Thrombin-Induced p65 Homodimer Binding to Downstream NF-κB Site of the Promoter Mediates Endothelial ICAM-1 Expression and Neutrophil Adhesion[1]

Arshad Rahman,[2] Khandaker N. Anwar, Andrea L. True, and Asrar B. Malik

We investigated the mechanisms by which proinflammatory mediator, thrombin, released during intravascular coagulation and tissue injury, induces ICAM-1 (CD54) expression in endothelial cells. Stimulation of HUVEC with thrombin resulted in dose- and time-dependent increases in ICAM-1 mRNA and cell surface expression and in ICAM-1-dependent endothelial adhesivity toward polymorphonuclear leukocytes. Transient transfection of endothelial cells with ICAM-1 promoter luciferase reporter gene (ICAM-1LUC) constructs indicated that deletion of upstream NF-κB site (−533 bases from translation start site) had no effect on thrombin responsiveness, whereas mutation/deletion of downstream NF-κB site (−223 bases from the translation start site) prevented the activation of ICAM-1 promoter, indicating that the downstream NF-κB site is critical for thrombin inducibility. NF-κB-directed luciferase activity increased ~3-fold when cells transfected with the plasmid pNF-κBLUC containing five copies of consensus NF-κB site linked to a minimal adenovirus E1B promoter-luciferase gene were exposed to thrombin, indicating that activation of NF-κB was essential for thrombin response. Gel supershift assays demonstrated that thrombin induced binding of NF-κBp65 (Rel A) to downstream NF-κB site of the ICAM-1 promoter. Thrombin receptor activation peptide, a 14-amino-acid peptide representing the new NH_2 terminus of proteolytically activated receptor-1, mimicked thrombin's action in inducing ICAM-1 expression. These data indicate that thrombin activates endothelial ICAM-1 expression and polymorphonuclear leukocyte adhesion by NF-κBp65 binding to the downstream NF-κB site of ICAM-1 promoter after proteolytically activated receptor-1 activation. *The Journal of Immunology,* **1999, 162: 5466–5476.**

Thrombin, a serine protease derived from the zymogen prothrombin, plays a critical role in hemostasis (1), and functions as an agonist for responses in a variety of cell types (2–4). In endothelial cells, the responses of thrombin can be classified into two general categories, type I and type II activation. Type I activation (5, 6) includes the events that occur rapidly and are independent of protein synthesis (7–10). Type II activation (6) refers to delayed events that are protein synthesis dependent and are transcriptionally regulated (11–15). Thrombin mediates most of its responses through activation of the G protein-coupled receptor PAR-1[3] that belongs to a new family of protease-activated receptors (16, 17). PAR-1 has a novel mechanism of activation as thrombin binds to the extracellular NH_2-terminal, hirudin-like domain (amino acids 53–64) of the receptor and catalyzes receptor proteolysis between arginine-41 and serine-42 (16). This enzymatic event unmasks a tethered ligand that interacts within sequences corresponding to extracellular loop 2 (amino acid 248–268) of the receptor (18), which in turn activates thrombin's cellular responses. Thrombin receptor activation peptide (TRAP; SFLLRNPNDKYEPF), a 14-amino-acid peptide corresponding to the newly exposed tethered ligand, reproduces many of the cellular responses characteristic of native thrombin (16).

Studies have shown that thrombin is an important regulator of polymorphonuclear leukocyte (PMN) adhesion to endothelial cells (9, 10). The basis of increased endothelial adhesivity may involve type I activation of adhesive proteins such as ICAM-1 (CD54) on the endothelial cell surface. However, there is no evidence indicating that thrombin mediates type II activation of ICAM-1 expression in endothelial cells. The interaction of ICAM-1 with its counter-receptors on the surface of leukocytes, CD11a/CD18 and CD11b/CD18 β_2 integrins, is a critical requirement for PMN adhesion and transendothelial PMN migration (19, 20). ICAM-1 is constitutively present in low levels, but its expression can be transcriptionally up-regulated by cytokines via the activation of NF-κB (21–23). NF-κB/Rel transcription factors are composed of five distinct DNA-binding subunits, called p50, p52, p65 (RelA), c-Rel, and Rel-B (24). The different family members can associate in various homo- or heterodimers through a highly conserved NH_2-terminal sequence, NRD (NF-κB/rel/Dorsal) (25) or Rel homology domain. Inactive NF-κB is sequestered in the cytoplasm by the inhibitory protein I-κB (IκB) and released after phosphorylation of IκB either on serine residues 32 and 36 of IκBα and serines 19 and 23 of IκBβ by IκB kinases α and β, respectively (26, 27), that regulates their ubiquitin-dependent degradation through the 26S proteasome (28–31), or on tyrosine residues of IκBα that does not involve its degradation (32). The activated NF-κB dimer then translocates to the nucleus and regulates transcription of genes such as ICAM-1 involved in inflammatory responses (33–35).

Department of Pharmacology, College of Medicine, University of Illinois, Chicago, IL 60612

Received for publication July 31, 1998. Accepted for publication February 9, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants HL27016, HL46350, and HL45638.

[2] Address correspondence and reprint requests to Dr. Arshad Rahman, Department of Pharmacology, College of Medicine, University of Illinois, 835 South Wolcott Avenue, Chicago, IL 60612-7343. E-mail address: ARahman@uic.edu

[3] Abbreviations used in this paper: PAR-1, protease-activated receptor-1; C/EBP, CAAT enhancer-binding protein; CHX, cycloheximide; COX-2, cyclooxygenase-2; EGM, endothelial growth medium; IκB, inhibitory protein-κB; LUC, luciferase; PDTC, pyrrolidinedithio-carbamate; PMN, polymorphonuclear leukocyte; RLU, relative light unit; Sp-1, promoter selective-1; TRAP, thrombin receptor activation peptide; TRE, tetradecanoylphorbol-13-acetate responsive element.

 0022-1767/99/$02.00

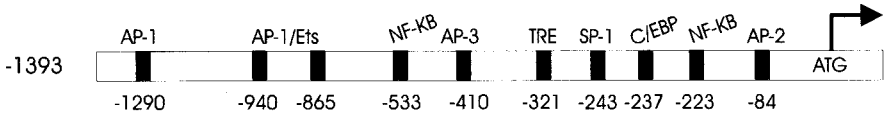

FIGURE 1. Schematic diagram of 5′ regulatory region of human ICAM-1 gene. Rectangles indicate the location of potential binding sites for transcription factors AP-1, AP-2, and AP-3, Ets, NF-κB, TRE, Sp-1, and C/EBP. The arrow above the initiation codon ATG indicates the translation start site.

Sequence analysis of ICAM-1 promoter has revealed the presence of two NF-κB sites (22, 23, 36): the upstream NF-κB (5′-CGGGAGGATTCCTGGGCC-3′, element underlined, within −542 to −524 bases from the translation start site) and the downstream NF-κB (5′-AGCTTGGAAATTCCGGAGCTG-3′, element underlined, within −231 to −211 bases from the translation start site) (Fig. 1). In the present study, we demonstrate that thrombin-induced expression of ICAM-1 is regulated at the level of transcription, and that this expression is mediated by binding of NF-κBp65 to the downstream NF-κB site of ICAM-1 promoter. These data provide evidence linking the activation of the procoagulant, thrombin, to induction of the inflammatory response.

Materials and Methods

Cell culture

HUVEC were obtained from Clonetics (La Jolla, CA) and grown on gelatin-coated flasks or plates in endothelial cell growth medium (EGM) containing 10% FCS, and 3 mg/ml of endothelial-derived growth factor from bovine brain extract protein. Human thrombin with an activity of 3170 NIH U/mg protein was purchased from Enzyme Research Laboratories (South Bend, IN). All experiments, except where indicated, were made using cells under eighth passage. Eahy926 cells, a hybrid cell line of HUVEC and A549 cell line (derived from human lung epithelial type II cells), were provided by Dr. C. J. Edgell (University of North Carolina, Chapel Hill) and cultured as described (38). Eahy926 cells retain endothelial morphology and express endothelial cell-specific marker human factor VIII-related Ag (38), and upon stimulation with TNF-α these cells also express the endothelial cell-specific adhesion molecule E-selectin (39). Confluent HUVEC or Eahy926 cells were starved for 2 h in EGM containing 1–2% FCS or in RPMI containing 0.5% FCS, respectively, and were then incubated in the same medium with thrombin or TRAP for the times and at concentrations indicated in each experiment.

Northern blot analysis

Total RNA was isolated from HUVEC with RNeasy kit (Qiagen, Chatsworth, CA), according to manufacturer's recommendations. Quantification and purity of RNA were assessed by A_{260}/A_{280} absorption, and an aliquot of RNA (20 μg) from samples with ratio above 1.6 was fractionated using a 1% agarose formaldehyde gel. The RNA was transferred to Duralose-UV nitrocellulose membrane (Stratagene, La Jolla, CA) and covalently linked by UV irradiation using a Stratalinker UV cross-linker (Stratagene). Human ICAM-1 (0.96-kb *Sal*I to *Pst*I fragment) (40) and rat GAPDH (1.1-kb *Pst*I fragment) were labeled with [α-^{32}P]dCTP using the random primer kit (Stratagene), and hybridization was conducted as described (41). Briefly, the blots were prehybridized for 30 min at 68°C in QuikHyb solution (Stratagene) and hybridized for 2 h at 68°C with random primed ^{32}P-labeled probes. After hybridization, the blots were washed twice for 30 min at room temperature in 2× SSC with 0.1% SDS, followed by two washes for 15 min each at 60°C in 0.1× SSC with 0.1% SDS. Autoradiography was performed with an intensifying screen at −70°C for 12–24 h. The signal intensities were quantified by scanning the autoradiograms with a laser densitometer (Howtek, Hudson, NH) linked to a computer analysis system (PDI Imageware Systems, Huntington Station, NY). The nitrocellulose membrane was soaked for stripping the probe with boiled water containing 0.1× SSC with 0.1% SDS.

