Thrombin Stimulation of Vascular Adhesion Molecule-1 in Endothelial Cells IsMediated by Protein Kinase C (PKC-δ-NF-κB and PKC-κ-GATA Signaling Pathways*

Received for publication, September 3, 2002, and in revised form, December 2, 2002
Published, JBC Papers in Press, December 18, 2002, DOI 10.1074/jbc.M208974200

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We recently demonstrated that thrombin induces the expression of vascular adhesion molecule-1 (VCAM-1) in endothelial cells by an NF-κB- and GATA-dependent mechanism. In the present study, we describe the signaling pathways that mediate this response. Thrombin stimulation of the VCAM-1 gene and promoter in human umbilical vein endothelial cells was inhibited by preincubation with the phosphatidylinositol 3-kinase inhibitor, LY294002, the protein kinase C (PKC)-δ inhibitor, rottlerin, a PKC-ζ peptide inhibitor, or by overexpression of dominant negative (DN)-PKC-ζ. In electrophoretic mobility shift assays, thrombin-mediated induction of NF-κB p65 binding to two NF-κB motifs in the upstream promoter region of VCAM-1 was blocked by LY294002 and rottlerin, whereas the inducible binding of GATA-2 to a tandem GATA motif was inhibited by LY294002 and the PKC-ζ peptide inhibitor. In co-transfection assays, thrombin stimulation of a minimal promoter containing multimerized VCAM-1 NF-κB sites was inhibited by DN-PKC-δ but not DN-PKC-ζ. In contrast, thrombin-mediated transactivation of a minimal promoter containing tandem VCAM-1 GATA motifs was inhibited by DN-PKC-ζ but not DN-PKC-δ. Finally, thrombin failed to induce VCAM-1 expression in vascular smooth muscle cells. Taken together, these data suggest that the endothelial cell-specific effect of thrombin on VCAM-1 expression involves the coordinate activity of PKC-δ-NF-κB and PKC-ζ-GATA signaling pathways.

Vascular adhesion molecule-1 (VCAM-1)† is a 110-kDa cell surface glycoprotein that is expressed in cytokine-activated endothelial cells (1). The VCAM-1 promoter, originally cloned and characterized in cultured endothelial cells (2), represents a potentially valuable tool for dissecting the molecular mechanisms of endothelial cell activation. Several studies have demonstrated the importance of two tandem NF-κB elements located at position –77 and –63, relative to the transcriptional start site, in transducing the response to inflammatory mediators (2–4). Other studies have provided evidence for the role of co-stimulators in mediating cytokine response, including Sp1 (5), activating protein-1 (6), and interferon regulatory factor-1 (7).

In a recent report, we showed that the incubation of endothelial cells with thrombin or the PAR-1 agonist, thrombin receptor activation peptide (TRAP), resulted in increased VCAM-1 mRNA levels and VCAM-1 promoter activity (8). Not surprisingly, a mutation of the tandem NF-κB motif blocked the response to thrombin. Moreover, in electrophoretic mobility shift assays, thrombin induced the binding of p65 homodimers to the two adjacent NF-κB sites in the VCAM-1 promoter. A more interesting finding was that a mutation of a tandem GATA motif (located at –244 and –259) also attenuated the thrombin response. Consistent with these results, thrombin treatment resulted in increased binding of GATA-2 to the VCAM-1 promoter. These data suggested that thrombin-mediated induction of VCAM-1 involves the coordinate activity of NF-κB p65 and GATA-2 and raised new questions as to how the PAR-1 receptor was linked to the downstream transcription factors and whether or not the PAR-1-VCAM-1 pathway was specific to endothelial cells.

In this study, we provide evidence for the importance of PKCs in coupling PAR-1 signaling to NF-κB- and GATA-dependent expression of VCAM-1 in endothelial cells. The PKCs comprise a family of structurally related serine/threonine protein kinase isozymes that play a key role in divergent signaling pathways and cellular functions (9–11). The PKCs are classified into three subgroups, based on structural differences in their regulatory domains and mode of activation. The conventional or classic PKCs (cPKCs-α, -β1, -β2, and -γ) are activated by Ca²⁺, diacylglycerol (DAG), and phorbol esters; the novel PKCs (nPKCs-δ, -ε, -η, and -θ) are activated by DAG and phorbol esters, but not Ca²⁺; and the atypical PKCs (aPKCs-ζ and -λ) are activated by Ca⁺⁺, DAG, and phorbol ester-independent mechanisms. PKC isozymes are differentially distributed between tissues, cell types, and subcellular compartments. Moreover, the various PKC isozymes vary in their response to extracellular signals, substrate specificities, and cellular functions.

Several isozymes of PKC have been identified in cultured endothelial cells, including PKC-α, -δ, -ε, -θ, and -ζ (12, 13). In contrast, PKC-β1 and PKC-λ are undetectable in endothelial cells.
The various PKC isoforms have been shown to differ in their spatial distribution both within endothelial cells and in response to extracellular mediators (12). PKCs have been implicated in multiple endothelial cell functions, including expression of adhesion molecules (14), and endothelin-1 (15), proliferative response to growth factors (16), shear stress signaling (17), and angiogenesis (18).

We show here that thrombin stimulates binding of NF-κB p65 homodimers via a PI3K- and PKC-δ-dependent pathway and binding of GATA-2 through a PI3K- and PKC-δ-dependent signaling cascade. Moreover, we demonstrate that thrombin induces VCAM-1 expression in endothelial cells but not vascular smooth muscle cells (VSMC). Taken together, these findings provide novel information about cell type-specific thrombin signaling.

