tryptase (40), it remains to be shown that either of these proteases is the primary activator of PAR2 on endothelial cells (6). We asked whether PAR2 could contribute to the thrombin response in human endothelial cells without being a direct substrate for thrombin. It has been shown previously that non-signaling (but cleavable) variants of PAR1 can "transactivate" PAR1 variants that can signal, but not be cleaved (41). Transactivation in that case was thought to involve the intermolecular donation of the tethered ligand domain of the cleavable receptor, allowing it to activate non-cleavable receptors. PAR2 is not a thrombin substrate and cannot be directly activated by thrombin (Ref. 8 and Fig. 6). However, peptides corresponding to the tethered ligand domain of human PAR1 (SFLLRN) are known to be able to activate PAR2 (42, 43), and Mirza and colleagues (33) have shown that the response of HUVEC to thrombin is reduced when PAR2 is activated first with a selective agonist peptide. Although the latter observation was attributed to heterologous desensitization of PAR1 by downstream mediators of the PAR2 response, an additional

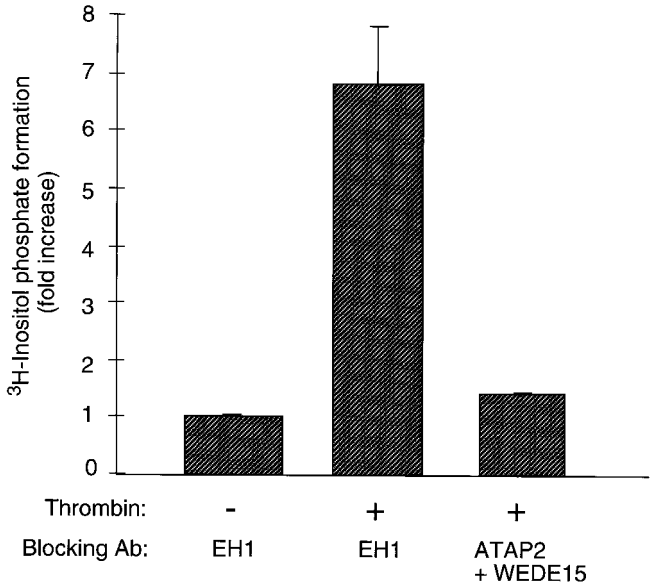

FIG. 5. **PAR1 blocking antibodies inhibit thrombin-induced phosphoinositide hydrolysis in HUVEC.** Adherent cells were loaded with [^{3}H]inositol and pretreated with control or PAR1 blocking antibodies as described under "Experimental Procedures." Cells were treated with thrombin for 45 min as indicated. The results shown are mean ± S.E. of three studies expressed as the -fold increase in total [^{3}H]inositol phosphate formation compared with the results obtained in each experiment in which control antibody-treated cells were stimulated with buffer.

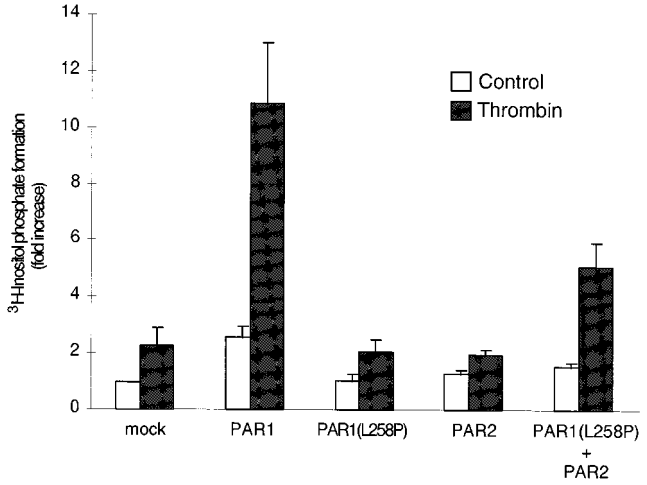

FIG. 6. **PAR1 transactivation of PAR2 in transfected COS-7 cells.** COS-7 cells were transfected with empty vector (*mock*) or the indicated plasmids, loaded with [^{3}H]inositol, and stimulated with thrombin (20 nM) as described under "Experimental Procedures." The results shown are mean ± S.E. of three studies expressed as -fold increase in total [^{3}H]inositol phosphate formation compared with the results obtained in each experiment with vector-transfected cells stimulated with buffer.

hypothesis is that part of the thrombin signal is mediated by the transactivation of PAR2 by thrombin-cleaved PAR1.