Reporter gene constructs, endothelial cell transfection, and luciferase assay

The ICAM-1LUC reporter plasmid and its 5′ deletion derivatives have been described (36). The constructs containing ~600 bp of ICAM-1 promoter with wild-type (pGL2-WT) and mutated versions of Sp-1 (pGL2-Sp-1-MU, C/EBP (pGL2-C/EBP-MU), and downstream NF-κB site (pGL2-NF-κB-MU) (22) were provided by Dr. Z. Cao (Tularik, San Francisco, CA). The plasmid pNF-κBLUC containing five copies of consensus NF-κB site linked to a minimal E1B promoter-luciferase reporter gene was purchased from Stratagene. HUVEC under the fifth passage or Eahy926 cells were plated into six-well Primaria culture dishes 18–24 h before transfection. Transfections of HUVEC (except for the experiment shown in Fig. 8*A*) were performed using superfect (Qiagen) according to manufacturer's recommendations. Briefly, reporter DNA (1 μg) was mixed with 5–7.5 μl of superfect in 100 μl serum-free EGM (Clonetics, La Jolla, CA). We used 0.2 μg pTKRLUC plasmid (Promega, Madison, WI) containing *Renilla* luciferase gene driven by a constitutively active thymidine kinase promoter to normalize transfection efficiencies. Since we did not observe any significant difference in transfection efficiencies in the initial experiments, we did not cotransfect the pTKRLUC construct in the later experiments. After a 5–10-min incubation at room temperature, 0.6 ml EGM containing 10% FCS was added, and the mixture was applied onto the cells that had been washed once with PBS. Two to three hours later, the medium was changed to EGM containing 10% FCS and the cells were grown to confluency.

Using this protocol, we achieved a transient transfection efficiency of 11 ± 2% (mean ± SD; $n = 3$) for HUVEC. To determine the transfection efficiency, HUVEC were transfected with an expression plasmid pGreen Lantern-1 containing green fluorescence protein gene (Life Technologies, Grand Island, NY). Transfected cells were subjected to FACS analysis for green fluorescence protein expression to determine the transfection efficiency. For transfection of Eahy926 cells, we used lipofectamine (Life Technologies), as described (42). Briefly, reporter DNA (1 μg) was mixed with 2 μl of lipofectamine in 200 μl of Opti-MEM I (Life Technologies). After a 30-min incubation at room temperature, Opti-MEM I (800 μl) was added, and the mixture was applied onto the cells that had been washed twice with Opti-MEM I. Three hours later, the medium was changed to RPMI containing 10% serum and the cells were grown to confluency. Twelve to 18 h before harvesting cells, the medium was replaced with EGM containing 1% FCS or RPMI containing 0.1% FCS, for HUVEC and Eahy926 cells, respectively, and cells were exposed to thrombin (2.5 or 5 U/ml) or TRAP (50 μM). Cell extract was prepared and assayed for luciferase activity using Promega Biotech dual luciferase repoter assay system either by TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) or Monolight 2010 lumionmeter (Analytical Luminiscence Laboratory, Ann Arbor, MI). Firefly luciferase activity was determined and expressed as relative light units (RLU)/μg of cell protein. The protein content was determined using a Bio-Rad protein determination kit (Bio-Rad, Hercules, CA).

Nuclear extract preparation

After appropriate treatments, cells were washed twice with ice-cold Tris-buffered saline (TBS) and resuspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). After 15 min, Nonidet P-40 was added to a final concentration of 0.6%. Nuclei were pelleted and resuspended in 50 μl of buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). After 30 min at 4°C, the lysates were centrifuged and supernatants containing the nuclear proteins were transferred to new vials. The protein concentration of the extract was measured using a Bio-Rad protein determination kit (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assays (EMSA)

EMSA were performed as described (42). Briefly, 10 μg of nuclear extract was incubated with 1 μg of poly(dI-dC) in a binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, and 10% glycerol (20 μl final volume)) for 15 min at room temperature. Then end-labeled double-stranded oligonucleotides containing the downstream NF-κB site of ICAM-1 promoter (30,000 cpm each) were added in the absence or presence of 25- or 100-fold molar excess cold competitor, and the reaction mixtures were incubated for 15 min at room temperature. In Ab supershift

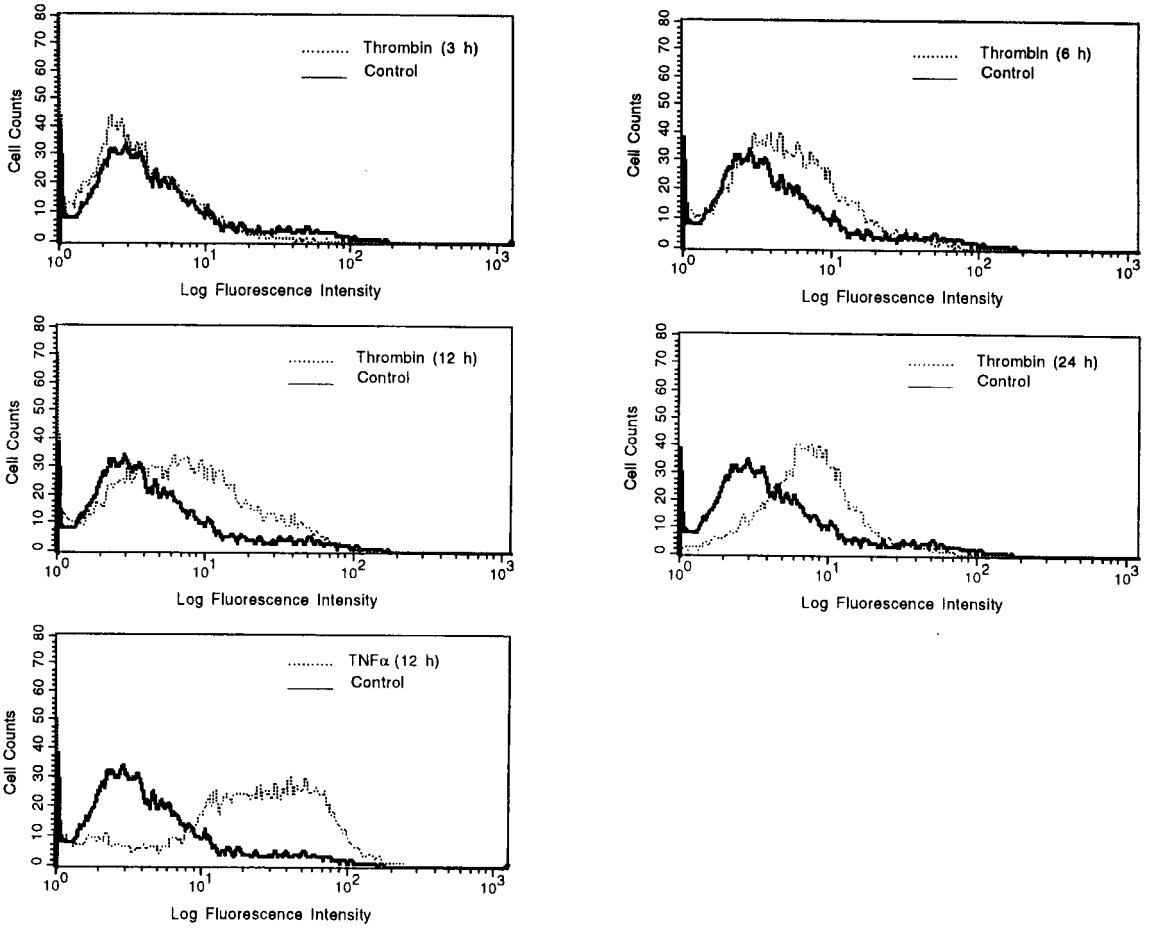

FIGURE 2. Thrombin induces ICAM-1 expression on endothelial cell surface. Confluent HUVEC monolayers were stimulated with thrombin (2.5 U/ml) for 3, 6, 12, and 24 h with thrombin (2.5 U/ml) or for 12 h with TNF-α (100 U/ml). ICAM-1 expression was quantitated by flow cytometry using mAb against ICAM-1 (BIRR0001) or mAb against IgG, as described in *Materials and Methods*. Comparative FACS profiles of control untreated (thick line), thrombin-, or TNF-α-treated (dotted line) cells are shown. A representative experiment of two performed is shown.

experiments, nuclear extracts were incubated for 15 min at room temperature with polyclonal rabbit Ab to human NF-κB proteins (p65 [RelA], p50, p52, c-Rel, and RelB) (obtained from Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 2 μg/20 μl before incubation with the labeled probe for another 15 min at room temperature. The DNA-protein complexes were resolved in 5% native polyacrylamide gel electrophoresis in low ionic strength buffer (0.25× Tris-borate-EDTA). Oligonucleotides used for the gel shift analysis were as follows: ICAM-1 NF-κB, 5′-AGCTTGGAAATTCCGGAGCTG-3′; mut-ICAM-1 NF-κB, 5′-AGC TTccAAATTCCGGAGCTG-3′.

The oligonucleotide designated as ICAM-1NF-κB represents a 21-bp sequence of ICAM-1 promoter encompassing the downstream NF-κB binding site located 223 bp upstream of translation initiation site (22, 23). The oligonucleotide mut-ICAM-1NF-κB is the same as ICAM-1NF-κB, except that it has 2-bp mutations in NF-κB site. Sequence motifs within the oligonucleotides are underlined and the mutations are shown in lower case.

