NF-κB and IκBα: An Inducible Regulatory System in Endothelial Activation

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Summary

Structural analysis of the promoters of several endothelial genes induced at sites of inflammatory or immune responses reveals binding sites for the transcription factor nuclear factor κB (NF-κB). Endothelial cells express transcripts encoding the p50/p105 and p65 components of NF-κB and the *rel*-related proto-oncogene c-*rel*; steady state levels of these transcripts are transiently increased by tumor necrosis factor α (TNF-α). Western blotting revealed that stimulation of endothelial cells with TNF-α resulted in nuclear accumulation of the p50 and p65 components of NF-κB. Ultraviolet crosslinking and immunoprecipitation demonstrated binding of the p50 and p65 components of NF-κB to the E-selectin κB site. Endothelial cells express an inhibitor of NF-κB activation, IκB-α (MAD-3). Protein levels of this inhibitor fall rapidly after TNF-α stimulation. In parallel, p50 and p65 accumulate in the nucleus and RNA transcript levels for IκB-α are dramatically upregulated. Recombinant p65 stimulates expression of E-selectin promoter–reporter constructs. IκB-α inhibits p65 or TNF-α–stimulated E-selectin promoter–reporter gene expression in transfected endothelial cells. The NF-κB and IκB-α system may be an inducible regulatory mechanism in endothelial activation.

At sites of inflammation and cell-mediated immune responses, endothelial cells develop a characteristic morphology and synthesize new proteins not present in resting vascular beds. These alterations in endothelial cell function are part of a process termed endothelial activation (for a review see reference 1). Many of these morphologic and functional changes can be induced in cultured endothelial cells by purified immune mediators, such as monokines, lymphokines, and bacterial LPS. Based on these in vitro studies, it has been proposed that resident tissue cells and infiltrating leukocytes release cytokines during inflammatory and immune responses that cause endothelial cells to become activated. The activated endothelial cells express new surface proteins and soluble mediators that subsequently recruit additional circulating cells to the peripheral tissues. Many inducible genes involved in endothelial activation, including vascular cell adhesion molecule 1 (2, 3), E-selectin (4, 5), tissue factor (6), IL-1 (7), IL-6 (8), G-CSF (9), IL-8 (10) and *c-myc* (11), contain elements in their promoter regions that could be recognized by the nuclear factor κB (NF-κB)[1]/Rel family of transcription factors. With some of these genes, deletion analysis has demonstrated that the NF-κB sites are functional elements in cytokine or LPS-induced endothelial expression (2–4).

NF-κB is a transcription factor associated with rapid-response activation mechanisms (for reviews see references 12–15). The NF-κB protein subunits, p50 (NFKB1, derived from its p105 precursor) and p65 (Rel A), belong to the Rel family of genes that also includes p52 (NFKB2 and its p100 precursor, also called lyt-10), the *v-rel* oncogene, *c-rel* protooncogene products, and the Drosophila morphogen *dorsal*. In resting cells, NF-κB binding proteins are in an inactive cytosolic form and are complexed to members of a family of inhibitory proteins referred to as IκB. Several IκB proteins have been cloned or purified including IκB-α (MAD3), IκB-β, p105, IκB-γ and bcl-3 (for reviews see references 12, 15). All IκB forms identified to date contain multiple ankyrin repeats, which are involved in interactions with NF-κB. NF-κB can be activated by a number of diverse agents, such as the cytokines IL-1β and TNF-α, LPS, oxidative stress and viral products. These agents initiate the nuclear translocation and subsequent DNA binding of NF-κB, possibly by posttranslational modifications that result in loss of inhibition by IκB proteins (12–15). One of these inhibitors, IκB-α, binds the p65 subunit preferentially and is released from p65 after phosphorylation in response to mediators which activate NF-κB (16–19). The existence of multiple Rel family members and IκB subunits emphasizes the need to examine the structure and regulation

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[1] *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; NF-κB, nuclear factor κB; VCAM-1, vascular cell adhesion molecule 1.

of the transcription factor in specific cell types and biologic contexts.

