Thrombin-induced Autoinhibitory Factor, Down Syndrome Critical Region-1, Attenuates NFAT-dependent Vascular Cell Adhesion Molecule-1 Expression and Inflammation in the Endothelium

Activation and dysfunction of the endothelium underlie many vascular disorders including atherosclerosis, tumor growth, and inflammation. We recently reported that thrombin and vascular endothelial growth factor, but not tumor necrosis factor-α, results in dramatic up-regulation of Down syndrome critical region (DSCR)-1 gene in endothelial cells, a negative feedback regulator of calcineurin-NFAT signaling. Constitutive expression of DSCR-1 in activated endothelial cells markedly impaired NFAT nuclear localization, proliferation, tube formation, and tumor growth. The goal of the present study was to elucidate the relative roles of NFAT/DSCR-1 and NF-κB/IκB in mediating thrombin-responsive gene expression in endothelial cells. DNA microarrays of thrombin-treated human umbilical vein endothelial cells overexpressing DSCR-1 or constitutive active IκBα revealed genes that were dependent on NFAT and/or NF-κB activity. Vascular cell adhesion molecule-1 was inhibited both by DSCR-1 and IκB at the level of mRNA, protein, promoter activity, and function (monocyte adhesion). Using a combination of transient transfections, electrophoretic mobility shift assays, and chromatin immunoprecipitation, thrombin was shown to induce time-dependent coordinate binding of RelA and NFATc to a tandem NF-κB element in the upstream promoter region of vascular cell adhesion molecule-1. Together, these findings suggest that thrombin-mediated activation of endothelial cells involves an interplay between NFAT and NF-κB signaling pathways and their negative feedback inhibitors, DSCR-1 and IκB, respectively. As natural brakes in the inflammatory process, DSCR-1 and IκB may lend themselves to therapeutic manipulation in vasculopathic disease states.

The endothelium is highly malleable cell layer constantly responding to changes within the extracellular environment and responding in ways that are usually beneficial but at times harmful to the organism. Several extracellular mediators activate gene transcription in endothelial cells, resulting in changes in hemostatic balance, increased leukocyte adhesion, permeability, migration, and proliferation. The tight control of these processes is essential for maintaining homeostasis; endothelial cell activation, if excessive, over-sustained, or spatially and temporally misplaced, may lead to vasculopathic disease such as pathological angiogenesis, inflammation, and atherosclerosis.

Under normal conditions, activation signals may be terminated by negative feedback inhibition of downstream transcriptional networks. Such a paradigm is well established for transcription factors, NF-κB, Egr-1, and Smad-2/3, which activate the self-inhibitory molecules, IκBα, nerve growth factor induced A-binding protein (NAB), and Smad-7, respectively (1–3). Moreover, we have recently demonstrated that vascular endothelial growth factor (VEGF)3 and thrombin-mediated activation of calcineurin-NFAT signaling in endothelial cells is autoinhibited by the induction of the Down syndrome critical region (DSCR)-1 gene (4).

In humans, the DSCR-1 gene (also known as calcipressin 1, MCIP-1, and Adapt 78) consists of 7 exons, of which exons 1–4 can be alternatively spliced, resulting in a number of different mRNA isoforms (5, 6). These isoforms have different expression patterns and are regulated by distinct transcriptional mechanisms. For example, the DSCR-1 exon 4–7 variant is regulated by a calcineurin- and GATA-dependent pathway (4), whereas the exon 1 variant is under the control of a Notch- and Hes-1-dependent pathway (7).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. I–VII and Tables I and II.

1 To whom correspondence should be addressed: The Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan.
2 Supported in part by National Institutes of Health Grant HL36028.
3 The abbreviations used are: VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; DSCR-1, down syndrome critical region-1; CA, constitutive active; VCAM-1, vascular cell adhesion molecule-1; PAR, protease-activated receptor; ICAM-1, intercellular adhesion molecule-1; HUVEC, human umbilical vein endothelial cells; FBS, fetal bovine serum; Ad, adenosine; EMSA, electrophoretic mobility shift assays; ChIP, chromatin immunoprecipitation; CsA, cyclosporine A; IRES, internal ribosome entry site; siRNA, small interfering RNA; Pol II, polymerase II.
DSCR-1 Negatively Regulates Vascular Inflammation

A

Thrombin treatment

0 h 4 h 4 h

Ad-Control

Ad-DCR-1

1 2 3

DSCR-1

β-Actin

B

Gene description

Interleukin-8

Endothelial cell family 7 (cationic amino acid transporter)

E-selectin

Human similar to TNF-induced protein (LOC28293)

VCAM-1

M-CSF

JAK-2

Integrin family, RNA polymerase II

Reid

TRAF-6

ICAM-1

MCP-1

CD44

UDP-Gal-p-GalNAc (1,4-galactosyltransferase)

Ras

Caspase and FADD-like apoptosis regulator

Nuclear receptor coactivator 7

TRAF-5

Homo sapiens cDNA FLJ22635 fhus, clone KAT01739

four and a half LIM domain 1

Human sequence with similarity to protein NM_002651

EH-domain containing 9

Neosin, plasminogen activator inhibitor type 1, member 1

cadherin

chromosome 8 open reading frame 68

Discothe family homolog 5 (Exosporium levis)