Flow cytometry analysis

Flow cytometry analysis was perfomed as described (42). Briefly, HUVEC monolayers in six-well Primaria tissue culture dishes were stimulated with thrombin for various time points. After completion of incubation period, cells were washed twice with cold PBS, removed by careful trypsinization, and washed again with Ca^{2+}/Mg^{2+}-free PBS before incubating with 20% horse serum for 30 min. Following two washes, cells were incubated with a mouse mAb directed against human ICAM-1, BIRR0001 (kindly provided by Dr. Robert Rothlein, Boeringer Ingleheim, Ridgefield, CT) (43), in Ca^{2+}/Mg^{2+}-free PBS containing 3% horse serum for 30 min at 4°C. Cells were then washed twice with PBS/horse serum and incubated for 30 min at 4°C with a goat anti-mouse IgG FITC-conjugated secondary Ab. Cells were then fixed with 2% paraformaldehyde, and analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA), and the results were gated for mean fluorescence intensity above the fluorescence produced by the secondary Ab alone.

PMN adhesion assay

PMN adhesion assay was performed as described (9). Briefly, confluent HUVEC monolayers in 24-well plates were incubated with thrombin for 2 or 6 h. At the end of each incubation period, HUVEC were fixed with 1% paraformaldehyde/PBS at room temperature for 15 min, and washed three times with DMEM without serum. The ^{51}Cr-labeled human PMN (2×10^6 cells/ml DMEM) were distributed at 1 ml/well over the HUVEC and co-incubated for 1 h at 37°C in 5% CO_2 and 98% humidity.

To determine the contribution of ICAM-1 in thrombin-induced PMN adherence, HUVEC were treated with RR1/1 (mAb to ICAM-1) 15 min before ^{51}Cr-labeled PMN administration at a concentration of 10 μg/ml. HUVEC monolayers were then gently washed three times with DMEM without serum to remove the nonadherent PMN. Endothelial monolayers were kept overnight in 1 ml of 1 N NaOH at 4°C. The cell lysates were scraped, collected in polypropylene tubes, and counted for radioactivity in a Tm analytical gamma counter. Phase-contrast microscopy confirmed HUVEC integrity and PMN adherence to HUVEC.

Results

Thrombin induces ICAM-1 cell surface expression and PMN adhesion to HUVEC

We previously showed that thrombin increased endothelial adhesivity toward PMN within 0.5 h and that this response was associated with type I activation of ICAM-1 expression, that is, this early response was protein synthesis inhibitor cycloheximide (CHX) insensitive and did not require increased ICAM-1 mRNA

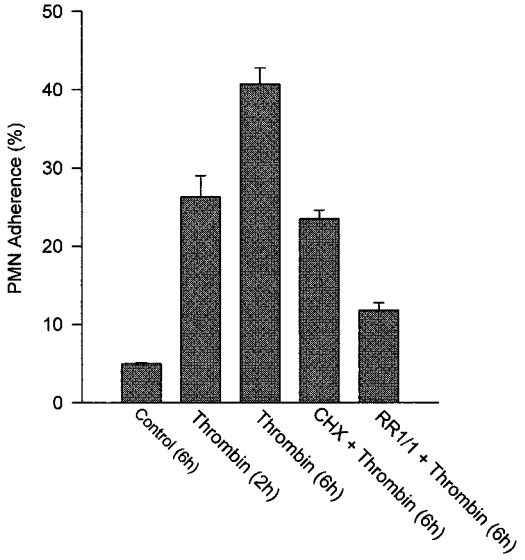

FIGURE 3. Thrombin induces endothelial adhesivity toward PMN. Confluent HUVEC monolayers were pretreated with or without CHX, and then stimulated with thrombin (2.5 U/ml) for 2 or 6 h, washed with DMEM, and fixed with 1% paraformaldehyde/PBS for 15 min at room temperature, after which unstimulated ^{51}Cr-labeled PMN (2×10^6) were layered onto endothelial monolayers for 1 h in 1 ml of DMEM with HEPES at 37°C, and adhesion was determined. In some experiments, thrombin-stimulated HUVEC were preincubated with mAb RR1/1 (anti-ICAM-1) (10 μg/ml) before applying PMN. Data are mean $\pm$ 1 SE; $n = 6$ for each condition.

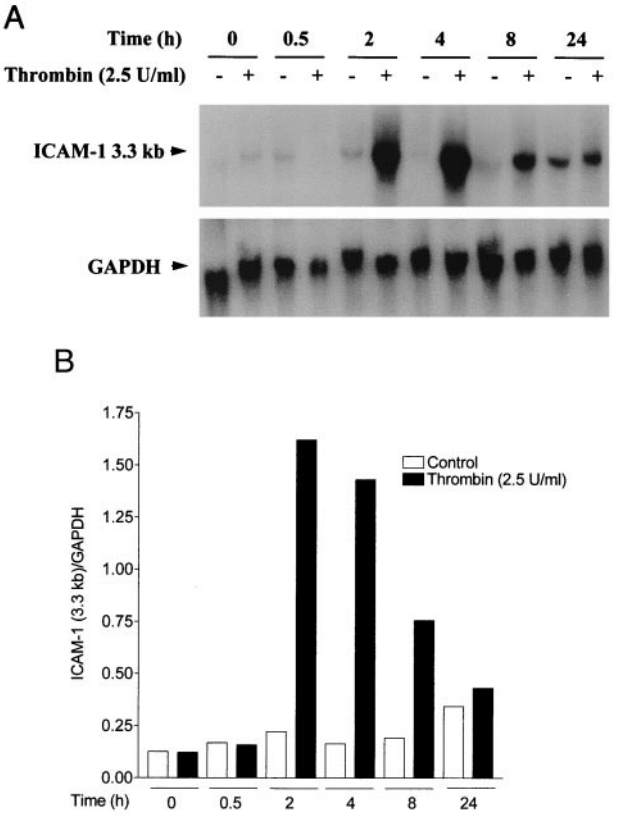

FIGURE 4. Thrombin induces ICAM-1 mRNA expression in HUVEC. Confluent HUVEC monolayers were stimulated with or without thrombin (2.5 U/ml) for the indicated time periods. Total RNA was isolated and analyzed by Northern hybridizations with human ICAM-1 or rat GAPDH cDNAs, which hybridize to a 3.3- or 1.3-kb transcript, respectively. *A*, Autoradiogram. *B*, Bar graph representing the relative intensities of ICAM-1 mRNA signals. A representative experiment of two performed is shown.

expression (9). To examine whether thrombin-induced ICAM-1 expression is initially independent of de novo ICAM-1 protein synthesis, but later becomes protein synthesis dependent, we determined the time course of thrombin-induced ICAM-1 expression on endothelial cell surface as assessed by flow cytometry (Fig. 2). ICAM-1 expression increased within 6 h of thrombin challenge and continued to increase further at 12 and 24 h (Fig. 2). TNF-α, used as a positive control, also induced cell surface expression of ICAM-1 (Fig. 2).

The time course of PMN adherence to HUVEC induced by thrombin challenge correlated with the kinetics of ICAM-1 cell surface expression (Fig. 3). Approximately 50% of the increased PMN adherence to HUVEC at 6 h was CHX sensitive (Fig. 3), consistent with the finding that 50% of ICAM-1 expression at 6 h was CHX sensitive (data not shown). The CHX sensitivity of PMN adhesion in the 6-h thrombin-stimulated cells (Fig. 3) was in contrast to the thrombin response at 2 h, which was insensitive to CHX (9). Fig. 3 shows that pretreatment of HUVEC with mAb RR1/1 reduced PMN adherence at 6 h by ~80%, indicating a role of ICAM-1 in mediating thrombin-induced PMN adhesion.

Given that type I activation of P-selectin and P-selectin-mediated PMN adherence is rapid and returns to the basal level within 2 h after thrombin challenge of HUVEC (9), it is unlikely that type I activation of P-selectin contributes to 6-h thrombin-induced PMN adherence. Furthermore, since studies have shown type II activation of P-selectin gene in murine, but not in human, endothelial cells (44, 45), it is unlikely that thrombin mediates type II activation of P-selectin expression, and therefore contributes to 6-h thrombin-induced PMN adherence to HUVEC. Thus, these data suggest an early protein synthesis-independent ICAM-1 expression induced by thrombin and a delayed and progressive protein synthesis-dependent response, which prolongs and stabilizes PMN adhesion.

Thrombin induces ICAM-1 mRNA expression

To determine whether protein synthesis-dependent expression of ICAM-1 is preceded by an increase in ICAM-1 mRNA expression, Northern blot analysis was performed to measure the abundance of ICAM-1 transcript, up to 24 h, after addition of thrombin (2.5 U/ml) to the medium. Thrombin induced ICAM-1 mRNA expression in a time-dependent manner, with maximum induction occurring between 2 and 4 h after thrombin challenge, followed by a ~55% decrease at 8 h, and returned to the basal level by 24 h (Fig. 4, *A* and *B*).

Thrombin-induced ICAM-1 gene transcription requires activation of PAR-1

We evaluated the effects of TRAP, a 14-amino-acid peptide representing the new NH_2 terminus of PAR-1 generated after thrombin cleavage, to determine whether the induction of ICAM-1 mRNA expression requires activation of PAR-1. Both thrombin and TRAP induced the expression of ICAM-1 transcript in a dose-dependent manner (Fig. 5, *A* and *B*). The 3.3-kb transcript increased slightly with 1 U/ml thrombin or 12.5 μM TRAP; the maximum induction occurred with 5 U/ml or 50 μM TRAP (Fig. 5, *A* and *B*), indicating that thrombin-induced ICAM-1 transcription occurs secondary to the cleavage of PAR-1.