A growing body of evidence suggests that the NF-κB/IκB system might play a regulatory role in endothelial activation (for a review see reference 20). NF-κB can be activated by a number of stimuli relevant to endothelial activation, and functional NF-κB sites are found in many genes whose expression is increased at sites of inflammatory responses. In support of this proposal, it has been reported that cytokine or LPS activation induces NF-κB–like DNA-binding activity in endothelial cells (e.g., 2–5, 21). However, these studies do not establish the repertoire of Rel family members expressed by endothelial cells, the identity of the family members involved in κB complex formation, or the role of IκB in regulating NF-κB activation in endothelial cells. Here we define the dynamic pattern of expression of the Rel proteins expressed by endothelial cells in response to TNF-α stimulation and show that specific members of the family are functionally important in the cytokine-induced expression of a leukocyte adhesion molecule, E-selectin. Furthermore, we provide evidence for a rapid loss of IκB-α in endothelial cells stimulated with the TNF-α. The time course of this loss correlates with the appearance of nuclear p50 and p65, as well as NF-κB binding activity. IκB-α is rapidly resynthesized after the loss, suggesting an autoregulatory mechanism for NF-κB regulation in endothelium.

Materials and Methods

Cell Cultures. Human endothelial cells obtained from collagenase-digested umbilical veins (22) were cultured in M199 with 20% fetal bovine serum (FBS), 100 μg/ml porcine intestinal heparin, 50 μg/ml endothelial mitogen, 50 U/ml penicillin and 50 μg/ml streptomycin, and 25 mM Hepes in gelatin-coated plates. Confluent monolayers (3–5 × 10^6 cells, passages 2–4) were exposed to recombinant human TNF-α (Genentech, San Francisco, CA) at a final concentration of 100 or 200 U/ml in complete media. Bovine aortic endothelial cells (BAEC) were isolated and maintained in culture using modifications of previously described procedures (2).

Northern Analysis. Total RNA from 10^7 cells was isolated by the guanidine isothiocyanate method (23). RNA was separated by electrophoresis on a 1% agarose formaldehyde gel, transferred to Hybond-N membrane (Amersham Corp., Arlington Heights, IL), and immobilized by UV irradiation with a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Blots were prehybridized for 6 h and hybridized overnight (24) at 42°C with ^{32}P-labeled probes (Megaprime kit; Amersham Corp.). cDNA clones for p65, p50/p105, c-Rel, and IκB-α were generously provided by Michael Lenardo (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), Gary Nabel (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI), and Stephen Haskill (University of North Carolina, Chapel Hill, NC).

Cell Extracts. After experimental treatment of endothelial cells, nuclear and cytosolic extracts were prepared as described by Moliter et al. (25) with the following modifications. Cell monolayers (3–5 × 10^6 cells) were harvested by scraping, washed in cold PBS, and incubated in 100 μl of buffer A (10 mM Hepes, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 200 mM sucrose, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5% NP-40) for 10 min at 4°C. The crude nuclei released by lysis were collected by microcentrifugation and the cytosolic extracts frozen on dry ice. The nuclear pellet was rinsed once in buffer A and resuspended in 100 μl of buffer B (20 mM Hepes, pH 8.0, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 1 μg/ml each leupeptin and aprotinin). Nuclei were sonicated for 10 s at 15% power output (Virsonic Cell Disruptor; Virtis, Gardner, NY) and clarified by microcentrifugation for 30 s. The resulting supernatants contained 1–2 mg/ml protein by Bradford assay (26) with BSA as the standard. Nuclear extracts were frozen on dry ice and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA). A double-stranded oligonucleotide containing the E-selectin NF-κB site (5′-AGCTTAGAGGGGATTTCCGAGAGGA-3′) was end-labeled with α-[^{32}P]ATP (50 μCi at 3,000 Ci/mmol, New England Nuclear, Boston, MA) and the Klenow fragment of *Escherichia coli* DNA polymerase I. Binding reactions in 20 μl contained 15 μg nuclear extract protein, binding buffer (10 mM Tris, pH 7.5, 20 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μg poly dI-dC, 1 μg single-stranded salmon testes DNA), and 100,000 cpm ^{32}P-labeled DNA. Reactions were incubated at room temperature for 20 min and analyzed by electrophoresis on a 4% nondenaturing polyacrylamide gel at 180 V for 2 h using the high ionic strength conditions described by Ausubel et al. (24). After electrophoresis, gels were dried and DNA–protein complexes localized by autoradiography for 18 h. Competition studies were performed by adding unlabeled double-stranded oligonucleotides to the binding reaction before the addition of labeled oligonucleotide. Competitor oligonucleotides included the wild-type NF-κB sequence (specified above), and an NF-κB 5′ mutant (5′-AGCTTAGATTTTATTTCCGAGAGGA-3′). Competition reactions were incubated for 10 min before addition of labeled oligonucleotide. For supershift analysis, nuclear extracts from TNF-α–treated endothelial cells were incubated with 2 μl of antisera or nonimmune rabbit serum for 15 min at room temperature before addition of binding buffer containing labeled oligonucleotide. Samples were subjected to electrophoresis as described above.