DSCR-1

NADP-dependent retinol dehydrogenase/reductase

Homo sapiens transcribed sequences

Protein disulfide isomerase A1, endoplasmic reticulum calcium-binding protein 1

Sterol regulatory element containing 4

Rhodopsin

Core promoter element binding protein

Phospholipid phosphatase, acid phosphatase type 2A

Endoplasmic reticulum calcium-binding protein 9

Sterol regulatory element-containing transcription factor 1 (SREBP-1)

PDE4A

cytosine-rich angiogenic inducer 61, Cyr 61

Hypothetical protein FLJ33948

Likely ortholog of mouse limb-bud and heart gene

Hypothetical protein FLJ32029

HIF-1α

TGF-β2 precursor

Homo sapiens cDNA FLJ21816 fhus, clone ADG64782

Regulator of S-phase kinase 3, RIKEN C3.1

Homo sapiens full length insert cDNA clone ZC3H46

Knaiz family member 13A

Tubulin β-1

Dedicator of cytokinesis protein 10

Hypothetical protein BC016658

AdAMTS-6

Chromokinin (C motif) ligand 8

Chromomodulin 8 open reading frame 17

Aldose dehydrogenase 1, family member 01

Etanercept

GCP3

Carboxylate reductase 3

Malignant fibrosarcoma histidinylamidase amplified sequence 1

Aldose dehydrogenase 1, family member 01

TGF-β2

Soluble cell factor 12 (Nakagi 2 transporters) member 2

CITED4

Homo sapiens cDNA FLJ28639 mRNA

Human, clone MGC:17708 IMAGE:3026959

Nucleosidase 68kDa

Phospholipid (1,2)-glycerol-3-phosphate: ethanolamine 3-acyltransferase

Human sequence with similarity to protein NP_007832

TNF-induced protein 9 (ADD)

Baculoviral IAP-repressed containing 3 (BIRC3)

docking protein 5

sL hirudin (Drosophila)

Type IV enzyme-linked binding protein

Metalloproteinase suppressor 1

Interaction 15 receptor

Soluble cell factor 12 (KCI transporters) member 7

UDP-GalNAc β-D Gal-1,3-N-acetylglucosaminyltransferase/peptidylprolyl isomerase F
DSCR-1 Negatively Regulates Vascular Inflammation

Thrombin is a multifunctional serine protease that is involved not only in mediating the cleavage of fibrinogen to fibrin in the coagulation cascade but also in activating a variety of cell types, including platelets, vascular smooth muscle, and endothelial cells (8–10). Thrombin signaling in the endothelium is mediated by a family of seven transmembrane G-protein-coupled receptors, termed protease activated receptors (PARs). Currently, four members of the PAR family have been isolated (PAR-1–4). In endothelial cells PAR-1 is thought to be the predominant thrombin receptor. Once activated, PAR-1 is linked to a number of signal intermediates (including mitogen-activated protein kinase, protein kinases C and A, phosphatidylinositol 3-kinase, and calcineurin), transcription factors (such as Egr-1, NF-κB, NFAT, SP-1, and AP-1), and downstream target genes (e.g., vascular adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, E-selectin, fractalkine, and monocyte chemoattractant protein-1, endothelial-specific molecule-1, bone morphogenetic protein-2, and HB-EGF) (4, 11–13).

In this study we employed global gene expression profiles to elucidate the relative roles of NFAT-DSCR-1 and NF-κB-1κB pathways in mediating thrombin signaling in endothelial cells. We identified several thrombin target genes that require both transcription factors for activation. Among these was the proinflammatory gene, VCAM-1, which is reported to play a critical role in firm leukocyte adhesion in diverse inflammatory disease states (14–17). Additional studies were carried out to determine the interaction between NFAT and NF-κB in mediating thrombin induction of VCAM-1. The results suggest that DSCR-1-mediated inhibition of NFAT signaling may be leveraged for therapeutic gain in inflammatory states.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—VEGF and tumor necrosis factor (TNF)-α were obtained from Peprotec (Rocky Hill, NJ). Human thrombin and cyclosporine A (CsA) were obtained from Calbiochem. Human umbilical vein endothelial cells (HUVEC) were grown in endothelial growth medium-2-MV (EGM-2-MV) BulletKit (Clonetics, San Diego, CA). HUVEC were used within the first 8 passages. Human embryonic kidney 293 cells (ATCC CRL-1573) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human U937 cells (JCRB-9021) were grown in RPMI medium plus 10% FBS.

Plasmids and Adenoviruses—Construction of wild type human VCAM-1 luc and −251NFAT-like-mut-luc was previously described (11). To generate −216NFAT-like-mut-luc and NF-κB-mut-luc, a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer’s instruction with the mutated oligonucleotides shown in supplemental Table I. To generate the mouse VCAM-1-luc plasmid, the mouse 2.5-kilobase promoter was amplified by PCR using the BAC clone (RPCI23 362K11) as template and subcloned into the pGL3-basic vector (Promega, Madison, WI). NFAT expression vector (pCMV-NFATc) was purchased from Invitrogen (IMAGE clone ID 5354603). The RelA expression plasmid (pCMV-RelA) was kindly provided by Dr. Mukesh Jain (Harvard Medical School, Boston, MA). For construction of pTNT-mNFATc, a SalI-NotI fragment containing mNFATc was subcloned into the SalI/NotI-digested pTNT vector (Promega). Human IκBα cDNA fragment was amplified using PCR from reverse-transcribed HUVEC total RNA and subcloned into the pGEM-T easy (Promega). Constructive active (CA; S32A and S36A) IκBα was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and mutated primer pairs (supplemental Table I). The EcoRI-digested CA-IκBα fragment was subcloned into pIRE2-EGFP (Clontech, Mountain View, CA) to generate pIRE2-CA-IκBα-EGFP. The generation of adenovirus (Ad)-Control, Ad-DSR-1, and Ad-CA-NFAT was previously described (4). For the construction of the Ad-CA-IκBα, pIRES2-CA-IκBα-EGFP was digested with NheI and AflIII, and the resulting fragment was subcloned into the pShuttle and Adeno-X DNA (Clontech) using the Adeno-X adenoviral expression system (Clontech). All cloned and subcloned constructs were confirmed by restriction enzyme digestions and automated DNA sequencing.