EXPERIMENTAL PROCEDURES

Materials—Human thrombin and TRAP (SFLRNPNDKYEFP) were obtained from Sigma (St. Louis, MO). Rottlerin, PD98059, SB203580, LY294002, BIM, Go6976, or myristoylated PKC-ζ peptide inhibitor was obtained from Calbiochem (San Diego, CA). Myristoylated PKC-ζ pseudosubstrate peptide inhibitor was obtained from BIOMOL (Plymouth, PA), and human IL-2 was from Peprotec (Rocky Hill, NJ).

Cell Culture—Human umbilical vein endothelial cells (HUVEC) and human coronary artery smooth muscle cells (HCASMC) (Clonetics, La Jolla, CA) were cultured in EGM-2 MV and SmGM-2 complete media, respectively. HUVEC and HCASMC were used within the first eight passages.

Plasmids—The construction of the VCAM-1-1uc plasmid and GATA-1 TK-luc was previously described (6). To generate NF-κB-TK-luc, a double-stranded oligonucleotide containing four copies of the tandem NF-κB motifs from the VCAM-1 promoter was cloned into XhoI-digested TK-luc plasmid vector. Insert direction was confirmed by automated DNA sequencing. The DN (kinase-dead)-PKC-δ and DN-PKC-δ expression plasmids were obtained from Fredig Mjumjed (Dana Farber Cancer Institute, Boston, MA) and Christopher Carpenter (Beth Israel Deaconess Medical Center, Boston, MA), respectively.

RNA Isolation, Northern Blot Analysis, and RNase Protection Assays—HUVEC were serum-starved in EBM-2 MV medium containing 0.5% FBS. 18 h later, HUVEC were pretreated for 30 min with PD98059, SB203580, LY294002, BIM, Go6976, or myristoylated PKC-ζ peptide at the doses indicated and then incubated in the absence or presence of 1.5 units/ml human thrombin for 4 h. Alternatively, HUVEC were infected with adenoviruses encoding the cDNAs of β-galactosidase, dominant negative Akt (CA-Akt), or dominant negative PKC-ζ (DN-PKC-ζ). The recombinant viruses DN-Akt and CA-Akt were a kind gift of Kohjiro Ueki (Joslin Diabetes Center, Boston, MA). The recombinant viruses DN-PKC-ζ and WT-PKC-ζ were generated as previously described (19). Infections were carried out at a multiplicity of infection of 20 for β-galactosidase, CA-Akt, and DN-Akt and a multiplicity of infection of 10 for green fluorescence protein, DN-PKC-ζ, and WT-PKC-ζ for 12 h. Infected cells were grown in complete medium for another 24 h, serum-starved in 0.5% FBS for 12 h, and then treated in the absence or presence of 1.5 units/ml thrombin for 4 h. HCASMC were serum-starved in SmGM-2 medium containing 0.5% FBS. 18 h later, cells were incubated in the absence or presence of 10 ng/ml human IL-2, 1.5 units/ml human thrombin, or 20 mmol/ml TRAP. HUVEC and HCASMC were harvested for total RNA at the times indicated, using the TRIzol reagent (Invitrogen, Gaithersburg, MD). 10 μg of total RNA was electrophoresed on the 1.4% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized for 18 h at 42 °C with 106 cpm/ml [32P]cDNA-labeled human VCAM-1 or tissue factor cDNA probes. The signals were quantitated with Image (National Institutes of Health), and statistical analyses were carried out using the Student t test. Cell migration assays were carried out by modified Boyden chamber, as previously described (20).

Transfection and Analysis of Luciferase Activity—HUVEC were transfected using FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (8). Briefly, HUVEC (1 × 105 cells) were plated in 12-well plates 24 h before transfection. 0.5 μg of the reporter gene construct and 50 ng of a control plasmid containing the Renilla luciferase reporter gene under the control of a cytomeglovirus (CMV) enhancer/promoter (pRL-CMV) (Promega, Madison, WI) were incubated with 2 μl of FuGENE 6. 24 h later, the cells were washed with phosphate-buffered saline two times and cultured for 18 h in serum-starved medium (EBM-2 plus 0.5% FBS). The serum-starved cells were preincubated for 30 min with rottlerin, PD98059, SB203580, LY294002, BIM, Go6976, or myristoylated PKC-ζ peptide inhibitor and then incubated with 1.5 units/ml thrombin for 6 h. The cells were lysed and assayed for luciferase activity using the Dual-luciferase reporter assay system (Promega) and Lumat LB 9507 luminometer (Berthold, Gautersburg, MD). For co-transfections, 0.4 μg of the reporter gene construct, 0.4 μg of the PKC expression vector, and 50 ng of pRL-CMV were incubated with 2 μl of FuGENE 6. The cells were then incubated in the presence or absence of 1.5 units/ml thrombin for 6 h, at which time cells were lysed and assayed for luciferase activity as described above.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as previously described (8, 21). Briefly, double-stranded oligonucleotides were labeled with [32P]dCTP and Klenow fragment and purified by spin column (Amersham Biosciences, Piscataway, NJ). 10 μg of HUVEC nuclear extracts was incubated with 10 fmol of 32P-labeled probe, 1 μg of poly(dI-dC), and 3 μl of 10× binding buffer (100 μM Tris-HCl, pH 7.5, 50% glycerol, 10 mM dithiothreitol, 10 μM EDTA) for 20 min at the room temperature, followed by 30 min at 4 °C. The following oligonucleotides sequences were used for probes: VCAM-1 NF-κB motifs, 5′-TGCGCTGTTTCCCCTTTGAAGTTTCTCCCGCGCTC-3′; VCAM-1 GATA motifs, 5′-ATGTCCTTTATCTTTCCGATAGCCCTTTT-3′. To test the effect of antibodies on DNA-protein complexes, nuclear extracts were preincubated with antibodies to p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature or with antibodies to GATA-2, GATA-3, or GATA-6 (Santa Cruz) for 1 h at 4 °C. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel containing 5% glycerol in 0.5× TBE (50 mM Tris, 50 mM boric acid, and 1 mM EDTA). The loaded gel was fixed with 10% methanol and 10% acetic acid then autoradiographed. Electrophoretic mobility shift assays were carried out in triplicate, using independent preparations of nuclear extracts. The signals were quantified with NIH Image, and statistical analyses were carried out using the Student t test.