As a first test of this hypothesis, we asked whether a non-signaling, but cleavable variant of human PAR1 would transactivate wild type human PAR2 in transfected COS-7 cells. PAR1(L258P) contains a Leu to Pro substitution in the second extracellular loop of the receptor near a domain needed for receptor activation. The data in Fig. 6 show that neither PAR1(L258P) nor intact PAR2 signals in response to thrombin when expressed alone in COS-7 cells. However, when the cells were transfected with both constructs, there was a 5-fold increase in [^{3}H]inositol phosphate accumulation in response to thrombin: nearly half of that seen in cells expressing wild type PAR1 alone.

This result suggests that cleaved PAR1 can donate its tethered ligand to PAR2 and transactivate PAR2, at least under conditions in which direct signaling through PAR1 cannot occur because of a mutation in a putative tethered ligand domain binding site. An alternative explanation for the rescue of thrombin responses in this experiment is that PAR2 can physically interact with PAR1(L258P) and complement the mutation to restore PAR1 signaling. However, although such interactions have been described for other G protein-coupled receptors (44–46), it seems less likely to be necessary here since it was previously shown by Chen *et al.* (41) that transactivation of non-cleavable PAR1 by PAR1 can occur when the ligand donor is the NH_2 terminus of PAR1 fused to the single transmembrane domain of CD8.

Since there are no known antagonists of PAR2 and since the antibodies that block PAR1 activation do so by blocking PAR1 cleavage, we used a different approach to ask whether transactivation of PAR2 by thrombin-cleaved PAR1 occurs on human endothelial cells at normal levels of expression of the native forms of the two receptors. Fig. 7 shows the response of Fura-2-loaded HUVEC to thrombin in the presence and absence of the PAR1 antagonist, BMS200261. This compound is a peptidomimetic based on the PAR1 activating peptide that prevents PAR1 signaling by PAR1 agonist peptides (47) and is thus thought to work by inhibiting the binding of the tethered ligand domain to sites in the body of the receptor. BMS200261 has been reported to completely inhibit human platelet responses to SFLLRN and to concentrations of thrombin that activate PAR1 but not PAR4 (21, 48). We found that BMS200261 also completely inhibits thrombin responses in the REH pre-B-cell line that expresses PAR1, but not other known PAR family members (see below). Despite the similarities between PAR1 and PAR2, BMS200261 is selective for PAR1 and did not inhibit the activation of PAR2 by SFLLRN in HEK-293T or COS-7 cells (data not shown). In contrast to the antibodies that block PAR1 cleavage, BMS200261 reduced, but did not abolish, the thrombin response when added to HUVEC (Fig. 7, compare *panels A* and *B*). To test whether any of the residual thrombin response could be due to transactivation of PAR2, HUVEC were also pretreated with the agonist peptide SLIGRL to first activate and then desensitize PAR2. Fig. 7*C* shows that BMS200261 does not prevent the PAR2-mediated response to SLIGRL, nor does it prevent the desensitization of PAR2 caused by SLIGRL (*i.e.* there is no response when SLIGRL is added a second time 5 min later). However, the combination of BMS200261 and SLIGRL essentially abolished a subsequent response to thrombin. In four such studies, the thrombin response was reduced by 74 ± 3% in the presence of BMS200261 alone and by 93 ± 6% when PAR2 was also desensitized ($p = 0.023$ by paired t test).