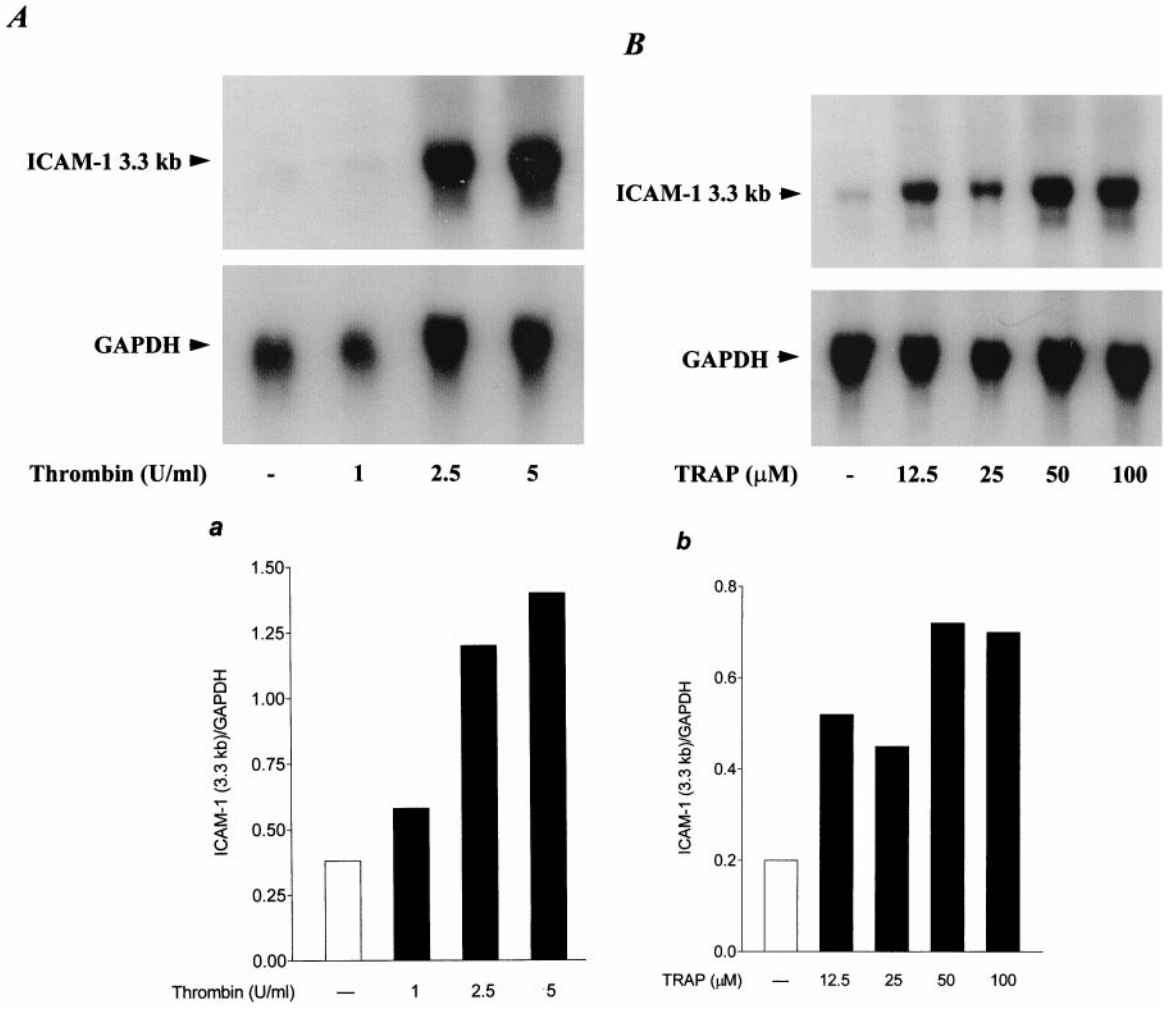

FIGURE 5. Thrombin-induced ICAM-1 mRNA expression requires activation of cell surface PAR-1. HUVEC were treated for 3 h with thrombin (*A*) or TRAP (*B*) at the indicated concentrations. ICAM-1 and GAPDH mRNA expression was determined, as described in *Materials and Methods*. *A* and *B*, Autoradiograms. *a* and *b*, Bar graphs representing the relative intensities of ICAM-1 mRNA signals in response to thrombin and TRAP, respectively (representative of two separate experiments).

Thrombin-induced ICAM-1 mRNA expression does not require novel protein synthesis

We used CHX to determine whether thrombin induction of ICAM-1 transcript was a direct effect of thrombin treatment or it required the synthesis of additional proteins. The presence of CHX in the medium during thrombin treatment of HUVEC did not prevent the thrombin-induced ICAM-1 mRNA expression (Fig. 6), suggesting that proteins necessary to mediate thrombin response were already present in cells. CHX alone caused a slight induction of ICAM-1 transcript (Fig. 6), a characteristic of NF-κB-dependent genes, since CHX is known to activate nuclear transport of NF-κB, presumably by inhibiting synthesis of relatively labile IκB proteins (24).

Inhibition of NF-κB activation prevents thrombin-induced ICAM-1 mRNA expression

To further assess the role of NF-κB in mediating ICAM-1 expression by thrombin, we used PDTC, an antioxidant that prevents NF-κB activation, and thereby its translocation to the nucleus through its ability to chelate metal ions and deliver thiol groups to cells (46). Confluent HUVEC monolayers were treated with PDTC for 0.5 h before stimulation with thrombin for 3 h. PDTC prevented thrombin-induced ICAM-1 mRNA expression in a dose-dependent manner (Fig. 7). These data suggest that reactive oxygen species, produced during inflammatory response, function as second messengers in activating NF-κB and may mediate ICAM-1 transcription in thrombin-stimulated endothelial cells.

Thrombin activates ICAM-1 gene promoter in endothelial cells

We assessed the effects of thrombin on transcriptional activity of the ICAM-1 promoter to demonstrate that thrombin was capable of activating ICAM-1 gene transcription. HUVEC were transfected with a full-length wild-type construct containing 1393 bp of the ICAM-1 promoter linked to the firefly luciferase gene (ICAM-1 LUC). Thrombin increased ICAM-1 promoter activity (Fig. 8*A*). A similar but more pronounced activation by thrombin or TRAP was observed in Eahy926 cells (Fig. 8*B*). TNF-α or PMA, used as positive controls, also activated the promoter in these cells (Fig. 8, *A* and *B*). Since transfection efficiency and the consequent luciferase activity were 2- to 3-fold higher in Eahy926 cells, we used these cells to localize the thrombin-responsive region within the ICAM-1 promoter (as described below).

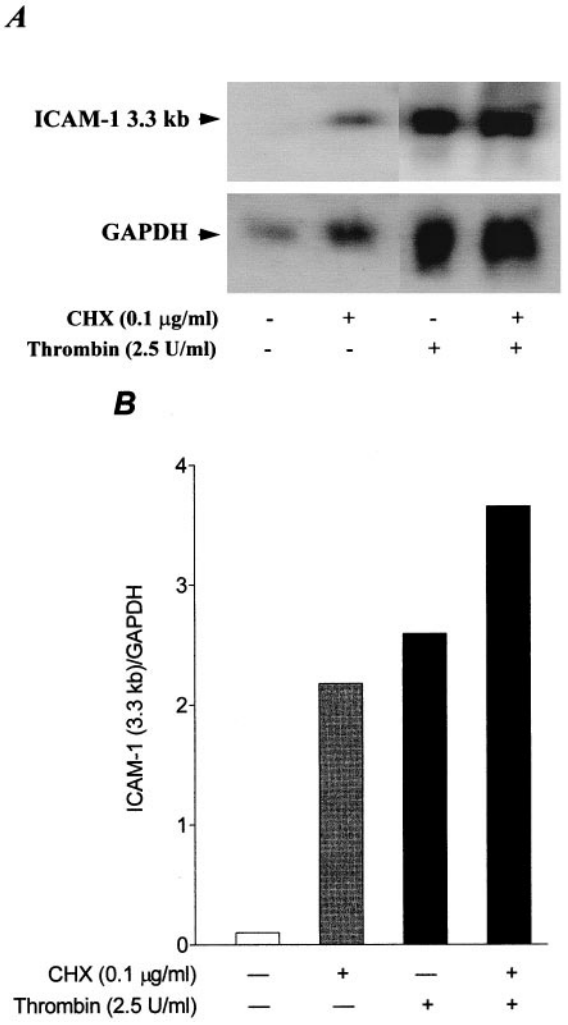

FIGURE 6. Thrombin-induced ICAM-1 gene transcription does not require novel protein synthesis. Confluent HUVEC monolayers were pretreated for 0.5 h with CHX, followed by stimulation with thrombin for a period of 3 h in continuous presence of CHX. ICAM-1 and GAPDH mRNA expression was determined by Northern blotting, as described in *Materials and Methods*. *A*, autoradiogram; *B*, bar graph representing the relative intensities of ICAM-1 mRNA signals (representative of two separate experiments).

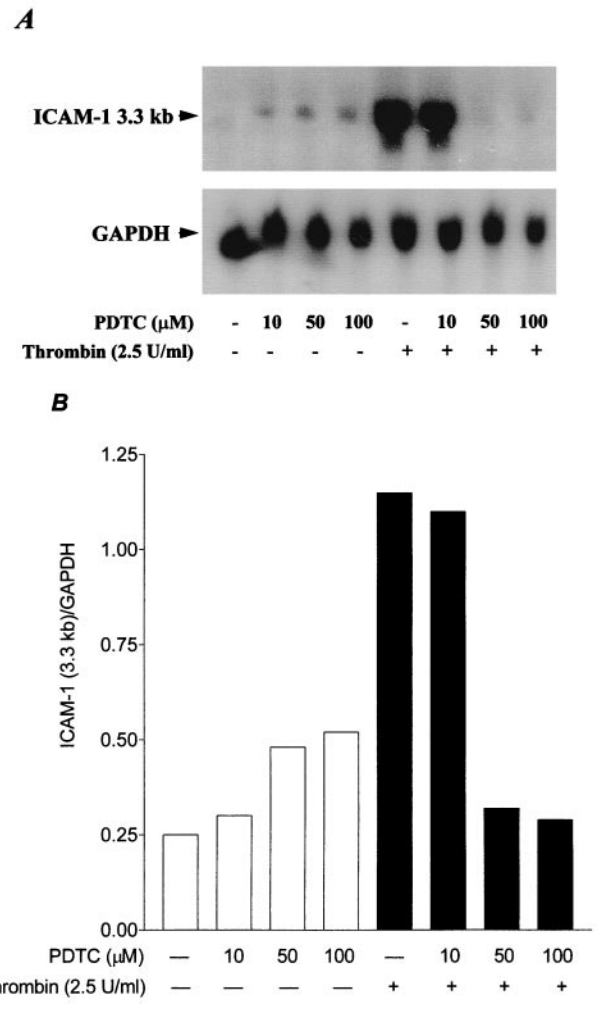

FIGURE 7. PDTC prevents thrombin-induced ICAM-1 gene transcription. Confluent HUVEC monolayers were preincubated with the indicated concentrations of PDTC for 0.5 h and then stimulated with thrombin (2.5 U/ml) for 3 h in continuous presence of PDTC. ICAM-1 and GAPDH mRNA expression was determined as described in *Materials and Methods*. *A*, Autoradiogram; *B*, bar graph representing the relative intensities of ICAM-1 mRNA signals. A representative experiment of two performed is shown.