RESULTS

Thrombin Induces VCAM-1 mRNA Expression in Endothelial Cells through PI3K, PKK, and p38 MAPK-dependent Signaling Pathways—In a previous study, we demonstrated that thrombin induces VCAM-1 mRNA levels and VCAM-1 promoter activity in primary human endothelial cells and that this effect is mediated by tandem NF-κB and GATA motifs (8). In this study, we wished to delineate the signaling pathways that couple the thrombin receptor to the inducible binding of NF-κB and GATA. To that end, we employed Northern blot analyses to assay for thrombin-mediated induction of VCAM-1 mRNA in the absence or presence of various protein kinase inhibitors. Thrombin-mediated induction of VCAM-1 mRNA expression was inhibited 53% by 1 μM BIM (Fig. 1, lane 4), 67% by 5 μM BIM (Fig. 1, lane 5), 89% by 10 μM LY294002 (Fig. 1, lane 7), 95% by 50 μM LY294002 (Fig. 1, lane 8), 22% by 2.5 μM SB203580 (Fig. 1, lane 10), and 53% by 20 μM SB203580 (Fig. 1, lane 11). In contrast, thrombin stimulation of VCAM-1 was unaltered by 10–50 μM PD98059 (Fig. 1, lanes 13 and 14). Consistent with these results, thrombin-mediated induction of the 1.8-kb human VCAM-1 promoter (spanning the region between −1716 and +119, relative to the start site of transcription) was inhibited 95% by LY294002, 65% by BIM and 67% by SB203580 but not by PD98059 (data not shown). Taken together, these findings suggest that thrombin stimulation of VCAM-1 is mediated by PI3K-, PKC-, and p38 MAPK-dependent, ERK1/2-independent signaling pathway(s).

Thrombin-mediated PI3K-dependent Induction of VCAM-1 mRNA in Endothelial Cells Is Mediated by PKC-δ and PKC-ζ but Not Akt—PI3K lipid products have been shown to activate several downstream substrates. Perhaps the best understood and most widely studied of these targets is Akt. To determine whether the thrombin signal was dependent on Akt, endothelial cells were infected with adenovirus expressing β-galactosidase, constitutively active Akt (CA-Akt), or dominant nega-
Thrombin-mediated Regulation of VCAM-1

Thrombin-mediated Induction of VCAM-1 was not inhibited by the overexpression of DN-Akt (Fig. 2A, lane 6). Moreover, the overexpression of CA-Akt failed to induce VCAM-1 expression (Fig. 2A, lane 3). As a positive control, overexpression of the DN-Akt adenovirus was shown to inhibit insulin-induced glycogen synthase kinase-3 in L6 myotendinous cells (data not shown). More importantly, DN-Akt inhibited vascular epidermal growth factor-mediated induction of endothelial cell migration, whereas CA-Akt was sufficient in inducing endothelial cell migration (Fig. 2B). These findings, which are similar to those of a previous study (22), suggest that the DN-Akt is functioning as a dominant negative in cultured endothelial cells. Therefore, we conclude that Akt is neither necessary nor sufficient for mediating the thrombin response.

In addition to Akt, a number of other signaling molecules lie downstream of PI3K. Some of these mediators, such as p90RSK, are activated by MAPK, and therefore should be inhibited by PD98059. In contrast, activation of PKC isoforms may be regulated in a PI3K/PDK-1-dependent, MAPK-independent manner. Indeed, overexpression of a dominant negative form of PKC-ζ resulted in significant attenuation (70%) of thrombin response (Fig. 2C, lane 6). Moreover, the overexpression of wild-type PKC-ζ resulted in super-induction of VCAM-1 by thrombin (1.8-fold) (Fig. 2C, lane 4). Consistent with these results, the preincubation of HUVEC with a myristoylated PKC-ζ peptide inhibitor resulted in a profound reduction (75%) of thrombin-mediated VCAM-1 stimulation (Fig. 2D, lane 8). Interestingly, the addition of the PKC-δ inhibitor, rottlerin, resulted in complete loss (97%) of thrombin-mediated induction of VCAM-1 (Fig. 2D, lane 6). Together, these data suggest that thrombin stimulation of VCAM-1 is mediated by PI3K-dependent, PKC-ζ and PKC-δ signaling pathways. Finally, the addition of the classic PKC inhibitor, Go6976, resulted in partial inhibition (38%) of the thrombin response (Fig. 2D, lane 4), supporting a role for one of the classic PKC isoforms, namely cPKC-α, cPKC-β1, cPKC-β2, or cPKC-γ.