Antibodies to NF-κB and IκB Proteins. Peptide-specific rabbit antisera to p65, p50, and c-Rel were generously provided by Warner Greene (Gladstone Institute of Virology and Immunology, University of California, San Francisco, CA) (27). Anti-IκB-α was generously provided by Stephen Haskill (16, 28).

UV-Crosslinking and Immunoprecipitation. Photoreactive labeled DNA probes were prepared by annealing coding strand template (5′-AGCTTAGAGGGGATTTCCGAGAGGA-3′) with a complementary 10-base primer, (5′-TCCTCTCGGA-3′) and filling in with the Klenow fragment of DNA polymerase I in the presence of α-[^{32}P]dATP, α-[^{32}P]dCTP, dGTP and 1:1 dTTP and 5-bromo-2′-deoxyuridine 5′ triphosphate (BrdU; Sigma Chemical Co., St. Louis, MO) (29). DNA binding reactions performed in 20 μl as described above but using the BrdU substituted probe, were UV irradiated for 15 min using a transilluminator (Fotodyne Inc., New Berlin, WI). For immunoprecipitation, radiolabeled adducts were diluted in 180 μl ELB buffer (50 mM Hepes-CI, pH 7.9, 250 mM NaCl, 0.1% NP-40, 5.0 mM EDTA, 0.5 mM DTT, and supplemented with 200 μg/ml BSA, as well as 1 mM PMSF and 1 μg/ml each of leupeptin and aprotinin) and incubated with 1 μl antisera (1 h on ice) followed by 20 μl protein A-Sepharose (1 h on ice). Immune complexes were washed three times with ELB buffer and analyzed on 8% SDS-polyacrylamide gels.

Western Blot Analysis. Nuclear and cytosolic extracts from cytokine-treated human umbilical vein endothelial cells were electrophoresed on 8% SDS polyacrylamide gels and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 5% methanol at

100 V for 1.5 h at 4°C. Western blots were analyzed for p50, p65, c-Rel, and IκB-α using antiserum at a 1:10,000, 1:10,000, 1:10,000, and 1:5000 dilution, respectively. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham) using 1:10,000 horseradish peroxidase–linked donkey anti–rabbit secondary antiserum. Blots were exposed to film for 1–15 min.

E-Selectin Promoter Growth Hormone Receptor Constructs and Expression Constructs. An E-selectin promoter fragment (30) was generated by PCR amplification and cloned into pOGH (Nichols Institute Diagnostics, San Juan Capistrano, CA) to obtain an E-selectin–GH hybrid reporter construct. The 613-bp PCR fragment (−578 to +35) was gel purified, treated with Klenow to assure blunt ends, phosphorylated by polynucleotide kinase and blunt-end ligated into XbaI-cut and end-filled pOGH. The internal deletion construct, pGH(−578)ΔκB was made using two rounds of PCR amplification. Briefly, subfragments were generated corresponding to −578 to −100 and −75 to +35 which included the SalI sites at the −100 and −75 ends. The subfragments were digested with SalI, ligated overnight, and used as the template for a second round of amplification with forward −578 and reverse +35 primers. The product of the second amplification was cloned into Xba-I-digested pOGH as above. The DNA sequences of the promoter–reporter constructs were confirmed by dideoxynucleotide sequencing. To generate expression vectors, NotI fragments of p65 and p105/p50 were cloned into the NotI site of pcDNA (Invitrogen, San Diego, CA). An EcoRI restriction fragment containing the complete open reading frame of IκB-α was cloned into the EcoRI site of pcDNA.