Localization of thrombin-responsive region within ICAM-1 promoter

The ICAM-1 promoter contains a number of *cis*-acting elements of potential importance in mediating the activation of ICAM-1 gene (Fig. 1). We used a set of 5′ deletion mutation constructs containing different lengths of the ICAM-1 promoter linked to the firefly luciferase reporter gene to localize the thrombin-responsive elements. Deletion of ICAM-1 promoter sequences 393 bp upstream of the ATG start codon decreased basal promoter activity (Fig. 9). AP-1, AP-1/Ets repeats, NF-κB, and AP-3 are the apparent elements in this region, indicating that these sites are important for basal expression of construct 393 ICAM-1 LUC. However, the expression of this construct was still strongly activated by thrombin and TRAP, indicating that AP-1, AP-1/Ets, AP-3, and the upstream NF-κB site (533 bp upstream of translation start site) are not essential for thrombin responsiveness (Fig. 9). Thrombin responsiveness was lost upon deletion from position 393 to 176 bp, suggesting that *cis*-regulatory elements responsible for thrombin response reside within this region (Fig. 9). Potential binding sites for AP-1 (tetradecanoylphorbol-13-acetate responsive element (TRE)), SP1, C/EBP, and NF-κB transcription factors are located within this region.

Since thrombin is known to stimulate AP-1 DNA binding and AP-1-mediated transactivation (47), we used a plasmid containing three copies of TRE from ICAM-1 gene linked to a minimal β-globin promoter-luciferase reporter gene to determine whether TRE could function as the thrombin response element. As shown in Table I, TRE-directed promoter activity did not increase significantly when the transfected cells were exposed to thrombin or TRAP.

Mutation of downstream NF-κB site prevents thrombin-induced ICAM-1 promoter activation

To ascertain the role of Sp-1, C/EBP, and downstream NF-κB sites in mediating ICAM-1 promoter activation by thrombin, we transfected HUVEC with pGL2-WT and -MU vectors containing wild-type and mutant versions of Sp-1 (pGL2-Sp-1-MU), C/EBP (pGL2-C/EBP-MU), and downstream NF-κB (pGL2-NF-κB-MU) site. Thrombin induced a ~4-fold increase in ICAM-1 promoter

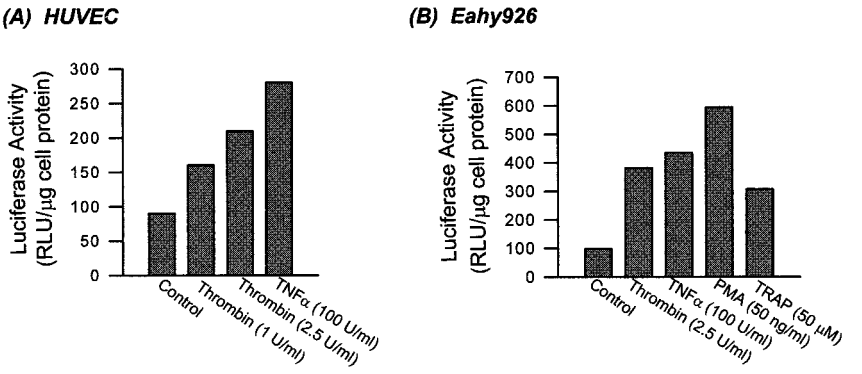

FIGURE 8. Activation of ICAM-1 promoter by thrombin in endothelial cells. The 1393-bp ICAM-1LUC construct was transfected into HUVEC (*A*) or Eahy926 (*B*) cells using lipofectamine, as described in *Materials and Methods*. At 24 h after transfection, the cells were left untreated (control) or stimulated with thrombin, TRAP, TNF-α, or PMA at the indicated concentrations, and were harvested 15 h after treatment, and cell extract was assessed for luciferase activity using a Monolight 2010 luminometer. Luciferase activity is expressed as RLU/μg of cell protein. Data shown are the average of two separate experiments performed in triplicates.

activity when pGL2-WT was used, whereas thrombin failed to increase ICAM-1 promoter activity in cells transfected with pGL2-NF-κB-MU, which is the same as pGL2-NF-κB-WT, except that it has 2-bp mutations in downstream NF-κB site (Fig. 10). However, Sp-1 and C/EBP sites do not appear to be important, as the mutations in these sites failed to prevent thrombin-induced luciferase activity in cells transfected with pGL2-Sp-1-MU and pGL2-C/EBP-MU vectors. These results indicate that the downstream NF-κB site is critical in mediating thrombin-induced activation of ICAM-1 promoter.

NF-κB activation is essential to mediate thrombin response

To determine whether activation of NF-κB site is necessary to confer thrombin inducibility of ICAM-1 gene, we transfected HUVEC with a plasmid pNF-κBLUC containing five copies of consensus NF-κB sequence from Ig gene linked to a minimal adenovirus E1B promoter-luciferase reporter gene. As shown in Fig. 11, NF-κB-directed promoter activity increased ~3-fold when the transfected cells were exposed to thrombin. These data indicate that NF-κB sequence alone is capable of mediating thrombin response.

Thrombin induces DNA-binding activity on downstream NF-κB site of ICAM-1 promoter

To determine whether the downstream NF-κB site of ICAM-1 promoter is capable of binding thrombin-activated DNA-binding proteins, we synthesized an oligonucleotide containing this sequence, prepared nuclear extracts from thrombin-stimulated HUVEC, and determined the binding activity by EMSA. Fig. 12 shows that thrombin-induced DNA-binding activities, when assayed on nondenaturing gel electrophoresis, resolved into three closely migrating bands. We performed competition experiments to determine the specificity of these binding activities. These DNA-binding activities were competed by specific oligonucleotide probe, ICAM-1NF-κB (*lane 4*), yet remained intact when challenged with an oligonucleotide (mut-ICAM-1NF-κB) bearing 2-bp substitution in the downstream NF-κB site of ICAM-1 promoter (*lane 5*). The fast migrating activities (bands 2 and 3) are nonspecific, as evidenced by the appearance of these bands when the same nuclear extract was tested for its ability to bind to the mutant version of downstream NF-κB site (*lane 6*).

We next performed supershift experiments using specific Abs to p50, p65 (Rel A), p52, c-Rel, and RelB to determine the identity of proteins in the thrombin-induced NF-κB-binding complex. Incubation with Ab to p65 abolished the slowest migrating complex (band 1), with a concomitant supershift, but did not affect the fast migrating protein:DNA complexes (bands 2 and 3) (Fig. 13*A*). Abs to p50, p52, c-Rel, or Rel B had no effect on any of these bands (Fig. 13*A*). As a positive control, we used TNF-α, which is known to induce NF-κBp65 binding to the downstream NF-κB site of ICAM-1 promoter (23). As in the case of thrombin, Ab to p65 also

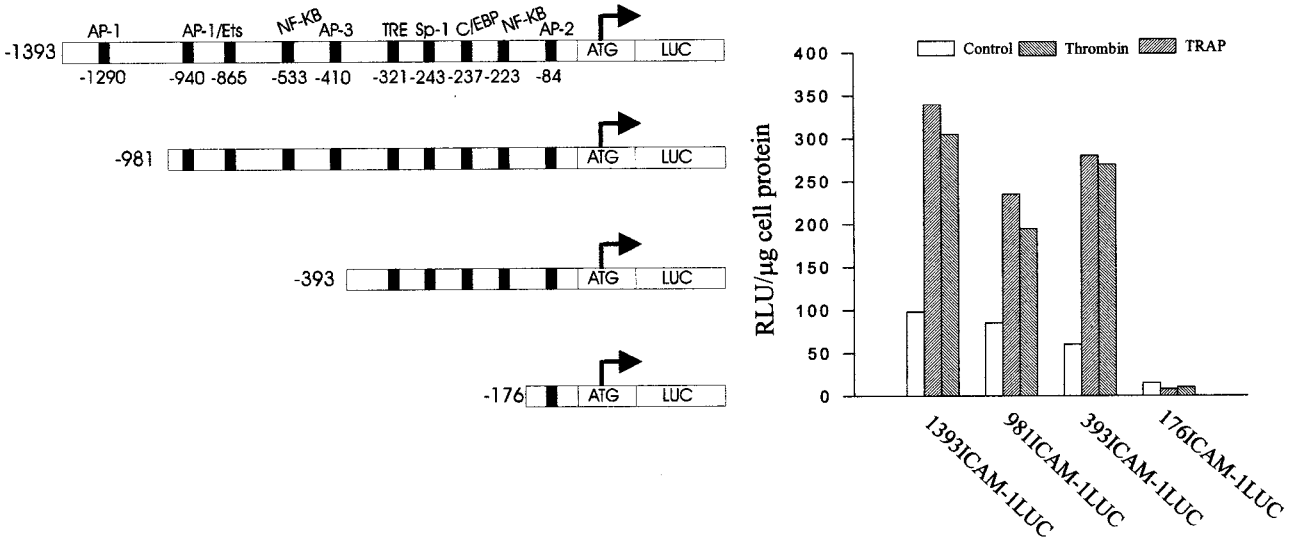

FIGURE 9. Localization of thrombin-responsive region within ICAM-1 promoter. The structure of different ICAM-1LUC constructs is shown at *left*. The nucleotide position of 5′ end of each construct is given relative to initiation codon of the gene. The relative luciferase activities per microgram of cell protein to each construct transiently expressed in Eahy926 cells untreated (control) or stimulated with thrombin (2.5 U/ml) or TRAP (25 μM) are given at *right*. Luciferase activity in these experiments was measured using a Monolight 2010 luminometer. Data shown are the average of three separate experiments performed in triplicates.