Microarray Analysis—Two independent lots of HUVEC were infected with either Ad-Control, Ad-DSR-1, Ad-CA-NFAT, or Ad-CA-IκBα (multiplicity of infection of 30) for 2 days, serum-starved overnight in medium containing EBM-2 (Clonetics) and 0.5% FBS, and then treated with or without 2 units/ml thrombin for 4 h. RNA was harvested and purified with Trizol (Invitrogen). Preparation of cRNA and hybridization of probe arrays were performed according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA). Each array experiment was performed duplicate. File maker software was used for the genes that demonstrated identical patterns in both experiments. Data were analyzed according to the minimum information about a microarray experiment (MIAME) rule. Annotation of the probe numbers and targeted sequences were shown in Affymetrix web page.

Quantitative Real-time PCR—RNA was extracted from endothelial cells with TRIzol reagent (Invitrogen). Two μg of total RNA was reverse-transcribed using SuperScript II enzyme using oligo-dT primer as specified by Invitrogen. Real-time PCR including SYBR Green PCR reagent was performed on an instrument according to instructions provided by the manufacturer (Applied Biosystems). Primer pairs are indicated in supplemental Table I.

Plasmid DNA and siRNA Transient Transfections and Luciferase Assay—HUVEC and human embryonic kidney 293 cells were transfected with plasmid DNA using FuGENE 6 reagent...
(Roche Applied Science), and luciferase activities were calculated using the dual-luciferase assay kit (Promega) as previously described (11). The serum-starved transfected HUVEC were incubated with 50 ng/ml VEGF, 2 units/ml thrombin, and/or 10 ng/ml TNF-α for 6 h. For siRNA transfection, HUVEC were incubated with a mixture of 40 nM siRNA and lipofectamine2000 (Invitrogen) for 6 h. Medium was replaced, and cells were cultured with EGM-2-MV for 24 h. After serum-starvation (EBM-2 plus 0.5% FBS) and agonist treatment, cells were processed for real-time PCR, Western blot, or adhesion assays. Targeted siRNA sequences (Invitrogen) are shown in supplemental Table I.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extraction and EMSA were carried out as previously described (18). To reduce nonspecific binding, 1 µg of poly(dI-dC) (Invitrogen) and 20 fmol of oligonucleotide spanning the flk-1/KDR SP1 motifs (supplemental Table I) were added to the reaction mixture. Double-stranded oligonucleotides were labeled with [α-βP]dCTP and Klenow fragment and purified by spin column (Amersham Biosciences). Oligonucleotide sequences used for probes and competitors are shown in supplemental Table I. To test the effect of antibodies on DNA-protein binding, nuclear extracts were preincubated with antibodies against NFATc (Affinity BioReagents, Golden, CO), Ets-1, RelA, and IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. Recombinant NFATc and RelA were generated using the TNT-coupled wheat germ extract systems (Promega) with pTNT-mNFATc and pCMV-RELA, respectively, according to the manufacturer’s instruction.

Chromatin Immunoprecipitation (ChIP) Analysis—HUVEC were treated with thrombin for 0, 1, 2, and 4 h, subsequently exposed to 1% formaldehyde (Wako Chemicals, Osaka, Japan) for 10 min at
RESULTS

Identification of NFAT and NF-κB-dependent Pathways in Thrombin-treated Human Primary Endothelial Cells—In a previous study we demonstrated that thrombin and VEGF treatment of HUVEC resulted in marked and rapid up-regulation of the DSCR-1 gene and secondary inhibition of NFAT signaling (4). Other studies have demonstrated an important role for NF-κB in thrombin-mediated gene expression (19, 20). To determine the relative contributions of these two signaling pathways (NFAT and NF-κB) and their respective endogenous inhibitors (DSCR-1 and IκBα) in thrombin signaling, we carried out DNA microarray analysis of control or thrombin-treated HUVEC overexpressing EGFP (control), DSCR-1, or constitutively active IκBα. High transduction efficiency was confirmed using fluorescent microscopy, real-time PCR, and Western blots (Fig. 1A and data not shown).

In duplicate arrays, thrombin induced a total of 172 genes more than 2-fold in Ad-Control-infected HUVEC, 52 and 59 of which were inhibited more than 40% by constitutive expression of DSCR-1 and CA-IκBα, respectively (see supplemental Table II for details). Clustering analyses revealed the existence of four groups of thrombin-inducible genes, namely those that were 1) reduced both by DSCR-1 and CA-IκBα (n = 19) (group 1), 2) reduced by DSCR-1 alone (n = 33) (group 2), 3) reduced by Ad-CA-IκBα (n = 35) (Group 3), and 4) unaffected by either inhibitor (group 4) (groups 1–3 are shown in Fig. 1B).