DNA Sequencing. Nucleotide sequence was determined by the dideoxynucleotide chain termination method with modified T-7 polymerase (United States Biochemical, Cleveland, OH) and α-[^{35}S]dATP (New England Nuclear). Oligonucleotide primers were synthesized on a oligonucleotide synthesizer (model 381A; Applied Biosystems, Foster City, CA) and used without purification.

Transfection of Vascular Cells and Transient Expression of Chimeric Constructs. Subconfluent human umbilical vein endothelial cultures (10^6 cells/100-mm culture dish) and bovine aortic endothelial cells were transfected by a modified calcium phosphate precipitation technique (23). 1 h before transfection, M199 media was replaced with DMEM supplemented with 10% FBS, penicillin-streptomycin, and L-glutamine. Cells were transfected with 20 μg of reporter plasmid and 0.5–1.0 μg p50 or p65 expression plasmids. All plates were balanced for total amount of DNA with an inert pBS vector. Relative transfection efficiency was determined by cotransfection with 5 μg pCATcontrol (Promega, Madison, WI). Bovine aortic endothelial cells were transfected with 10 μg of IκB-α expression plasmid, 10 μg reporter plasmid, and 2.5 μg of PDGF B chain promoter-CAT vector (31). After transfection, cultures were washed twice with HBSS. Complete endothelial media and TNF-α were added 2 h later. 48 h after washing, samples of the medium were collected and assayed for growth hormone by using a commercially available RIA kit (Nichols Institute Diagnostics, Geneva, Switzerland). Cells were harvested by trypsin dissociation, centrifuged (650 *g* for 5 min), washed once in PBS, and resuspended in 0.10 ml of 0.25 M Tris-HCl (pH 7.8) with 10 μg/ml aprotinin (Sigma Chemical Co.). Cells were lysed by sonication and one freeze-thaw cycle. Supernatants containing the cell extracts were obtained after centrifugation for 10 min at 14,000 *g* and stored at −20°C. Transfection of the E-selectin promoter-GH reporter constructs was performed in duplicate for each experiment and the experiments were repeated at least three times using at least two independent plasmid preparations. Each experiment included a negative control, the promoterless plasmid, pOGH, and a positive control, pTKGH.

Conversion of radiolabeled acetyl-coenzyme A to acetylated chloramphenicol was assayed by the two phase fluor diffusion technique (23). For each sample, 30–50 μl of cell extract was incubated at 65°C for 15 min to inactivate endogenous transacetylases. The assay was performed in a reaction mixture of 1.25 mM chloramphenicol (Sigma Chemical Co.), 125 mM Tris-HCl, pH 7.8, and 0.1 μCi of [^{3}H]-acetyl coenzyme A (New England Nuclear) in a reaction volume of 0.2 ml. The reaction mixture was overlaid with 5 ml of water immiscible scintillation fluid (Econofluor; Dupont, Wilmington, DE) and incubated at 37°C for 2 h. The activity of the [^{3}H]-acetylated chloramphenicol was measured on a Rackbeta scintillation counter (LKB, Piscataway, NJ).

Results

Cytokine-induced Expression of NF-κB Transcripts in Cultured Endothelial Cells. Fig. 1 shows Northern blot analysis of RNA from endothelial cells activated by TNF-α. Transcript levels from two NF-κB–dependent leukocyte adhesion molecules, E-selectin and VCAM-1, are shown for comparison (Fig. 1 *A*). p65 mRNA (2.6 kb [32]) was present constitutively and increased slightly after cytokine activation. p105/p50 tran-