Table I. *Effect of thrombin and TRAP on ICAM-1-TRE driven luciferase expression*[a]

Agonist	Relative Luciferase Activity (RLU/μg cell protein)
None	50 ± 0.37
Thrombin (2.5 U/ml)	66 ± 0.36
TRAP (25 μM)	74 ± 0.30

[a] Three copies of TRE element from the ICAM-1 promoter linked to β-globin TATA box luciferase construct (pICAMTRE1) (36) was transfected into the Eahy926 cell using lipofectamine. Cells were exposed to thrombin or TRAP at the indicated concentrations. At 18 h, cells were harvested and luciferase activity was determined using a Monolight 2010 luminometer as described in *Materials and Methods*. Data are mean ± 1 SE; $n = 4$ for each condition.

disrupted TNF-α-induced band 1, with a concomitant supershift, whereas Abs to p50 or p52 did not have an effect (Fig. 13*B*). To demonstrate that the failure of p50 or other Abs to elicit a supershifted complex is the result of the absence of these proteins in the NF-κB complexes interacting at the ICAM-1 NF-κB site rather than the inability of these Abs to supershift under the conditions used, we performed another control experiment in which anti-p50 indeed caused a supershift when mixed with purified p50 protein (data not shown). Thus, these data indicate that thrombin exposure of HUVEC results in a binding complex (as evidenced by band 1) containing the p65 homodimer.

Discussion

In the present study, we demonstrate for the first time that the serine protease thrombin, through the activation of its cell surface receptor PAR-1, induces ICAM-1 gene expression in vascular endothelial cells via the NF-κB-dependent pathway. Thrombin stimulation of HUVEC resulted in increased ICAM-1 mRNA and cell surface expression, and consequently ICAM-1-dependent PMN adhesion to endothelial cells. The expression of ICAM-1 message did not require novel protein synthesis, as it was insensitive to CHX. The mRNA expression peaked between 2–4 h after thrombin challenge, and returned to the basal level by 24 h. EMSA, mutation/deletion construct reporter gene transfection assays, and NF-κB inhibition experiments indicated that binding of NF-κB p65 to the downstream NF-κB site of ICAM-1 promoter mediated thrombin-induced ICAM-1 transcription.

NF-κB complexes containing the p65 subunit are known to play an important role in the generation of an inflammatory response.

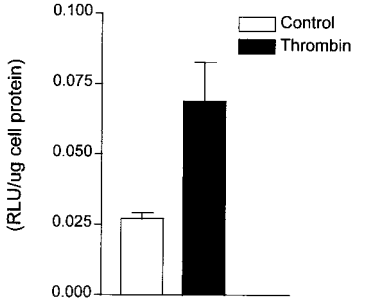

FIGURE 11. NF-κB is essential to confer thrombin inducibility. HUVEC were transfected with pNF-κB-LUC plasmid containing five copies of NF-κB sites linked to firefly luciferase reporter gene. At 24 h after transfection, cells were left untreated (control) or stimulated with thrombin (5 U/ml) and were harvested 6 h after treatment, and cell extract was assessed for luciferase activity using a Turner TD 20/20 luminometer. Luciferase activity is expressed as RLU/μg of cell protein. Data are mean ± 1 SE; $n = 6$ for each condition.

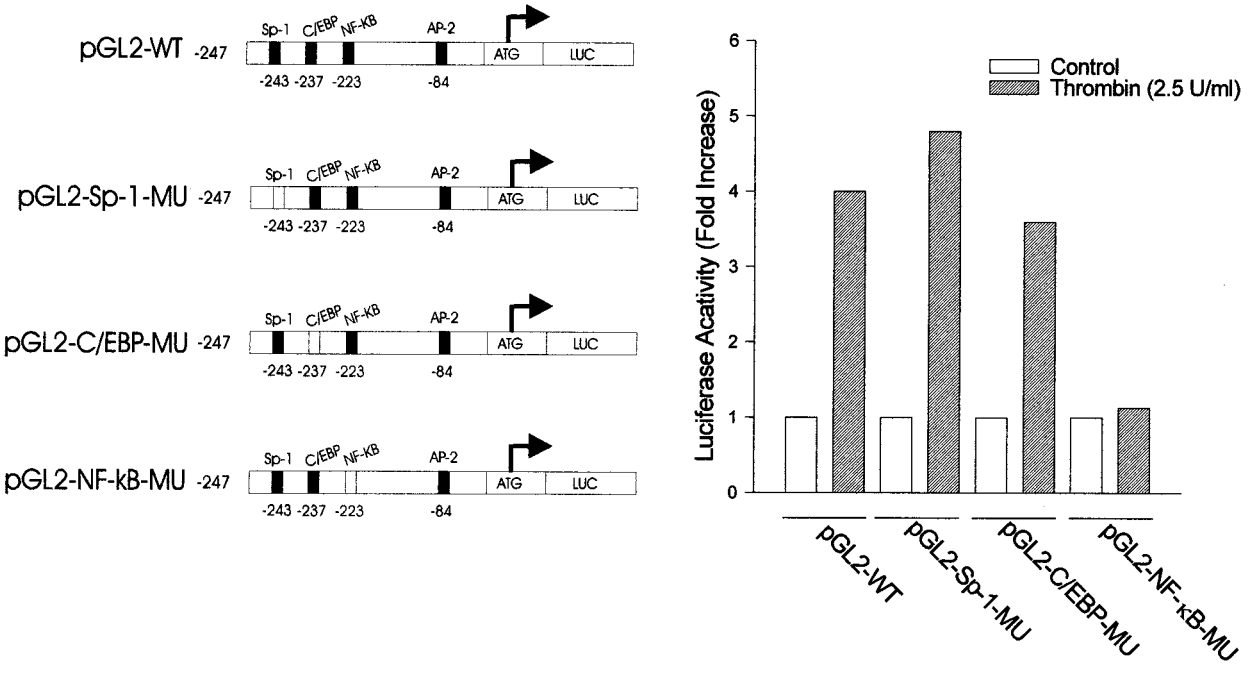

FIGURE 10. Mutation of downstream NF-κB site prevents thrombin-induced ICAM-1 promoter activation. The pGL2-WT or -MU vectors containing wild-type (solid rectangles) and mutant versions (open rectangles) of Sp-1 (pGL2-Sp-1-MU), C/EBP (pGL2-C/EBP-1-MU), and the downstream NF-κB (pGL2-NF-κB-MU) site and pTKRLUC plasmid were cotransfected into HUVEC using superfect, as described in *Materials and Methods*. The pTKRLUC plasmid contains a *Renilla* luciferase reporter gene driven by the constitutively active thymidine kinase promoter. At 24 h after transfection, cells were left untreated (control) or stimulated with thrombin at the indicated concentration, and were harvested 15 h after treatment, and cell extract was assessed for luciferase activity using a TD 20/20 luminometer. Firefly luciferase activity normalized to *Renilla* luciferase activty is expressed as fold increase relative to the untreated medium control of each construct. Values shown are the average of three separate experiments performed in triplicates.

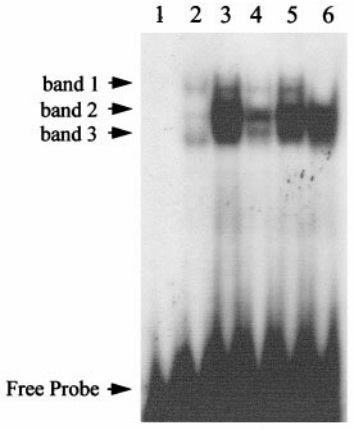

FIGURE 12. Thrombin induces NF-κB binding to ICAM-1 promoter. EMSA were performed as described in *Materials and Methods*. Nuclear extracts prepared from HUVEC stimulated for 1 h with (*lanes 3–6*) or without (*lane 2*; control) thrombin (2.5 U/ml) were incubated in the absence or presence of 75-fold molar excess of cold wild-type (*lane 4*), or mutant ICAM-1 NF-κB (*lane 5*) before the addition of radiolabeled wild-type (*lanes 1–5*) or mutant ICAM-1 NF-κB probes (*lane 6*). *Lane 1*, no extract. A representative experiment of four performed is shown.

Several lines of evidence suggest that activation of downstream NF-κB site is necessary for thrombin induction of the ICAM-1 gene. The inability of CHX, as well as the ability of PDTC to prevent the thrombin-induced increase in ICAM-1 mRNA expression, is consistent with a NF-κB-dependent signaling pathway. Moreover, the slight induction of ICAM-1 mRNA by CHX alone may be due to inhibition of IκB synthesis by CHX and is characteristic of NF-κB-dependent genes (24). EMSA showed that thrombin induced nuclear NF-κB translocation and DNA-binding activity. The binding complex consisted of NF-κB p65 homodimer, as shown by the supershift experiments. Inhibition of NF-κB, either by blocking its activation and thereby its nuclear translocation or by mutating the downstream NF-κB site, and thereby preventing binding of activated NF-κB to the ICAM-1 promoter, abolished thrombin-induced increase in ICAM-1 mRNA expression and ICAM-1 promoter activity, respectively.