To confirm that BMS200261 completely antagonizes the PAR1 response at the concentrations that were used, we also tested its ability to prevent thrombin responses in the REH pre-B-cell line (49). As shown in the *lower part* of Fig. 7, these cells were found to lack responses to PAR2 and PAR4 agonist peptides (*panel D*), and their response to thrombin was eliminated by PAR1 blocking antibodies (data not shown), suggesting that PAR1 is the only thrombin receptor expressed. In REH cells, 10 μM BMS200261 blocked the calcium response to 20 nM thrombin without inhibiting a subsequent response to the chemokine SDF-1α (Fig. 7, compare *panels D* and *E*). Taken together, these results show that significant transactivation of PAR2 by cleaved PAR1 can occur on endothelial cells, at least in the presence of a PAR1 antagonist.

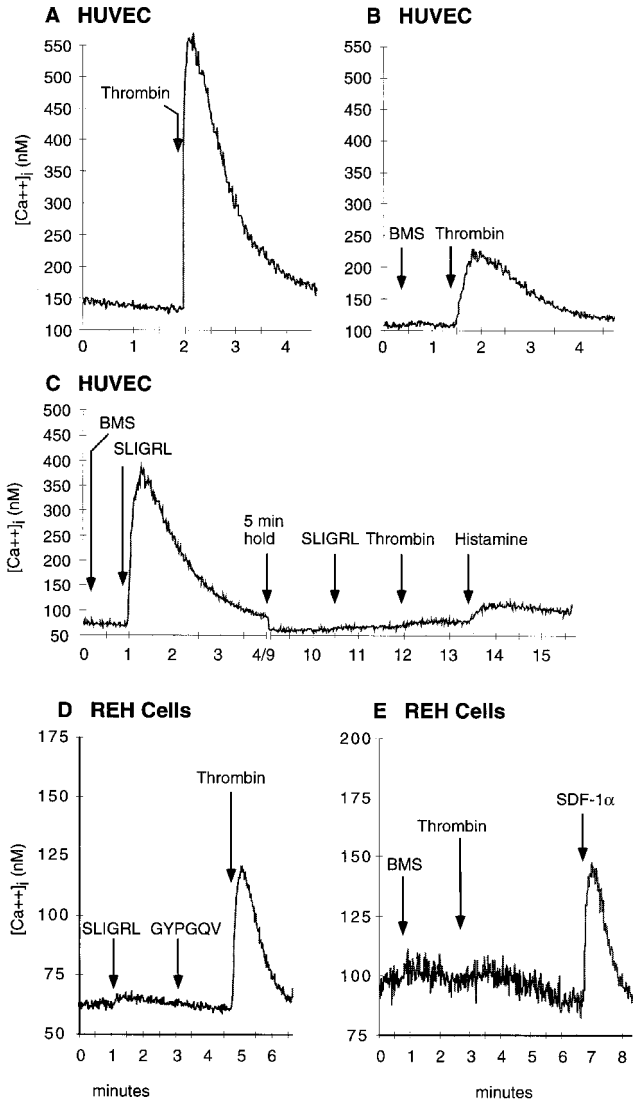

FIG. 7. **Contribution of PAR2 to thrombin responses.** *A* and *B*, Fura-2-loaded HUVEC were stimulated with 20 nM thrombin or with 10 μM BMS200261 followed by thrombin. In *C*, cells were treated with BMS200261, followed by the PAR2 agonist, SLIGRL (600 μM). After 5 min in which data collection was suspended (*5 min hold*), the cells were restimulated with SLIGRL, followed by thrombin and 15 μM histamine. The results of four such experiments are summarized in the text. *D* and *E*, the cytosolic free Ca^{2+} concentration was measured in Fura-2-loaded REH cells treated with 100 μM SLIGRL, then 1.5 mM GYPGQV, followed by 20 nM thrombin (*D*), or with 10 μM BMS200261, followed by thrombin and 10 μM SDF-1α, the agonist for CXCR4 (*E*).