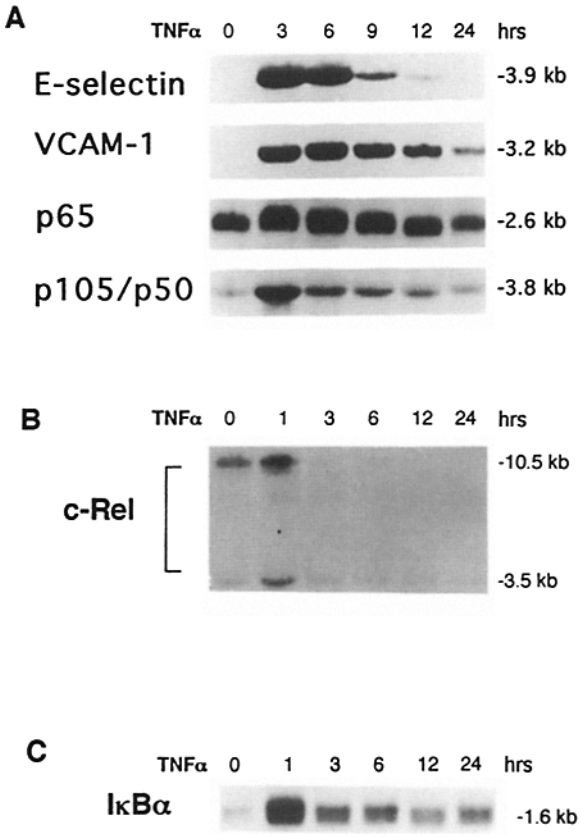

Figure 1. Expression of Rel family members by TNF-α–activated endothelial cells. Human umbilical vein endothelial cells were incubated in medium alone or treated with TNF-α (200 U/ml) for the times indicated. Total RNA was isolated and Northern blots prepared as described in Materials and Methods. (*Right*) Transcript sizes (*kb*).

script (3.8 kb [33, 34]) was constitutively present at low levels, increased at 3 h, and gradually returned to basal levels. The two transcripts for c-Rel, >10.5 and 3.5 kb (35) were present in control cultured endothelial cells (Fig. 1 *B*). Levels of both transcripts increased transiently in parallel after TNF-α stimulation and then decreased to below control levels. IκB-α transcript (28) levels increased dramatically in response to TNF-α and remained elevated (Fig. 1 *C*). Thus, TNF-α activation of endothelial cells caused a significant but transient increase in p65, p105/p50, and c-Rel, as well as a rapid increase in the transcript levels of a potential inhibitor of NF-κB, IκB-α.

NF-κB Binding to the E-selectin κB Site in Cultured Endothelial Cells. The preceding observations establish that endothelial cells express transcripts for Rel family members and that levels of these transcripts can be modulated by TNF-α. Of the many induced genes at sites of endothelial activation which have κB sites, we have elected to further characterize the κB site from the E-selectin gene (4, 30) as an example of an NF-κB–dependent gene in endothelium. Nuclear extracts were prepared and assayed for NF-κB binding activity using the EMSA. TNF-α specifically induced two NF-κB–DNA binding complexes in endothelial cells (Fig. 2 *A*, lanes *1* and *2*). Competition of complexes A and B by unlabeled wild-type oligonucleotide, but not by a mutant oligonucleotide, verified the specifically of binding to DNA (Fig. 2 *A*, lanes *3–6*). A third complex (C) was constitutively expressed (Fig. 2 *A*, lane *1*) and was not competed by the highest concentration of unlabeled wild-type oligonucleotide, or by mutant NF-κB oligonucleotides (Fig. 2 *A*, lanes *3–6*). Similar complexes in endothelial cells have been seen in response to LPS and with other κB sites (e.g., 2, 3, 21).