Studies have shown that NF-κB p65 mediates a number of thrombin-induced cellular responses such as proliferation and cytokine production in vascular smooth muscle cells (37, 48). Activation of NF-κB requires the phosphorylation either on serine or tyrosine residues of IκB, which sequesters NF-κB in the cytosol. Many, if not all, activators of NF-κB induce serine phosphorylation of IκB that targets it for rapid polyubiquitination, followed by degradation through 26S proteasome (28–31). However, in certain settings such as reoxygenation of hypoxic cells, tyrosine phosphorylation of IκBα leads to NF-κB activation without proteolytic degradation of IκBα (32). We do not exclude any of these possible mechanisms for NF-κB activation following thrombin stimulation of endothelial cells.

The fact that TNF-α or IL-1β activates ICAM-1 expression via NF-κB, coupled with the observation that TNF-α- or IL-β-induced ICAM-1 expression is rapid, with mRNA being detectable as early as 0.5 h, peaking at steady state level by 2 h (49) as opposed to thrombin-induced ICAM-1 mRNA expression, which peaks between 2–4 h, raises the possibility that the thrombin response may be secondary to TNF-α or IL-1β expression. However, such a possibility is ruled out by the findings that thrombin response was not prevented by the protein synthesis inhibitor CHX (Fig. 6). In addition, Kaplanski et al. (12) have recently shown that receptor antagonist, Abs, or antisense oligonucleotides to IL-1β, TNF-α, or IL-1α failed to inhibit thrombin-induced E-selectin and IL-8 gene expression in HUVEC. Furthermore, IL-1α, IL-1β, and TNF-α were not detected in the supernatants of thrombin-activated HUVEC (12).

Analysis of ICAM-1 promoter showed that deletion of upstream NF-κB site (−533 bases from translation start site) did not prevent the thrombin response. Thus, we did not examine whether this site can bind p65 homodimer in response to thrombin or TRAP. We found that the thrombin-responsive region (spanning from −393 to −176 bases from the start codon) contained AP-1/TRE, SP1, C/EBP, and downstream NF-κB binding sites. Further analysis of this region revealed that TRE by itself is not functionally important for thrombin activation of ICAM-1 gene; however, we do not exclude the possibility that it may cooperate with other *cis*-acting elements such as NF-κB to mediate thrombin response. Interestingly, the close arrangement of C/EBP and NF-κB binding sites is reminiscent of the IL-8 gene promoter (50). These sites have also been shown to be critically involved in the mediation of TNF-α signal to the ICAM-1 promoter (22). However, the mutation of C/EBP or Sp-1 site failed to prevent thrombin-induced activation of ICAM-1 promoter, suggesting that these sites are not necessary to confer thrombin inducibility of ICAM-1 gene. Mutation of downstream NF-κB site, on the other hand, abrogated the response, indicating that the downstream NF-κB site is critical for

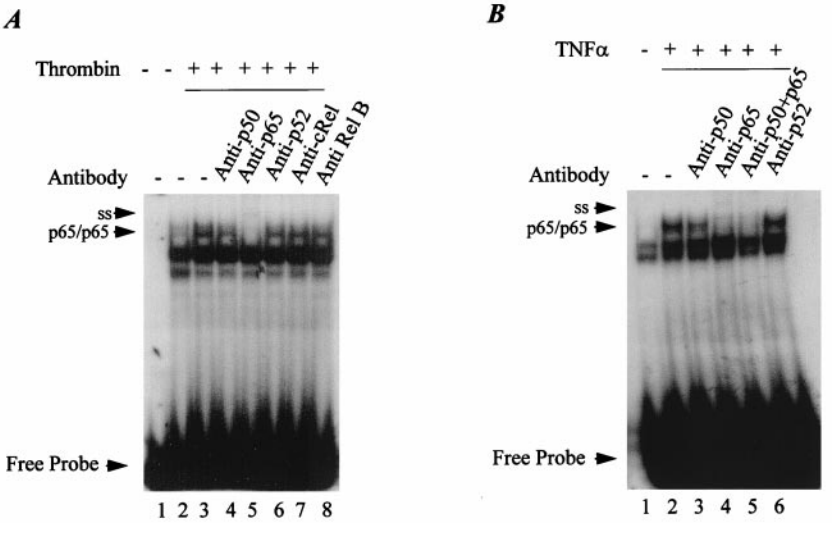

FIGURE 13. Thrombin induces binding of NF-κB p65 to the downstream NF-κB site of ICAM-1 promoter. EMSA were performed as described in *Materials and Methods*. Nuclear extracts prepared from HUVEC stimulated for 1 h with thrombin (2.5 U/ml) (*A*, *lanes 3–8*) or TNF-α (100 U/ml) (*B*, *lanes 2–6*) were incubated with rabbit Abs specific for *A*, p50 (*lane 4*), p65 (RelA) (*lane 5*), p52 (*lane 6*), cRel (*lane 7*), and RelB (*lane 8*); or *B*, p50 (*lane 3*), p65 (RelA) (*lane 4*), p50 + p65 (*lane 5*), and p52 (*lane 6*) for 15 min at room temperature before addition of radiolabeled ICAM-1 NF-κB probe. *A*, *Lane 1*, no extract; *lane 2*, extract from control untreated cells. *B*, *Lane 1*, extract from control untreated cells. A representative experiment of five performed is shown.

thrombin-mediated ICAM-1 gene transcription in endothelial cells. Furthermore, the ability of thrombin to induce NF-κB-mediated activation of a minimal heterologous promoter established that NF-κB is essential for ICAM-1 expression in endothelial cells (Fig. 11).

The downstream NF-κB site is also necessary for hypoxia-induced cyclooxygenase-2 (COX-2) expression in HUVEC. Human COX-2 promoter is characterized by the presence of two NF-κB sites: the upstream NF-κB site (5′-GGGGATTCCC-3′, −445 bp relative to transcriptional start site) and the downstream NF-κB site (5′-GGGGACTACC-3′, −223 bp relative to transcriptional start site). Schmedtj et al. (51) have reported recently that the downstream NF-κB site of COX-2 promoter is necessary for hypoxia-mediated COX-2 gene transcription in endothelial cells, whereas the upstream NF-κB site has no effect.

PMN sequestration into the extravascular space is mediated by not only the action of chemotactic agents, but also by PMN adhesion to endothelial cells (52). We and others have shown that thrombin is a potent activator of PMN adhesion to endothelial cells through P-selectin (CD62P) and ICAM-1 expression. However, this phenomenon is rapid (occurring within 0.5 h) and does not involve induction of mRNAs for ICAM-1 and P-selectin, and occurred in the presence of CHX (9). In the present study, we demonstrate that the delayed thrombin response (occurring after 2 h) resulted in further increase in PMN adhesion and required de novo protein synthesis of ICAM-1. Thrombin also induces both IL-8 and E-selectin (CD62E) gene expression in endothelial cells, with similar kinetics as that of ICAM-1 (12). It should be noted that in the sequence of adhesion events, E-selectin mediates PMN rolling, whereas ICAM-1 contributes to firm arrest and IL-8 promotes transmigration of PMN across endothelial cells; hence, the ability of thrombin to induce these genes correlates with the findings that thrombin infusion produced PMN sequestration in pulmonary microvessels secondary to the attachment of PMN to vascular endothelial cells (53, 54). The resultant vascular injury and tissue inflammation were critically dependent on ICAM-1-mediated PMN adhesion to microvessel endothelial cells (55).

In summary, we demonstrate that in addition to its role in intravascular coagulation, thrombin serves as a critical mediator of the inflammatory process through its ability to induce activation of NF-κBp65 and the expression of ICAM-1 and ICAM-1-dependent endothelial adhesivity toward PMN. Thus, thrombin-induced ICAM-1 expression is an important linkage between the coagulation cascade and inflammatory response, which may be important in the mechanism of vascular endothelial adhesivity, PMN adhesion, and PMN migration across the endothelial barrier.

Acknowledgments

We thank Dr. Christian Stratowa for plasmids containing 5′ deletion mutation of intercellular adhesion molecule-1 promoter, and Dr. Z. Cao for constructs with mutated nuclear factor-κB, CAAT enhancer-binding protein, or promoter selective-1 site in intercellular adhesion molecule-1 promoter. We also thank Drs. Michael Karin and Elizabeth Nabel for their useful suggestions. We are grateful to Robert Rothlein for anti-intercellular adhesion molecule-1 antibodies (RR1/1 and BIRR0001).