Thrombin-mediated PI3K-dependent Induction of VCAM-1 Promoter Activity in Endothelial Cells Is Mediated by PKC-δ and PKC-ζ—To establish a role for PKC-δ and PKC-ζ in mediating thrombin stimulation of the VCAM-1 promoter, HUVECs were transiently transfected with the VCAM-1-luc plasmid and treated with thrombin in the presence or absence of PKC isoform-selective inhibitors. As shown in Fig. 3, thrombin-mediated induction of VCAM-1 promoter activity was almost completely inhibited by rottlerin and the myristoylated PKC-ζ peptide inhibitor (93 and 80% inhibition, respectively) and partially inhibited by Go6976 (53% inhibition). These findings are consistent with those of the Northern blot assays. Together, these data suggest that PKC-δ and PKC-ζ lie downstream of PI3K in the PAR-1-VCAM-1 signaling pathway.
Thrombin-mediated Induction of GATA Binding to the VCAM-1 Promoter—In the next set of experiments, a radiolabeled double-stranded oligonucleotide probe encompassing the tandem GATA motif in the VCAM-1 promoter was incubated with nuclear extracts derived from untreated and thrombin-treated HUVEC. As expected, the incubation of probe with nuclear extract from thrombin-treated cells resulted in increased GATA binding (closed and open arrows, Fig. 5). The DNA-protein complexes were specific as defined by inhibitor studies (data not shown). Moreover, the complexes were inhibited by preincubation with the anti-GATA-2 antibody from Santa Cruz Biotechnology (Fig. 5, lane 13) but with anti-GATA-3 or anti-GATA-6 antibodies (data not shown). Thrombin stimulation of GATA-2 DNA-binding activity was inhibited ~82% by preincubation of cells with the PKC-ζ peptide inhibitor and LY294002 (Fig. 5, lanes 5 and 7) and 25% by preincubation with SB203580 (Fig. 5, lane 8). In contrast, GATA binding activity was unaltered by preincubation of cells with thrombin-treated HUVECs resulted in specific DNA-protein complexes (open arrows, Fig. 4). These DNA-protein complexes were inhibited by the addition of a 50-fold molar excess of unlabeled self-competitor but not by the same concentration of unlabeled NF-κB mutant competitor (data not shown). The faster migrating DNA-protein complex (closed arrow) was nonspecific, because it was inhibited by the addition of both wild type and mutant competitors (data not shown). As previously reported (8), the addition of thrombin resulted in a marked increase in DNA binding activity (Fig. 4; compare lane 3 with lane 2). Also consistent with our previous results, the addition of anti-p65 antibody resulted in a supershift of the specific DNA-protein complexes (asterisk, Fig. 4; lane 13). Thrombin-mediated induction of NF-κB binding was inhibited ~75% by preincubation of cells with rottlerin or LY294002 (Fig. 4, lanes 4 and 7), and 22–24% by preincubation with Go6976 or BIM, respectively (Fig. 4, lanes 8 and 9). In contrast, NF-κB DNA-protein complexes were unaffected by preincubation with the PKC-ζ peptide inhibitor, PD98059, or SB203580 (Fig. 4, lanes 5, 6, and 10). Together, these results suggest that thrombin-mediated binding of NF-κB to the VCAM-1 promoter is regulated by a PI3K, PKC-ζ-dependent pathway.

Thrombin-mediated Induction of GATA Binding to the VCAM-1 Promoter Is Regulated by a PI3K- and PKC-ζ-dependent Signaling Pathway —Thrombin stimulation of GATA-2 DNA-binding activity was inhibited ~82% by preincubation of cells with the PKC-ζ peptide inhibitor and LY294002 (Fig. 5, lanes 5 and 7) and 25% by preincubation with SB203580 (Fig. 5, lane 8). In contrast, GATA binding activity was unaltered by preincubation of cells with a phosphatidylinositol 3-kinase (PI3K) inhibitor and a protein kinase C (PKC-ζ) inhibitor. As shown in Fig. 6A, NF-κB transcriptional activity was inhibited ~75% by preincubation of cells with a PI3K inhibitor and a PKC-ζ inhibitor. Together, these results suggest that thrombin-mediated induction of GATA binding is regulated by a PI3K and PKC-ζ-dependent signaling pathway.

Thrombin-mediated Transactivation of a Minimal Promoter Containing Tandem VCAM-1 NF-κB or GATA Motifs Is Inhibited by DN-PKC-ζ and DN-PKC-ζ, Respectively —To more definitively establish a connection between the PKC isoforms and downstream transcription factor activity, HUVEC were co-transfected with a luciferase reporter plasmid containing either two copies of the tandem VCAM-1 GATA motif or four copies of the tandem VCAM-1 NF-κB motif linked to the minimal herpes simplex virus TK promoter (GATAζ-TK-luc and NF-κBζ-TK-luc, respectively) and expression plasmids for either DN-PKC-ζ or DN-PKC-ζ. As shown in Fig. 6A, NF-κBζ-
TK-luc activity was stimulated 5.3-fold by the thrombin treatment. Thrombin-mediated induction of NF-κB-TK-luc was significantly inhibited (64%) by DN-PKC-δ/H9254, but not by DN-PKC-ζ/H9256. In contrast, GATA 4-TK-luc was stimulated 2.1-fold by thrombin, an effect that was completely inhibited by DN-PKC-ζ/H9256, but not the DN-PKC-δ/H9254 (Fig. 6B). Together with the previous results, these findings suggest that thrombin activates the PKC-δ/NF-κB and PKC-ζ/GATA signaling pathways in endothelial cells.