Next, we employed real-time PCR to validate the results of two representative genes from each of the above groups. In group 1, thrombin treatment resulted in significant induction of the cell adhesion molecules, ICAM-1 and E-selectin by 52- and 832-fold, respectively (Fig. 2A and B) (a third adhesion molecule belonging to group 1 gene, VCAM-1, is discussed in detail below). Clustering analyses revealed the existence of four groups of thrombin-inducible genes, namely those that were 1) reduced both by DSCR-1 and CA-IκBα (n = 19) (group 1), 2) reduced by DSCR-1 alone (n = 33) (group 2), 3) reduced by Ad-CA-IκBα (n = 35) (Group 3), and 4) unaffected by either inhibitor (group 4) (groups 1–3 are shown in Fig. 1B).

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DSCR-1 Negatively Regulates Vascular Inflammation

A. VCAM-1 mRNA

B. VCAM-1 mRNA

C. DSCR-1 mRNA

D. DSCR-1 protein

E. VCAM-1 mRNA

F. VCAM-1 protein

G. RelA mRNA

H. RelA

I. NFATc mRNA
fected with siRNA against DSCR-1. As shown in Fig. 3, down on VCAM-1 expression. To that end HUVEC were trans-

of VCAM-1, we carried out real-time PCR of thrombin-stimulated HUVEC infected with Ad-Control, Ad-DSCR-1, and/or Ad-CA-IκBα. As shown in Fig. 3A, thrombin treatment resulted in a 452-fold up-regulation of the VCAM-1 mRNA at 4 h; an effect that was inhibited by Ad-DSCR-1 and Ad-CA-IκBα (63 and 89%, respectively). Combined overexpression of DSCR-1 and CA-IκBα resulted in a nearly complete (97%) inhibition of the thrombin effect. Consistent with the effect of Ad-DSCR-1, overexpression of constitutive nuclear-localized NFAT (CA-NFAT) in HUVEC resulted in an 18-fold induction of VCAM-1 expression (Fig. 3B).

Next, we wished to determine the effect of DSCR-1 knockdown on VCAM-1 expression. To that end HUVEC were transfected with siRNA against DSCR-1. As shown in Fig. 3, C and D, two independent siRNAs inhibited thrombin-stimulated DSCR-1 mRNA (87 and 69%, respectively) as well as DSCR-1 protein. Importantly, both siRNAs resulted in a super-induction of VCAM-1 mRNA and protein levels in thrombin-treated HUVEC (Fig. 3E and F). Similarly, siRNAs against IκBα accentuated the effect of thrombin on VCAM-1 mRNA expression (supplemental Fig. 1). In contrast, siRNA against RelA and NFATc (whose effectiveness in inhibiting their target genes is shown in Fig. 3, G–J) virtually abrogated thrombin-mediated induction of VCAM-1 mRNA (Fig. 3K). Taken together, these data strongly suggest that thrombin stimulation of VCAM-1 is mediated by RelA- and NFATc-dependent signaling pathways and that these pathways are in turn autoinhibited by IκBα and DSCR-1, respectively.

**NF-κB Activation Pathways**—To confirm the above results at the level of the VCAM-1 promoter, HUVEC were infected with Ad-Control, Ad-DSCR-1, Ad-CA-IκBα, or Ad-CA-NFAT and then transiently transfected with constructs containing the thymidine kinase promoter (control), the human VCAM-1 promoter (1800 bp 5′-flanking sequence), or the mouse VCAM-1 promoter (2500-bp 5′-flanking sequence) coupled to luciferase cDNA. In contrast to an absence of effect on the thymidine kinase promoter, overexpression of CA-NFAT resulted in a 3-fold induction of mouse and human VCAM-1 promoter activity (Fig. 4A). In co-transfection assays overexpression of NFATc or RelA resulted in a 1.67- and 24.5-fold induction of VCAM-1 promoter activity, respectively. Combined overexpression of NFATc and RelA had a synergistic effect on luciferase activity (42.1-fold induction) (Fig. 4B). Thrombin induced human VCAM-1 promoter activity by 4.5-fold, an effect inhibited by 64 and 92% in cells overexpressing DSCR-1 or CA-IκBα, respectively (Fig. 4C). Taken together with the results of DNA microarrays, real-time PCR, Western blots, and transient transfections, these data support a role for both NF-κB/IκBα and NFAT/DSCR-1 feedback pathways in the temporal regulation of VCAM-1 gene expression.