DISCUSSION

Since there are at least three PAR family members in addition to PAR1, the response of any given cell to thrombin can potentially be mediated by more than one receptor. This has already been shown to be the case for platelets. In human platelets PAR1 and PAR4 mediate aggregation and secretion in response to thrombin (48), while PAR3 and PAR4 do so in mouse platelets (11). In the present studies, we examined the possibility that more than one receptor might be needed for thrombin responses in endothelial cells and asked whether endothelial cell PAR2 could be transactivated by thrombin-cleaved PAR1, allowing it to contribute to thrombin signaling. The existence of additional endothelial thrombin receptors was initially suggested by recent reports showing that HUVEC can express PAR3 (20) and that antibodies directed against PAR1 cause only an incomplete blockade of thrombin signaling in HUVEC (18, 19). Our results confirm that PAR3 mRNA is present in HUVEC, and that it is less abundant than mRNA encoding PAR1 and PAR2. We were not, however, able to detect message encoding PAR4, and a PAR4 agonist peptide that activates human platelets was unable to stimulate an increase in cytosolic Ca^{2+} in HUVEC. To determine whether PAR3 (or any undiscovered thrombin receptors) contributes to the thrombin response in endothelial cells, we took advantage of two previously developed monoclonal antibodies with defined epitopes within the PAR1 NH_2 terminus. When added together, these antibodies were able to completely prevent PAR1 cleavage, even at high thrombin concentrations over prolonged periods of time. Preincubating HUVEC with the antibodies completely inhibited thrombin responses encompassing multiple signaling pathways. These results suggest that, at least for the effects that were measured, PAR1 cleavage is required for thrombin responses in HUVEC and that if other thrombin receptors are present, they require cleavage of PAR1 for their function. Although we cannot completely exclude the existence of another thrombin receptor that couples exclusively to an entirely different set of effectors, there is at the moment no reason to believe that this is the case.

Why might the antibodies used to block PAR1 cleavage in the present study completely inhibit thrombin responses, while those used previously (18, 19, 48, 50) caused only partial inhibition? One possible explanation may lie in the location of the epitopes for the monoclonal antibodies that we used, which separately target the tethered ligand domain and the domain of PAR1 that interacts with thrombin's exosite. We found that neither antibody was sufficient by itself to completely prevent PAR1 signaling and that complete blockade occurred only when the two antibodies were added together. The results described here are the first demonstration that we are aware of in which anti-receptor antibodies can produce complete and long-lasting inhibition of cleavage of native PAR1 by relatively high concentrations of thrombin.

The ability of the PAR1 antibodies to block thrombin responses in HUVEC shows that PAR1 cleavage is required for thrombin responses in these cells. It does not rule out the possibility that there are other receptors whose activation is dependent, either directly or indirectly on PAR1 cleavage. To examine this possibility, we used the PAR1 antagonist BMS200261 to block PAR1 signaling without inhibiting receptor cleavage, reasoning that the detection of a co-receptor for thrombin would depend upon the elimination of the PAR1 signal. Indeed, in the presence of BMS200261, a residual thrombin response was seen in HUVEC that averaged about 26% of the response in the absence of BMS200261. Since human endothelial cells express PAR2, we asked whether activation of PAR2 might contribute to thrombin signaling in endothelial cells, perhaps mediating the residual thrombin response in the presence of the PAR1 antagonist. We and others had observed that activation of PAR2 with peptides that do not activate PAR1 causes a reduced response when thrombin is added later (17, 33). This decrease in the thrombin response had been attributed to heterologous desensitization of PAR1, but other possibilities were not excluded. Second, PAR2 is structurally closely related to PAR1 and peptides that correspond to the human PAR1 tethered ligand domain sequence (SFLLRN) activate human PAR2 as well as activating PAR1 (42, 43). Prior work by other investigators has shown that PAR1 variants that are cleavable but not capable of signaling can transactivate non-cleavable mutants of PAR1 (41). What was not established in those studies was whether transactivation might extend to PAR2 and could occur at native receptor densities. Since we found that BMS200261 does not inhibit the

PAR2 response to SFLLRN, such transactivation could lead to the residual thrombin response described above.