Thrombin Fails to Induce VCAM-1 mRNA Expression in Vascular Smooth Muscle Cells—It is well established that thrombin signaling occurs in VSMC. Moreover, IL-4 has been shown to induce VCAM-1 mRNA in VSMC (23, 24). In the next set of experiments, we wished to determine whether thrombin induces VCAM-1 expression in VSMC. As shown in Fig. 7A, the incubation of HCASMC with 10 ng/ml IL-4, but not 1.5 units/ml thrombin, resulted in increased levels of VCAM-1 mRNA. In contrast to these results, thrombin and TRAP, but not IL-4, resulted in a marked increase in tissue factor expression in HCASMC, with maximal levels occurring at 4 h (Fig. 7B–D). Taken together, these results suggest that thrombin-mediated induction of VCAM-1 mRNA is specific to endothelial cells and that the absence of response in VSMC is attributable to a defect at the post-receptor level.

Thrombin Fails to Induce NF-κB and GATA Binding to the VCAM-1 Promoter in Vascular Smooth Muscle Cells—NF-κB is expressed ubiquitously and has been shown to transduce extracellular signals in endothelial cells and VSMC alike. In contrast, GATA-2 is a marker for endothelial cells and is not

![Fig. 5](image-url)  
Thrombin induces binding of GATA-2 to the VCAM-1 promoter via a PI3K, PKC-ζ-dependent signaling pathway. A, electrophoretic mobility shift assays were performed with 32P-labeled GATA probe in the absence (lane 1) or presence of 10 μg of nuclear extract from HUVECs preincubated for 30 min with vehicle (lanes 2 and 3), 10 μM rottlerin (lane 4), 10 μM myristoylated PKC-ζ inhibitor (lane 5), 50 μM PD98059 (lane 6), 50 μM LY294002 (lane 7), 20 μM SB203580 (lane 8), 1 μM G6976 (lane 9), and 5 μM BIM (lane 10) and then treated in the absence (–) or presence (+) of 1.5 units/ml thrombin. The open arrow indicates specific DNA-protein complexes. The closed arrow indicates the nonspecific DNA-protein complex. For supershift analysis, thrombin-treated HUVECs were incubated with antibody against GATA-2 (lane 13). The asterisk indicates the super-shifted complex. B, quantification of GATA binding. The results show the means ± S.D. of specific signal (relative to untreated cells) obtained from three independent experiments. *, p < 0.05 compared with thrombin treatment, no inhibitor.

![Fig. 6](image-url)  
Thrombin-mediated induction of NF-κB-TK- and GATA-2-TK-promoter activities is inhibited by DN-PKC-δ and DN-PKC-ζ, respectively. A, HUVECs were transiently co-transfected with 0.4 μg of NF-κB-TK-luc and 0.4 μg of expression plasmid for either DN-PKC-δ or DN-PKC-ζ, then treated in the absence (–) or presence (+) of 1.5 units/ml thrombin for 6 h. B, HUVECs were transiently co-transfected with 0.4 μg of GATA-2-TK-luc and 0.4 μg of expression plasmid for DN-PKC-δ or DN-PKC-ζ. The results show the means ± S.D. of luciferase light units (relative to untreated cells) obtained in triplicate from four independent experiments.
The observation that thrombin stimulates VCAM-1 through a PKC-δ-NF-κB p65 pathway is consistent with the results described for the intercellular adhesion molecule (ICAM)-1 gene (25). Together, these data contrast with the majority of published reports, in which PKC-ζ is implicated as the principal activator of NF-κB (26, 27). For example, in NIH-3T3 cells the expression of wild type or activated PKC-ζ was shown to stimulate IKKβ and increase NF-κB activity (28, 29), whereas the overexpression of DN-PKC-ζ had the opposite effect (29). In addition to its role as an IKKβ kinase, PKC-ζ may directly phosphorylate and activate p65 and or-c-Rel via an interaction with the transactivation domain (27). Tumor necrosis factor (TNF)α was shown to induce the expression of ICAM-1-dependent endothelial cells via a PKC-ζ-NFκB-p65-homodimer-dependent mechanism (13). Genetic evidence for the role of PKC-ζ in mediating NF-κB activation was obtained from studies of mice that are null for the PKC-ζ gene (30). The targeted disruption of PKC-ζ resulted in impaired cytokine (TNFα and IL-1)-induced phosphorylation of p65 and NF-κB transcriptional activity in embryonic fibroblasts and reduced IKK activation in lung tissue (30). Together with the results of the ICAM-1 study, our findings suggest that the link between PKC-δ and NF-κB may be relatively specific to PAR-1 signaling in endothelial cells.

Rottlerin, which has been widely used as a PKC-δ-selective inhibitor, was recently reported to uncouple mitochondria and thereby reduce the levels of intracellular ATP in a variety of non-endothelial cell types (31). The latter finding emphasizes the need for caution in interpreting studies with this compound. However, it should also be pointed out that rottlerin was shown to indirectly inhibit stimulus-induced phosphorylation of PKC-δ (31). Moreover, the finding that rottlerin inhibited thrombin-induced binding of p65 NF-κB, but not GATA-2, suggests that this compound exerts some degree of specificity in HUVEC at the level of DNA-protein interactions. Finally, and most importantly, the results of the co-transfection experiments with DN-PKC-δ and DN-PKC-ζ add strong support to our conclusion that thrombin induces VCAM-1 expression via a PKC-δ-NFκB-dependent signaling pathway.