**NFAT Stimulation of the VCAM-1 Promoter Is Mediated by the NF-κB Motif**—The human VCAM-1 promoter contains NFAT consensus motifs ((A/T)GGAAA) at −443, −251, and −216 relative to the transcriptional start site (Fig. 5). To determine whether one or more of these sites is required for NFAT-mediated transactivation of the VCAM-1 promoter, human embryonic kidney 293 cells were co-transfected with an NFATc expression plasmid (pCMV-NFATc) and VCAM-1 promoter-
DSCR-1 Negatively Regulates Vascular Inflammation

FIGURE 4. Thrombin-mediated induction of the VCAM-1 promoter in HUVEC is mediated by NFAT and NF-κB. A, HUVEC were transiently transfected with human (h) or mouse (m) VCAM-1-luc or with the TK-luc plasmid and infected with Ad-Control or Ad-CA-NFAT. Data represent the mean and S.D. (relative to Ad-Control-treated cells) obtained in triplicate from at least four independent experiments. B, HUVEC were transiently co-transfected with human VCAM-1-luc and NFATc and RelA expression vectors alone or in combination. Data represent the mean and S.D. (relative to pcDNA3-transfected cells) obtained in triplicate from at least three independent experiments. C, HUVEC were transiently transfected with human VCAM-1-luc plasmid and infected with Ad-Control, Ad-DSCR-1, or Ad-CA-1xBux, then exposed to 2 units/ml thrombin for 6 h. Data represent the mean and S.D. (relative to thrombin treated-Ad-Control cells) obtained in triplicate from at least three independent experiments.

 luciferase constructs containing various mutations/deletions. As shown in Fig. 5A, NFATc expression resulted in a 9-fold induction of the wild type promoter. This effect was unaltered by deletion of the 5′-most NFAT element (Δ-289-luc) or mutation of the −251 or −216 NFAT sites. In contrast, a promoter containing mutated NF-κB sites (NF-κB mut-luc) was unresponsive to NFAT (Fig. 5A). In other co-transfection studies, overexpression of RelA resulted in significant induction (58-fold) of the wild type promoter but not NF-κB mut-luc (Fig. 5B). Taken together, these results suggest that the NF-κB motifs are necessary for NFAT- and NF-κB-mediated transactivation of the VCAM-1 promoter.

Thrombin Induces Binding of NFAT to NF-κB Motifs in the VCAM-1 Promoter—EMSAs were carried out to characterize NFAT and NF-κB DNA binding interactions. To that end, a radiolabeled probe containing the two adjacent NF-κB sites was incubated with nuclear extracts derived from control or thrombin-treated HUVEC. As shown in Fig. 6A, thrombin treatment resulted in the appearance and/or induction of three DNA-protein complexes (lanes 2 and 3; open arrow, closed arrow, and arrowhead). These DNA-protein complexes were inhibited by the addition of a 20-fold molar excess of unlabeled self-competitor, confirming their specificity (Fig. 6A, lane 4). The fastest of the three complexes (delineated by the open arrow) was inhibited by the addition of a 20-fold molar excess of unlabeled DNA probe containing the consensus NFAT binding site at the −220 bp of the DSCR-1 promoter (Fig. 6A, lane 5).

To identify the protein(s) in the thrombin-inducible binding complex, the binding reactions were preincubated with antibodies to RelA or NFATc. Anti-RelA antibody inhibited the formation of the slow and intermediate complexes (delineated by the arrowhead and closed arrow, respectively) and resulted in the appearance of a strong supershifted band (Fig. 6B, lane 7, asterisk). Preincubation with anti-NFATc antibody resulted in marked reduction of the slow and fast DNA-protein complexes (delineated by the arrowhead and open arrow, respectively) and the appearance of a faint supershifted band (Fig. 6B, lane 8, double asterisk), which was more clearly visualized with longer exposure (data not shown). Preincubation with both anti-RelA and anti-NFATc antibodies resulted in a supershift of all three migrating complexes (Fig. 6B, lane 9). In contrast, control IgG had no effect on DNA binding (Fig. 6, lane 10).

To provide further evidence for binding of NFAT to the NF-κB sites, EMSAs were carried out with nuclear extracts derived from HUVEC overexpressing DSCR-1 or pretreated with the NFAT inhibitor, CsA. As shown in Fig. 6B, thrombin treatment of HUVEC infected with Ad-Control resulted in the induction of three specific DNA-protein complexes (lanes 1 and 2). In contrast, nuclear extracts from thrombin-treated HUVEC overexpressing DSCR-1 or pretreated with CsA demonstrated marked reduction in the fastest and slowest migrating complexes (Fig. 6B, lanes 3 and 4).

Finally, EMSAs were performed using recombinant NFATc and RelA. As predicted from the above studies, NFATc resulted in a fast migrating specific DNA-protein complex and RelA in a more slowly moving complex (Fig. 6C, lanes 2 and 3, open and closed arrows). When added together, the two proteins yielded an additional higher DNA-protein complex (Fig. 6C, lane 4, arrowhead). Collectively, these findings suggest that thrombin induces cooperative binding of NFATc and RelA to the VCAM-1 NF-κB motifs.