References

1. Fenton, J. W. II. 1988. Thrombin bioregulatory functions. *Adv. Clin. Enzymol. 6:186.*
2. Siess, W. 1989. Molecular mechanisms of platelet activation. *Physiol. Rev. 69: 58.*
3. Bachhuber, B. G., I. J. Sarenbock, L. W. Gimple, C. A. McNamara, and G. K. Owens. 1995. Thrombin-induced mitogenesis in cultured aortic smooth muscle cells requires prolonged thrombin exposure. *Am. J. Physiol. 268:C1141.*
4. Bar-Shavit, R., A. J. Khan, K. G. Mann, and G. D. Wilner. 1986. Chemotactic responses of monocytes to thrombin. *J. Cell Biol. 32:261.*
5. Pober, J. S., and R. S. Cotran. 1990. The role of endothelial cells in inflammation. *Transplantation 50:537.*
6. Bach, F. H., S. C. Robson, C. Ferran, H. Winkler, M. T. Millan, K. M. Stuhlmeier, B. Vanhove, M. L. Blakeley, W. J. Van der Werf, E. Hofer, R. De Martin, and W. W. Hancock. 1994. Endothelial cell activation and thromboregulation during xenograft rejection. *Immunol. Rev. 141:5.*
7. Jaffe, E. A., A. J. Grulich, B. B. Weksler, G. Hampel, and K. Watanabe. 1987. Correlation between thrombin-induced prostacyclin production and inositol triphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J. Biol. Chem. 262:8557.*
8. Malik, A. B., and J. W. Fenton. 1992. Thrombin-mediated increase in endothelial permeability. *Semin. Thromb. Hemost. 18:193.*
9. Sugama, Y., C. Tirruppathi, K. Janakidevi, T. T. Anderson, J. W. Fenton II, and A. B. Malik. 1992. Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. *J. Cell Biol. 199:935.*
10. Zimmerman, G. A., T. M. McIntyre, and S. M. Prescott. 1985. Thrombin stimulates the adherence of neutrophils to endothelial cells in vitro. *J. Clin. Invest. 76:2235.*
11. Ueno, A., K. Murakani, K. Yamanouchi, M. Watanabe, and T. Kondo. 1996. Thrombin stimulates production of interleukin-8 in human umbilical vein endothelial cells. *Immunology 88:76.*
12. Kaplanski, G., M. Fabrigoule, V. Boulay, C. A. Dinarello, P. Bougrand, S. Kaplanski, and C. Farnarier. 1997. Thrombin induces endothelial type II activation in vitro: IL-1 and TNFα-independent IL-8 secretion and E-selectin expression. *J. Immunol. 158:5435.*
13. Marsen, T. A., S. A. Simonson, and M. J. Dunn. 1995. Thrombin induces the preproendothelin-1 gene in endothelial cell by a protein tyrosine kinase-linked pathway. *Circ. Res. 76:987.*
14. Scarpati, E. M., and P. E. DiCorleto. 1996. Identification of a thrombin response element in the human platelet derived growth factor B-chain (c-*sis*) promoter. *J. Biol. Chem. 271:3025.*
15. Anrather, D., M. T. Milan, A. Palmetshofer, S. C. Robson, C. Geczy, A. J. Ritchie, F.-H. Bach, and B. M. Ewenstein. 1997. Thrombin activates NF-κB and potentiates endothelial cell activation by TNF. *J. Immunol. 159:5620.*
16. Vu, T. K. H., D. T. Hung, V. I. Wheaton, and S. R. Coughlin. 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell 64:1057.*
17. Coughlin, S. R. 1994. Protease-activated receptors start a family. *Proc. Natl. Acad. Sci. USA 91:9200.*
18. Gerszten, R. E., J. Chen, M. Ishii, K. Ishii, L. Wang, T. Nanevicz, C. W. Truck, T. K. H. Vu, and S. R. Coughlin. 1994. Specificity of thrombin receptor for agonist peptide is defined by its extracellular surfaces. *Nature 369:648.*
19. Smith, C. W., S. D. Marlin, R. Rothlein, C. Toman, and D. C. Anderson. 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J. Clin. Invest. 83:2008.*
20. Smith, C. W., R. Rothlein, B. J. Hughes, M. M. Mariscalo, H. E. Rudloff, F. C. Schmalsteig, and D. C. Anderson. 1988. Recognition of an endothelial determinant for CD-dependent human neutrophil adherence and transendothelial migration. *J. Clin. Invest. 82:1746.*
21. Collins, T., M. A. Read, A. S. Neish, M. Z. Whitley, D. Thanos, and T. Maniatis. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J. 9:899.*
22. Hou, J., V. Baichwal, and C. Zao. 1994. Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding ICAM-1. *Proc. Natl. Acad. Sci. USA 91:11641.*
23. Ledebur, H. C., and T. P. Parks. 1995. Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells: essential roles of a variant NF-κB site and p65 homodimers. *J. Biol. Chem. 270:933.*
24. Liou, H.-C., and D. Baltimore. 1993. Regulation of NF-κB/*rel* transcription factor and I κB inhibitor system. *Curr. Opin. Cell Biol. 5:477.*
25. Grimm, S., and P. A. Baeuerle. 1993. The inducible transcription factor NF-κB: structure-function relationship of its protein subunits. *Biochem. J. 290:297.*
26. DiDonato, J. A., M. Hayakawa, D. M. Rathwarf, E. Zandi, and M. Karin. 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature 388:548.*
27. Zandi, E., D. M. Rathwarf, M. Delhase, M. Hayakawa, and M. Karin. 1997. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for I κB phosphorylation and NF-κB activation. *Cell 91:243.*
28. Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist. 1995. Control of IκBα proteolysis by site-specific, signal-induced phosphorylation. *Science 267:1485.*
29. Chen, Z., J. Hagler, V. J. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis. 1995. Signal-induced, site-specific phosphorylation targets I κB to the ubiquitinin-proteasome pathway. *Genes Dev. 9:1586.*
30. Chen, Z. J., L. Parent, and T. Maniatis. 1996. Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. *Cell 84:853.*
31. Traenckner, E. B., H. L. Pahl, T. Henkel, K. N. Schmidt, S. Wilk, and P. A. Bauerle. 1995. Phosphorylation of human IκB on serine 32 and 36 controls IκB proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J. 14:2876.*
32. Imbert, V., R. A. Rupec, A. Livolsi, H. A. Pahl, E. B. M. Traenckner, C. Muller-Dieckmann, D. Farahifar, B. Rossi, P. Auberger, P. A. Bauerle, and J. F. Peyron. 1996. Tyrosine phosphorylation of IκBα activates NF-κB without proteolytic degradation of IκBα. *Cell 86:787.*

33. Bauerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol. 12:141.*
34. Baldwin, A. S. 1996. The NF-κB and I κB proteins: new discoveries and insights. *Annu. Rev. Immunol. 14:649.*
35. Barnes, P. J., and M. Karin. 1997. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med. 336:1066.*
36. Voraberger, G., R. Schafer, and C. Stratowa. 1991. Cloning of the human gene for intercellular adhesion molecule-1 and analysis of its 5′-regulatory region. *J. Immunol. 147:2777.*
37. Maruyama, I., K. Shigeta, H. Miyahara, T. Nakajima, H. Shu, S. Ide, and I. Kitajima. 1997. Thrombin activates NF-κB through thrombin receptor and results in proliferation of vascular smooth muscle cells: role of thrombin in atherosclerosis and restenosis. *Ann. NY Acad. Sci. 811:429.*
38. Edgell, C. J., C. C. McDonald, and J. B. Graham. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA 80:3734.*
39. Arai, T., S. A. Kelly, M. L. Brengman, M. Takano, E. H. Smith, P. J. Goldschmidt-Clermont, and G. B. Bulkley. 1988. Ambient but not incremental oxidant generation affects intercellular adhesion molecule-1 induction by tumor necrosis factor α in endothelium. *Biochem. J. 331:853.*
40. Staunton, D. E., S. D. Marlin, C. Stratowa, M. L. Dustin, and T. A. Springer. 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene family. *Cell 52:925.*
41. Roebuck, K. A., A. Rahman, V. Lakshminarayan, K. Janakidevi, and A. B. Malik. 1995. H_2O_2 and tumor necrosis factor-α activate intercellular adhesion molecule-1 (ICAM-1) gene transcription through distinct *cis*-regulatory elements within ICAM-1 promoter. *J. Biol. Chem. 270:18966.*
42. Rahman, A., J. Kefer, M. Bando, W. D. Niles, and A. B. Malik. 1998. E-selectin expression in human endothelial cells by TNFα-induced oxidant generation and NF-κB activation. *Am. J. Physiol. 19:L533.*
43. Rothlein, R., R. M. Dustin, S. D. Dustin, and T. A. Springer. 1986. A human intercellular adhesion molecule 1 (ICAM-1) distinct from LFA-1. *J. Immunol. 137:1270.*
44. Pan, J., L. Xia, and R. P. McEver. 1988. Comparison of promoters for murine and human P-selectin gene suggests species-specific and conserved mechanisms for transcriptional regulation in endothelial cells. *J. Biol. Chem. 273:10058.*
45. Pan, J., L. Xia, L. Yao, and R. P. McEver. 1988. Tumor necrosis factor-α- or lipopolysaccharide-induced expression of the murine P-selectin gene in endothelial cells involves novel κB sites and a variant activating transcription factor/cAMP response element. *J. Biol. Chem. 273:10068.*
46. Shreck, R., B. Meier, D. Manne, W. Droge, and P. A. Bauerle. 1992. Dithiocarbamate as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med. 175:1181.*
47. Trejo, J., J. C. Chambard, M. Karin, and J. H. Brown. 1992. Biphasic increase in c-*jun* mRNA is required for induction of AP-1 mediated gene transcription: differential effects of muscarinic and thrombin receptor activation. *Mol. Cell. Biol. 12:4742.*
48. Nakajima, T., I. Kitajima, H. Shin, I. Takasaki, K. Shigeta, K. Abeyama, Y. Yamashita, T. Tokioka, Y. Soejima, and I. Maruyama. 1994. Involvement of NF-κB activation in thrombin-induced human vascular smooth muscle cell proliferation. *Biochem. Biophys. Res. Commun. 204:950.*
49. Wertheimer, S. J., C. L. Myers, R. W. Wallace, and T. P. Parks. 1992. Intercellular adhesion molecule-1 gene expression in human endothelial cells. *J. Biol. Chem. 267:12030.*
50. Mukaida, Y., Y. Mahe, and K. Matsushima. 1990. Cooperative interactions of nuclear factor-κB and *cis*-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. *J. Biol. Chem. 265:21128.*
51. Schmedtje, J. F., Y. S. Ji, W. L. Liu, R. N. DuBois, and M. S. Runge. 1997. Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. *J. Biol. Chem. 272:601.*
52. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell 76:301.*
53. Cooper, J. A., S. J. Solano, R. Bizios, J. E. Kaplan, and A. B. Malik. 1984. Pulmonary neutrophil kinetics after thrombin-induced intravascular coagulation. *J. Appl. Physiol. 57:826.*
54. Mailk, A. B., and M. J. Horgan. 1987. Mechanisms of thrombin-induced lung vascular injury and edema. *Am. Rev. Respir. Dis. 136:464.*
55. Malik, A. B. 1983. Pulmonary microembolism. *Physiol. Rev. 63:1114.*