The mechanism by which PKC-δ activates NF-κB and VCAM-1 expression was not specifically addressed in this study. In the case of the ICAM-1 gene, thrombin-mediated activation of PKC-δ was shown to stimulate p38 MAPK, thereby resulting in increased transcriptional activity of NF-κB (25). The importance of p38 MAPK in mediating the transactivation potential of p65 is supported by studies in fibrosarcoma cells, in which TNFα-mediated induction of IL-6 involved a p38 MAPK-sensitive NF-κB pathway (32). In the present study, thrombin stimulation of VCAM-1 was partly inhibited by SB203580, suggesting that p38 MAPK also plays a role in the PAR-1-VCAM-1 pathway.

Thrombin stimulation of NF-κB-dependent TK-luc was not completely abolished (64% inhibition) by co-transfection with the DN-PKC-δ. These results raise the possibility that other signaling pathways are involved in transducing the thrombin signal at the level of NF-κB activity. Consistent with this hypothesis is the observation that inhibition of classic PKC with Gö6976 partially blocked thrombin induction of VCAM-1 mRNA and promoter activity as well as NF-κB binding. An alternative explanation is that the dominant negative (kinase-dead) PKC-δ may directly inhibit endogenous PKC-δ activity in the co-transfection assays.

In contrast to the established function of NF-κB in transducing extracellular signals, GATA binding proteins are widely viewed as constitutively active transcription factors involved in mediating cell type-specific gene expression and lineage determination. Only recently has it become evident that the GATA family of proteins may also play a role in mediating inducible gene expression. GATA DNA-binding activity and/or GATA mRNA expression has been shown to increase in response to a number of mediators, including insulin-like growth factor 1 (33), follicle stimulating hormone (34), endothelin-1 (35), IL-3

Fig. 7. Thrombin fails to induce VCAM-1 mRNA expression in vascular smooth muscle cells. Northern blot analyses of VCAM-1 (A) or tissue factor (B–D) in serum-starved HCASMC treated with 10 ng/ml IL-4 (A and D), 1.5 units/ml thrombin (A and B), or 20 nmol/ml TRAP (C) for the indicated times. The results are representative of two independent experiments.
(36), IL-4 (37), and thrombin (8). GATA activity has been reported to decrease in response to other mediators such as estrogen (38) and transforming growth factor-β (39). Together, these observations suggest that GATA-2 may function as a signal transducer or immediate early gene, coupling short term changes in the extracellular environment to long term changes in gene expression.

The present study is the first to establish a link between PKC- and the GATA family of transcription factors. The mechanism by which PKC- activates GATA binding remains to be determined. Although PKC- has been shown to activate ERK1/2 in response to a variety of signals (40–43), the inability of PD98059 to prevent thrombin-mediated induction of VCAM-1 argues against a role for ERK1/2 in the thrombin-VCAM-1 pathway. The preincubation of HUVEC with SB203580 resulted in partial inhibition of inducible GATA binding, suggesting that MAPK p38 may play a role in the PKC-GATA-2 pathway. It is conceivable that PKC- induces GATA binding activity by altering the redox state of the cell. Alternatively, PKC- may directly phosphorylate GATA-2, resulting in increased DNA-binding and transcriptional activity.

It is interesting to note that PKC- has been located in both the cytosol and the nucleus in resting endothelial cells, in contrast to PKC-, which was exclusively localized to the cytosol (12). The addition of thrombin resulted in a rapid nuclear translocation of PKC-, an effect that was not observed with basic fibroblast growth factor (12). These results suggest that thrombin-activated PKC- may translocate to the nuclear compartment where it activates GATA-2.

Several lines of evidence argue that PI3K-PDK1 lies upstream of thrombin-mediated PKC- NF-κB and PKC- GATA-2 interactions. First, the response of VCAM-1 to thrombin was abrogated in the presence of the PI3K inhibitor, LY294002. Second, the addition of this inhibitor resulted in a significant reduction of NF-κB and GATA-2 binding. Third, the overexpression of dominant negative or constitutively active Akt failed to alter VCAM-1 expression in the absence or presence of thrombin, pointing to the involvement of another downstream substrate. Finally, although PDK1 has been shown to

![Fig. 8. Thrombin fails to induce NF-κB and GATA binding to the VCAM-1 promoter in vascular smooth muscle cells.](image)

**A**
Nuclear Extract - + Thrombin - + GATA2 GATA3 GATA4

![Fig. 9. Model. Schematic shows the endothelial cell-specific effect of thrombin on VCAM-1 expression.](image)