Thrombin Induces NFAT and RelA Binding to the VCAM-1 NF-κB Region in Chromatin Immunoprecipitation Assays—To further demonstrate a role for NFAT in binding to the VCAM-1 promoter, we carried out sequential ChIP analysis (26) using the genomic VCAM-1 region. HUVEC were treated with
thrombin for 0, 1, 2, and 4 h and then harvested for formalin-fixed genomic DNA. The samples were immunoprecipitated with antibodies to RelA, NFATc1, or Pol II, and the resulting products were used as a template in a PCR reaction containing two sets of primers, one specific for the NF-κB region (Fig. 7A, region I) and the other specific for a region immediately down-
stream of the transcriptional start site (Fig. 7A, region II) of the VCAM-1 promoter. Binding intensities were calculated using real-time PCR and ethidium bromide density. Thrombin induced RelA binding to region I with maximal levels (16-fold) occurring at 1–2 h, whereas RelA binding efficiency on region II was weak (only detected 4 h after the thrombin treatment) (Fig. 7A, left). In contrast, maximal NFATc binding on regions I and II occurred at 1 h (7.8- and 7.9-fold, respectively) (Fig. 7A, middle). Thrombin treatment resulted in a gradual induction of Pol II to chromatin DNA spanning both regions of VCAM-1 (Fig. 7A, right). We also performed ChIP analysis in HUVEC transfected with siRNA against DSCR-1 or control siRNA. Compared with Sc-Control, Sc-DSCR-1 treatment resulted in a ∼2.5-fold increase of the NFATc binding levels at 0, 1, and 2 h after the thrombin stimulation (supplemental Fig. IIA). In addition, similar NFATc-chromatin DNA association patterns were detected in ChIP analysis using the human DSCR-1 promoter spanning the multi-NFAT binding regions, whereas RelA-chromatin DNA interactions were not detected in that region (supplemental Fig. IIIB).

To confirm the temporal pattern of thrombin-inducible NFAT and NF-κB binding, we carried out a time course study using EMSA. Consistent with the ChIP results, thrombin-mediated induction of NFAT-DNA binding occurred earlier and was more sustained compared with NF-κB binding (Fig. 7B). Taken together, these findings suggest that thrombin results in a dynamic, sequential, and temporally regulated interaction between RelA and NFAT at the level of the VCAM-1 NF-κB region in endothelial cells.

**Agonist-mediated Induction of VCAM-1 in Human Primary Endothelial Cells**—We have previously shown that in HUVEC, NFAT is more strongly activated by VEGF and thrombin compared with TNF-α, whereas NF-κB is more strongly activated by TNF-α and thrombin compared with VEGF (4). To validate these findings at the level of the promoter, HUVEC were transfected the VCAM-1 reporter plasmid, treated with various agonist combinations, and then assayed for luciferase activity. As shown in Fig. 8A, 50 ng/ml VEGF, 2 units/ml thrombin, and 10 ng/ml TNF-α activated the promoter by 1.9-, 4.7-, and 9.2-fold, respectively. The combined addition of VEGF and thrombin or VEGF and TNF-α resulted in synergistic stimulation of promoter activity. Co-incubation with thrombin and TNF-α resulted in significantly higher luciferase levels compared with either agonist alone. EMSAs were performed with nuclear extracts from HUVEC treated with various combinations of agonists for 4 h. As shown in Fig. 8B, VEGF, thrombin, or TNF-α each induced RelA and NFAT binding (lanes 3–5). The relative effects of these agonists on RelA binding were TNF-α > thrombin > VEGF and for NFAT binding were VEGF > thrombin > TNF-α. Co-incubation of HUVEC with pairs of agonists resulted in increased intensity of RelA and NFATc binding compared with either agonist alone (Fig. 8B, lanes 4–6).

To evaluate the role for agonist-mediated NF-κB and NFAT activation in stimulating VCAM-1 protein expression, we carried out Western blot analyses of Ad-Control- or Ad-DSCR-1-infected HUVEC treated with or without VEGF, thrombin, and/or TNF-α. As shown in Fig. 8C, VEGF, thrombin, and TNF-α resulted in a 21-, 42-, and 61-fold increase in VCAM-1 protein, respectively (lanes 1, 2, 4, and 6). Combinations of agonists resulted in additional stimulation of VCAM-1 protein expression (lanes 8, 10, and 12). Overexpression of DSCR-1 attenuated the effect of VEGF and thrombin (alone or in combination) but failed to inhibit TNF-α-mediated induction of VCAM-1 (lane 7).

**DSCR-1 and IkBα Attenuate VEGF- and Thrombin-mediated Monocyte Adhesion to Human Primary Endothelial Cells**—We next wished to determine the functional role for DSCR-1 and IkBα autoinhibitory pathways in attenuating endothelial cell activation. To that end, we assayed for adhesion of fluorescent (PKH-26)-labeled U937 monocytes to HUVEC. Treatment of HUVEC with 50 ng/ml VEGF and 2 units/ml thrombin for 4 h resulted in a 75-fold induction in monocyte adhesion compared with untreated controls (Fig. 9, A and B). This effect was almost completely blocked (90%) by antibodies against VCAM-1 but not E-selectin or ICAM-1 (Fig. 9, A and B). As reported previously (27), pretreatment with phosphatidylinositol 3-kinase inhibitor (LY294002) resulted in marked (97%) reduction of agonist-mediated monocyte adhesion. Phosphatidylinositol 3-kinase inhibition blocked the effect of VEGF and thrombin on expression levels of VCAM-1 but not E-selectin and ICAM-1 (supplemental Fig. III). Consistent with the VCAM-1 protein data (Fig. 8C), combined treatment with VEGF and thrombin had a far greater effect on monocyte adhesion compared with either agonist alone (data not shown). Taken together, these findings suggest that the effect of VEGF and thrombin on U937 monocyte adhesion is mediated primarily through the inducible expression of VCAM-1 expression on endothelial cells.