Evidence that PAR1 can transactivate PAR2 was obtained with COS-7 cells co-expressing PAR2 with a PAR1 variant that can be cleaved, but not activated by thrombin. The signaling that arose by transactivation was surprisingly robust, averaging about 40% of the signal observed with wild type PAR1 alone (Fig. 6). This is more than was seen in the previous studies on PAR1 transactivation by PAR1 (41). Since there are no reported antagonists of PAR2 signaling, we were obliged to use an indirect approach to dissect out any contribution of PAR2 to thrombin responses in HUVEC. When added at concentrations that completely block PAR1 activation in cells that express no other known PAR, the PAR1 antagonist BMS200261 caused only incomplete inhibition of the thrombin response in HUVEC. The remaining response to thrombin was blocked by desensitizing PAR2. Cells treated in this manner no longer responded to thrombin or to a PAR2-selective agonist peptide, but did respond to an unrelated agonist. Taken together, these observations suggest that PAR2 can be activated by cleaved PAR1 and that such transactivation might recruit PAR2 into the thrombin response in HUVEC, at least when a PAR1 antagonist such as BMS200261 is present. Since PAR2 is not a substrate for thrombin, transactivation of PAR2 by PAR1 fits with the observed requirement for PAR1 cleavage for thrombin responses in HUVEC. The fact that, even in the absence of the antagonist, selective activation and desensitization of PAR2 reduces subsequent responses to thrombin suggests that transactivation of PAR2 by cleaved PAR1 may also occur normally. Confirmation of this hypothesis may ultimately require the development of a potent and selective PAR2 antagonist.

Transactivation of PAR2 by PAR1 has several implications. First, it suggests that PAR1 and PAR2 are located sufficiently closely to each other in the endothelial cell plasma membrane that the cleaved NH_2 terminus of PAR1 can access PAR2. PAR1 and PAR2, like other receptors, are likely to be clustered in caveolae or other membrane microdomains (reviewed in Ref. 51). If so, the density of the receptors may be sufficiently high to crowd them close to one another.

Transactivation also has implications for the development of PAR1 antagonists. One approach to such antagonists has been to find molecules that selectively inhibit PAR1 activation by peptide agonists. In other words, to look for inhibitors of the interaction between the tethered ligand and the body of the receptor that do not necessarily affect cleavage of the receptor NH_2 terminus by thrombin. BMS200261 is one such antagonist. Others have been described recently (47, 52). On platelets, the efficacy of this type of inhibitor is limited by the presence of PAR4. The present studies predict that on cells such as endothelial cells where both receptors are present, PAR1 tethered ligand antagonists will be limited by the presence of PAR2 even if other thrombin receptors are not present. If the contribution of transactivation is great enough, then other strategies will be required to prevent thrombin responses in target cells that express PAR2 along with PAR1.

In conclusion, the results of this study show that 1) PAR1 is the predominant thrombin receptor expressed in HUVEC and that cleavage of PAR1 is required (but not necessarily sufficient) for endothelial cell responses to thrombin; 2) despite the presence of PAR3 mRNA in HUVEC (which we confirmed), PAR3 is either not expressed on the cell surface or is unable to support a thrombin response on its own; 3) PAR4 is not present; and 4) transactivation of PAR2 by cleaved PAR1 provides an additional mechanism by which cells that express both receptors can respond to thrombin, particularly in the presence of PAR1 antagonists.

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Thrombin Responses in Human Endothelial Cells: CONTRIBUTIONS FROM RECEPTORS OTHER THAN PAR1 INCLUDE THE TRANSACTIVATION OF PAR2 BY THROMBIN-CLEAVED PAR1

Peter J. O'Brien, Nicolas Prevost, Marina Molino, M. Katherine Hollinger, Marilyn J. Woolkalis, Donna S. Woulfe and Lawrence F. Brass

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