![Thrombin-mediated Regulation of VCAM-1](image)
phosphorylate the activation loop of all PKCs (44, 45), only PDK1-mediated activation of PKC-ζ and PKC-δ is dependent on PI3K (46–51). In contrast to our results, Rahman et al. (52) reported a role for Akt in PAR-1-mediated induction of PKC-δ-NF-κB. It is difficult to reconcile the differences between these two studies. Our investigation focused on the role of DN-Akt in inhibiting endogenous VCAM-1 mRNA levels in endothelial cells, whereas the previous study employed co-transfections with DN-Akt and a synthetic NF-κB or ICAM-1 promoter construct (52). It is formally possible that different signaling pathways mediate PAR-1 stimulation of endogenous VCAM-1 mRNA and NF-κB promoter activity. Indeed, we found that the overexpression of DN-Akt in HUVECs failed to abrogate thrombin stimulation of ICAM-1 (data not shown). It is interesting to point out that many of the co-transfection experiments employed in the previous report involved a construct that contains 5 NF-κB elements from the long terminal repeat of the human immunodeficiency virus (HIV) promoter (52). Previous studies have demonstrated important differences between the VCAM-1- and HIV-containing NF-κB DNA sequences (53). For example, the NF-κB elements from the VCAM-1 promoter are transactivated by p65 homodimers, whereas those from the HIV promoter are transactivated by p65-p50 heterodimers (53). It is conceivable that thrombin signals through different pathways induce NF-κB binding to the HIV and VCAM-1 consensus sites. Another interpretation of the discrepancy in results between these two studies is that our DN-Akt lacked inhibitory activity. However, several lines of evidence argue against this hypothesis. First, previous studies have demonstrated that the inactive phosphorylation mutant of Akt functions effectively as a dominant-negative (22, 54, 55). Moreover, overexpression of the DN-Akt adenovirus inhibited insulin-induced glycosyn thase kinase-3 in L6 myoblast cells. Finally, we demonstrated that DN-Akt inhibited vascular epidermal growth factor-mediated induction of endothelial cell migration, whereas CA-Akt was sufficient in inducing endothelial cell migration. In summary, our data argue strongly against a role for Akt in mediating the thrombin response of VCAM-1.

Although receptor tyrosine kinases are known to activate PI3K-α or PI3K-β, G protein-coupled receptors (GPCR) have been shown to stimulate a distinct subclass of PI3K, PI3K-γ, through an interaction of heterotrimeric G proteins with the catalytic subunit p100y (56, 57). Importantly, GPCR-coupled PI3K-γ is capable of activating PKC-ζ (40). Based on these observations, we propose that the PI3K-γ isomorph may be responsible for mediating thrombin’s effect on the PKC-δ-NF-κB and PKC-ζ-GATA-2 pathways.

The failure of thrombin to induce VCAM-1 expression in VSMC suggests that the PAR-1-VCAM-1 signaling pathway is specific to endothelial cells. Previous studies have established the presence of functional thrombin receptors on VSMC (58–64). Consistent with these reports, we demonstrated that thrombin stimulated tissue factor mRNA levels in human coronary artery VSMC, an effect that was mimicked by the PAR-1 agonist, TRAP. These data suggest that the discordance in VCAM-1 response to thrombin in endothelial cells and VSMC is attributed to differences at the post-receptor level. Because GATA-2 expression is restricted to endothelial cells, and because GATA-2 binding is both necessary and sufficient for thrombin-mediated induction of VCAM-1 in endothelial cells (8), we predicted that the lack of response in VSMC would be attributable to the absence of GATA-2. However, in electrophoretic mobility shift assays, thrombin failed to induce the binding not only of GATA-2 but also NF-κB p65 homodimers to the VCAM-1 promoter. Taken together, these results suggest that thrombin induces VCAM-1 mRNA specifically in endothelial cells and that the failure to stimulate VCAM-1 in VSMC is attributable to a “defect” in the pathway somehow downstream of the PAR-1 receptor and upstream of the NF-κB and GATA transcription factors. It is interesting to speculate that VSMCs express low levels of the GPCR-coupled PI3K-γ isomorph or lack the appropriate repertoire of G proteins to activate PKC-ζ-NF-κB and PKC-ζ-GATA-2 pathways. In support of the former hypothesis, thrombin has been shown to induce only a weak and transient phosphorylation of Akt compared with PDGF in VSMC (65).

An analysis of the differences between the regulation of the VCAM-1 and ICAM-1 genes and between thrombin and TNF-α signaling offers interesting contrasts in signaling mechanisms. In the case of ICAM-1, both thrombin and TNF-α stimulate expression through the inducible binding of NF-κB p65 homodimers. However, thrombin exerts its effect via PKC-δ (and to a lesser extent, PKC-ε), whereas TNF-α signals through PKC-ζ (25). In the case of VCAM-1, thrombin and TNF-α induce expression through the coordinate interaction of NF-κB p65 homodimers and GATA-2 (8). Here we show that thrombin stimulation of NF-κB binding is mediated by PKC-δ, whereas that of GATA-2 binding is dependent on PKC-ζ. It will be interesting to determine whether TNF-α, like thrombin, induces VCAM-1 expression through PKC-δ-NF-κB and PKC-ζ-GATA-2 pathways or whether, like the ICAM-1 gene, TNF-α mediates its effect predominantly through the PKC-ζ isomorph. Moreover, it would be valuable to study endothelial cells that are null for PKC-δ or PKC-ζ. Based on the existing data, one might predict that a deficiency in PKC-δ would lead to impaired ICAM-1 and VCAM-1 response to thrombin, whereas an absence of PKC-ζ might prevent TNF-α-mediated induction of ICAM-1 (via NF-κB) and thrombin stimulation of VCAM-1 (through GATA-2).

Acknowledgments—We thank Christopher Carpenter for helpful suggestions, Alex Toker for critical review of the manuscript, Kohjiro Ueki for invaluable input, and Katherine Spokes for technical assistance.