Overexpression of DSCR-1 or CA-IkBα alone inhibited agonist-mediated monocyte adhesion by 82 and 73%, respectively. Combined expression of both DSCR-1 and CA-IkBα completely blocked VEGF and thrombin-mediated monocyte adhesion (Fig. 9, C and D). Overexpression of CA-NFAT resulted in increased monocyte adhesion both to unstimulated and agonist-treated HUVEC (Fig. 9, C and D). HUVEC overexpressing DSCR-1, CA-NFAT, and CA-IkBα did not demonstrate evidence of toxicity (Fig. 9C, upper panels). Compared with VEGF...
FIGURE 7. Dynamic and temporally synchronized interaction of RelA and NFATc at the level of the VCAM-1 NF-κB region in thrombin-stimulated HUVEC. A, sequential chromatin immunoprecipitation analysis was performed with HUVEC genomic DNA after treatment with thrombin for 0, 1, 2, and 4 h. Formalin-fixed chromatin was immunoprecipitated with antibodies against RelA (left), NFATc (middle), and Pol II (right). Precipitated genomic DNA and preimmunoprecipitated chromatin (Input) were amplified by PCR (30 amplification cycles) using the specific primer pairs as described in the upper panel. The bottom bar graphs indicate the quantification of the binding levels. The results show the mean and S.D. of specific signal (relative to the whole input) obtained from three independent experiments. hVCAM, human VCAM. B, EMSAs were performed with 32P-labeled probe in the absence (lanes 1) or presence of nuclear extract from HUVEC untreated (lanes 2) or treated with 2 units/ml thrombin for 1, 2, 4, and 6 h (lanes 3–6).
and thrombin, TNF-α triggered similar levels of monocyte adhesion. However, consistent with the Western blot results, overexpressing DSCR-1 resulted in only a slight reduction (18%) of TNF-α-mediated monocyte adhesion, whereas overexpression of CA-IκBα had a far greater effect (78% reduction) (supplemental Fig. IV). siRNA-mediated knockdown of DSCR-1 and IκBα in HUVEC resulted in increased monocyte adhesion (Fig. 9, E and F). In contrast, knockdown of NFATc and RelA markedly reduced agonist-mediated monocyte adhesion (Fig. 9, E and F). Treatment of siRNAs to HUVEC did not demonstrate any morphological abnormalities (supplemental Fig. V). Taken together, these findings suggest that both NF-κB and NFAT play critical roles in regulating VEGF and thrombin-mediated cell adhesion to endothelial cells.

**DISCUSSION**

Activation of endothelial cells by extracellular stimuli, including VEGF, thrombin, and TNF-α, is a key step underlying many vascular diseases. In a previous study, we demonstrated that VEGF and thrombin resulted in the rapid and pronounced induction of DSCR-1 (4). DSCR-1, in turn, markedly attenuated calcineurin-dependent NFAT signaling, resulting in inhibition of proliferation and angiogenesis. In the present study, we have extended these findings by demonstrating that NFAT, the primary target of DSCR-1, cooperates with NF-κB to up-regulate many inflammatory genes and that DSCR-1 and IκBα function as autoinhibitory factors in these respective pathways.

NF-κB family is widely believed to play a critical role in mediating the inflammatory response in endothelium (28). We, and
Both DSCR-1 and IκBα attenuate and NFATc and RelA enhance monocyte adhesion to the activated HUVEC treated with VEGF and thrombin. A, HUVEC were serum-starved for 18 h, pretreated with or without 50 μM LY294002 for 30 min, and incubated in the absence (minus stimulus) or presence of 50 ng/ml VEGF and 2 units/ml thrombin for 4 h (plus stimulus). Either 10 μg of mouse anti-E-selectin, anti-ICAM-1, anti-VCAM-1 antibodies, or control IgG was added into the culture media for 1 h. PKH-26-stained U937 monocytes were plated on the HUVEC monolayer and incubated for 1 h. After washing, adherent cells were observed under the fluorescent microscopy. B, quantification of the adherent monocytes. The mean and S.D. values were calculated using the MetaMorph and cell image analyzer derived from four arbitral optical images with three independent experiments. C, HUVEC were infected with either Ad-Control, Ad-DSCR-1, Ad-CA-κBα, Ad-CA-NFAT, or both Ad-DSCR-1 and Ad-CA-κBα and then serum-starved for 18 h and incubated in the absence (minus stimulus) or presence of VEGF and thrombin for 4 h (plus stimulus). PKH-26-stained U937 monocytic cells were plated, and adherent cells were observed under the bright field (upper panels) or fluorescence (lower panels). D, quantification of the adherent monocytes. E, HUVEC were transfected with either 40 nm Si-Control, Si-DSCR-1, Si-κBα, Si-NFATc, or Si-RelA and then serum-starved for 18 h. Adhesion assays were performed as described above. F, quantification of the adherent monocytes. The means and S.D. values were calculated using the MetaMorph and cell image analyzer derived from three arbitral optical images with three independent experiments.
DSCR-1 Negatively Regulates Vascular Inflammation

**FIGURE 9—continued**

![Image showing the effects of Ad-Control, Ad-DSCR-1, and Ad-CA-IFAT on vascular inflammation in response to a stimulus.](image-url)
others have previously shown thrombin leads to nuclear translocation of RelA and subsequent transactivation of multiple proinflammatory target genes, including VCAM-1, ICAM-1, E-selectin, MCP-1 monocyte chemotactic protein-1, and fracc talkine (12, 19). In addition, the NF-κB family induces the early expression of its inhibitor, IκBα, which serves to dampen further RelA activity (1). IκBα is susceptible to IKK-mediated phosphorylation and subsequent ubiquitination and degradation. Thus, the non-phosphorylated mutant form of the IκBα (S32A and S36A; CA-IκBα) functions as a dominant negative factor for the RelA-mediated signaling cascade (29). In our DNA microarrays of HUVEC, overexpression of CA-IκBα inhibited one-third (54/172) of thrombin-stimulated genes. These data are consistent with an important role for NF-κB in thrombin signaling.

In addition, our study strongly supports a role for NFAT in mediating thrombin-responsive gene expression. First, overexpression of NFAT in HUVEC resulted in up-regulation (>4-fold) of 51 genes, including many that were categorized as mediators in cell-growth, inflammation, and signaling (data not
shown). Second, overexpression of DSCR-1 resulted in down-regulation of approximately one-third (52/172) of thrombin-stimulated genes in HUVEC. Half of these transcripts (including VCAM-1) were also inhibited by CA-IκBα, suggesting that many thrombin-responsive genes are under the dual regulation of NFAT and NF-κB.

VCAM-1 is believed to play a major role in mediating leukocyte adhesion in diverse disease states such as acute sepsis, early step of the atherosclerosis and chronic rheumatoid arthritis (14–17). We recently reported that thrombin increases the expression of VCAM-1 in endothelial cells by a mechanism that involves the coordinate binding of RelA and GATA to neighboring tandem consensus motifs in the upstream promoter (25). The current study provides compelling evidence for the role of NFAT-DSCR-1 and NF-κB-IκB autoinhibitory pathways in mediating thrombin induction VCAM-1 mRNA, protein, and promoter activity. Specifically, the thrombin response was inhibited by overexpression of DSCR-1 and CA-IκBα and by siRNA against RelA or NFATc, super-induced by siRNA against DSCR-1 or IκBα, and mimicked by overexpression of NFATc and RelA. Importantly, the role for NFAT-DSCR-1 and NF-κB-IκB in mediating the effect of thrombin (and VEGF) on VCAM-1 expression was confirmed in functional assays of monocyte adhesion.

Previous studies have shown that agonist-mediated Ca2+-calcineurin-dependent dephosphorylation of NFAT results in the translocation of the transcription factor into the nucleus and secondary induction of multiple target genes. In many cases, NFAT has been shown to cooperate with other transacting factors to promote target gene expression, including GATA, AP-1, Maf, and Mef-2 (30–33). In addition to binding to classic NFAT consensus elements, NFAT has been shown to bind to an NF-κB motif in the interleukin-8 or human immunodeficiency virus promoters (34–36). Using a combination of transient transfection, EMSA, and ChIP assays, we have shown that NFAT similarly binds to the tandem NF-κB motif in the VCAM-1 promoter and positively regulates its expression. However, rather than replacing or competing with RelA, NFAT appears to associate with NF-κB function, as evidenced by the DNA complexes in EMSA, sequential ChIP, and the additive/synergistic effects of Ad-CA-IκBα and Ad-DSCR-1 on VCAM-1 expression and monocyte adhesion. Further studies using in vivo (co-immunoprecipitation) and in vitro (GST pull-down) assays will be required to identify and characterize the precise nature of the dynamical regulated protein-protein and protein-DNA interaction.

Calcineurin/NFAT signaling has been implicated in cell growth, differentiation and contractile state neuronal cells, cells of the immune system, cardiac or smooth muscle cells, and endothelial cells. CsA and FK506 are calcineurin-specific inhibitors that are approved for clinical use to prevent graft rejection after organ transplantation (37, 38). However, widespread use of these drugs is limited by their neurotoxic and nephrotoxic effects (39, 40). In endothelial cells, high concentrations of CsA lead to toxic effects, including increased lactate dehydrogenase release (41). We found that whereas 50 nM CsA inhibited thrombin-mediated induction of VCAM-1 but not E-selectin and ICAM-1, treatment with 10 μΜ CsA actually increased basal levels of E-selectin (supplemental Fig. VI). In contrast to CsA, DSCR-1 is an endogenous inhibitor of calcineurin, with a Ki in the low nanomolar range (42). Chan et al. (43) recently reported the C-terminal 57 residues of DSCR-1 encoded by exon 7 binds calcineurin with high affinity and inhibits its activity with potency similar to full-length DSCR-1 (43). A 31-amino acid stretch similar to serine-proline repeats of NFAT also inhibits calcineurin activity by acting as a competitive inhibitor (44). Thus, DSCR-1 (and its fragments) appears to hold promise as a more selective and less toxic calcineurin inhibitor for therapy in inflammatory conductions, including vascular disease.

Vascular diseases, including atherosclerosis, thrombosis, and tumor angiogenesis represent complex pathological phenomena. Disease-associated changes in the extracellular environment may have profound effects on endothelial cells, with net signal inputs governing cellular phenotype in a time-dependent manner. Here, we show that thrombin, VEGF, and TNF-α serve to induce VCAM-1 expressions via overlapping yet distinct signaling pathways. An important goal is to further understand the relative role of intracellular signaling pathways and transcriptional networks in mediating endothelial cell activation and to use this information for tailoring anti-inflammatory therapies according to the nature of the signal input and the degree of desired attenuation.

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