REFERENCES

1. Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowsky, S., Chi-Rosso, G., and Lobb, R. (1989) Cell 59, 1203–1211
2. Indermaur, M. P., McQuillan, J. J., Rosen, G. D., and Dean, D. C. (1992) J. Biol. Chem. 267, 16323–16329
3. Neish, A. S., Williams, A. J., Palmer, H. H., Whitley, M. Z., and Collins, T. (1995) J. Exp. Med. 181, 1593–1595
4. Shu, H. B., Agrawal, A. B., Nabel, E. G., Leung, K., Dukeett, C. S., Neish, A. S., Collins, T., and Nabel, G. J. (1993) Mol. Cell. Biol. 13, 6283–6289
5. Neish, A. S., Khachigian, L. M., Park, A., Baichwal, V. R., and Collins, T. (1995) J. Biol. Chem. 270, 28003–28009
6. Ahmad, M., Theocharis, P., and Medford, R. M. (1998) J. Biol. Chem. 273, 4616–4621
7. Neish, A. S., Read, M. A., Thanos, D., Pine, R., Maniatis, T., and Collins, T. (1995) Mol. Cell. Biol. 15, 2558–2569
8. Minami, T., and Aird, W. C. (2001) J. Biol. Chem. 276, 47632–47641
9. Nishizuka, Y. (1989) FASEB J. 3, 484–496
10. Dempsey, E. C., Newton, A. C., Mochly-Rosen, D., Fields, A. P., Reyland, M. E., and Lindschau, C. (1998) Am. J. Physiol. 275, C906–C914
11. Liu, W. S., and Heckman, C. A. (1998) Cell. Signaling 10, 529–542
12. Haller, H., Ziegler, W., Lindschau, C., and Luft, F. C. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 678–686
13. Rahman, A., Anwar, K. N., and Malik, A. B. (2000) Am. J. Physiol. 279, C306–C314
14. Deisher, T. A., Haddix, T. L., Montgomery, K. F., Fahimi, T. H., Kaushansky, K., and Harlan, J. M. (1993) FEBS Lett. 331, 283–290
15. Kuchan, M. J., and Frangos, J. A. (1993) Am. J. Physiol. 264, H150–H156
16. Wellner, M., Maasch, C., Kupprion, C., Lindschau, C., Luft, F. C., and Haller, H. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 178–185
17. Misumi, M., FisHEL, R. S., Alexander, R. W., and Berk, B. C. (1993) Am. J. Physiol. 265, I31–I38
18. Davis, C. M., Danelhower, S. C., Laurens, A., and Molony, J. L. (1993) J. Biol. Chem. 268, 206–218
19. Suzuki, K., Takahara, N., Suzuki, I., Ishikii, K., Ueki, K., Leitges, M., Aoi, L. P., and King, G. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 721–726
20. Aizle, M. R., Tsao, J. C., Spokes, R. C., Deshpande, S. S., Imani, K., and Aird, W. C. (2001) FASEB J. 15, 2548–2550
21. Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983) Methods Enzymol. 101, 582–588
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22. Morales-Ruiz, M., Fulton, D., Sowa, G., Languino, L. R., Fujio, Y., Walsh, K., and Sessa, W. C. (2000) Circ. Res. 86, 892–896
23. Wright, P. S., Cooper, J. R., Kropp, K. E., and Busch, S. J. (1999) J. Cell. Physiol. 180, 381–389
24. Li, H., Cymbalsky, M. I., Gimbrone, M. A., Jr., and Libby, P. (1993) Am. J. Pathol. 143, 1551–1559
25. Rahman, A., Anwar, K. N., Uddin, S., Xu, N., Ye, R. D., Platanias, L. C., and Malik, A. B. (2001) Mol. Cell. Biol. 21, 5554–5565
26. Anrather, J., Csizmadia, V., Soares, M. P., and Winkler, H. (1999) J. Biol. Chem. 274, 13594–13603
27. Martin, A. G., San-Antonio, B., and Fresno, M. (2001) J. Biol. Chem. 276, 15840–15849
28. Diaz-Meco, M. T., Lallena, M. J., Monjas, A., Frutos, S., and Moscat, J. (1999) J. Biol. Chem. 274, 19606–19612
29. Lallena, M. J., Diaz-Meco, M. T., Bren, G., Paya, C. V., and Moscat, J. (1999) Mol. Cell. Biol. 19, 2180–2188
30. Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D., and Moscat, J. (2001) Mol Cell 8, 771–780
31. Suhoff, S. P. (2001) J. Biol. Chem. 276, 37986–37992
32. Vanden Berghe, W., Pleasance, S., Boone, K., De Bosscher, K., Schmitz, M. L., Fiers, W., and Haegeman, G. (1998) J. Biol. Chem. 273, 3285–3290
33. Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N., and Rosenthal, N. (1999) Nature 400, 581–585
34. Heinkemo, M., Ermolaeva, M., Bielinska, M., Rahman, N. A., Narita, N., Huhtaniemi, I. T., Tapanainen, J. S., and Wilson, D. B. (1997) Endocrinology 138, 3505–3514
35. Morin, S., Paradis, P., Aries, A., and Nemer, M. (2001) Mol. Cell. Biol. 21, 1036–1044
36. Towatari, M., May, G. E., Marusi, R., Perkins, G. R., Marshall, C. J., Cowley, S., and Enver, T. (1995) J. Biol. Chem. 270, 4101–4107
37. Lee, Y. W., Kuhn, H., Kaiser, S., Hennig, B., Daughtery, A., and Toborek, M. (2001) J. Lipid Res. 42, 785–791
38. Blohe, G. A., Sieff, C. A., and Orkin, S. H. (1995) Mol. Cell. Biol. 15, 3147–3153
39. Minami, T., Rosenberg, R. D., and Aird, W. C. (2001) J. Biol. Chem. 276, 5395–5402
40. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Libby, P. (2002) Circ. Res. 91, 398–405
41. Ahmad, M., Marui, N., Alexander, R. W., and Medford, R. M. (1995) J. Biol. Chem. 270, 8976–8983