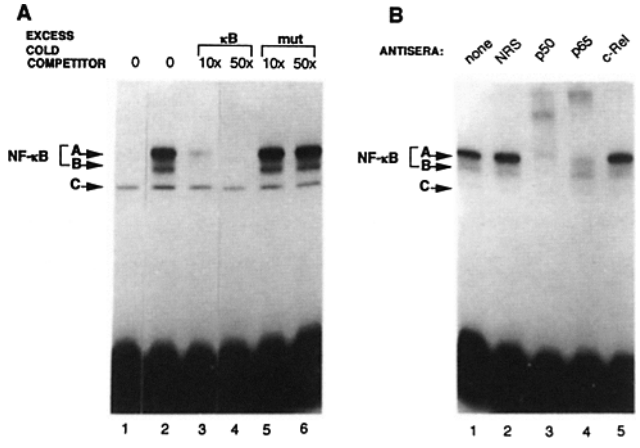

Figure 2. Activation of NF-κB in endothelial cells and the effects of antisera on NF-κB DNA binding. (*A*) Competition of DNA binding by unlabeled wild-type (*κB*), but not mutant (*mut*) oligonucleotides. Endothelial cells were incubated in medium alone (lane *1*) or TNF-α (200 U/ml) for 1 h (lanes *2–6*). Nuclear extracts were prepared and assayed for NF-κB binding activity in the absence or presence of indicated excess cold competitor oligonucleotides. (*Arrows*) Position of the induced complexes A and B, and the constitutive complex C. (*B*) Reactivity of the induced κB binding complexes with anti-p50, anti-p65, and anti-c-Rel antisera. Nuclear extracts from TNF-α–treated (200 U/ml, 1 h) endothelial cells were incubated with normal rabbit serum or antiserum before the assay for NF-κB binding activity. Lane *1*, no antisera; lane *2*, normal rabbit serum (*NRS*); lane *3*, anti-p50; lane *4*, anti-p65; and lane *5*, anti-c-Rel.

To determine which proteins were present in the E-selectin κB–DNA complexes induced by TNF-α, human umbilical vein endothelial cell nuclear extracts were incubated with 2 μl of normal rabbit serum or antisera to p50, p65, and c-*rel* before the EMSA. As shown in Fig. 2 *B*, normal rabbit serum did not affect complex formation (lane *2*). Anti-p50 eliminated and/or caused a supershift in both induced complexes A and B (Fig. 2 *B*, lane *3*). Anti-p65 caused a supershift of complex A, but did not affect formation of complex B (Fig. 2 *B*, lane *4*). Anti-c-Rel did not alter formation of either complex A or B (Fig. 2 *B*, lane *5*), although this antibody did recognize c-Rel expressed by a human monocytic cell line (THP-1) (18) and in extracts of Jurkat cells (data not shown) in a similar κB supershift analysis. None of the antisera affected formation of the nonspecific complex (C). Thus, the induced complexes contain p50 and p65 (complex A) and p50 (complex B) NF-κB DNA-binding proteins, but not c-Rel.

Biochemical characterization of E-selectin κB binding proteins present in nuclear extracts of control and TNF-α–treated human umbilical vein endothelial cells was accomplished by UV crosslinking analysis. A photoreactive form of the E-selectin κB probe was incubated with nuclear extracts from uninduced and TNF-α–treated endothelial cells. After UV irradiation, the resulting protein-DNA adducts were directly resolved by SDS-PAGE (Fig. 3 *A*) or immunoprecipitated with antisera to p50, p65, and c-Rel, and then resolved by SDS-PAGE (Fig. 3 *B*). TNF-α induced the formation of three major DNA protein adducts which migrated at 75 kD and as a 50–55-kD doublet (Fig. 3 *A*, lane *4*). These adducts were specifically competed with unlabeled wild-type oligonucleotide but not mutant oligonucleotide (Fig. 3 *A*, lanes *5* and *6*). The molecular weights of these adducts are consistent with previously described p65 and p50 UV crosslinked adducts (25). Low levels of these adducts were observed in nuclear extracts from uninduced cells (Fig. 3 *A*, lanes *1–3*). Immunoprecipitation analysis was used to identify these adducts. From TNF-α–induced nuclear extracts, anti-p65 identified a 75-kD adduct, corresponding to the predicted mobility of p65 covalently bound to the oligonucleotide (Fig. 3 *A*, lane 7) and the 50–55-kD adducts (lane 7). Anti-p50 coimmunoprecipitated a 75-kD adduct (p65) and two 50–55-kD adducts (Fig. 3 *B*, lane *6*). Denaturation by heating in SDS before immunoprecipitation confirmed that anti-p50 recognized only the 50–55-kD adducts, and that anti-p65 recognized only the 75-kD adduct (data not shown). Anti-c-Rel failed to immunoprecipitate the expected 85-kD adduct, consistent with the lack of immunoreactivity by gel shift analysis. From uninduced nuclear extracts, p50 immunoprecipitated small amounts of 50–55-kD adducts (Fig. 3 *B*, lane *2*).

Cytokine-induced Changes in IκB-α and NF-κB Protein Levels in Cultured Endothelial Cells. To assess the dynamics of IκB-α protein expression occurring during endothelial activation, human umbilical vein endothelial cells were treated with TNF-α, and subcellular fractions were subjected to immunoblotting (Fig. 4 *A*) and gel shift analysis (Fig. 4 *B*). Cyto-

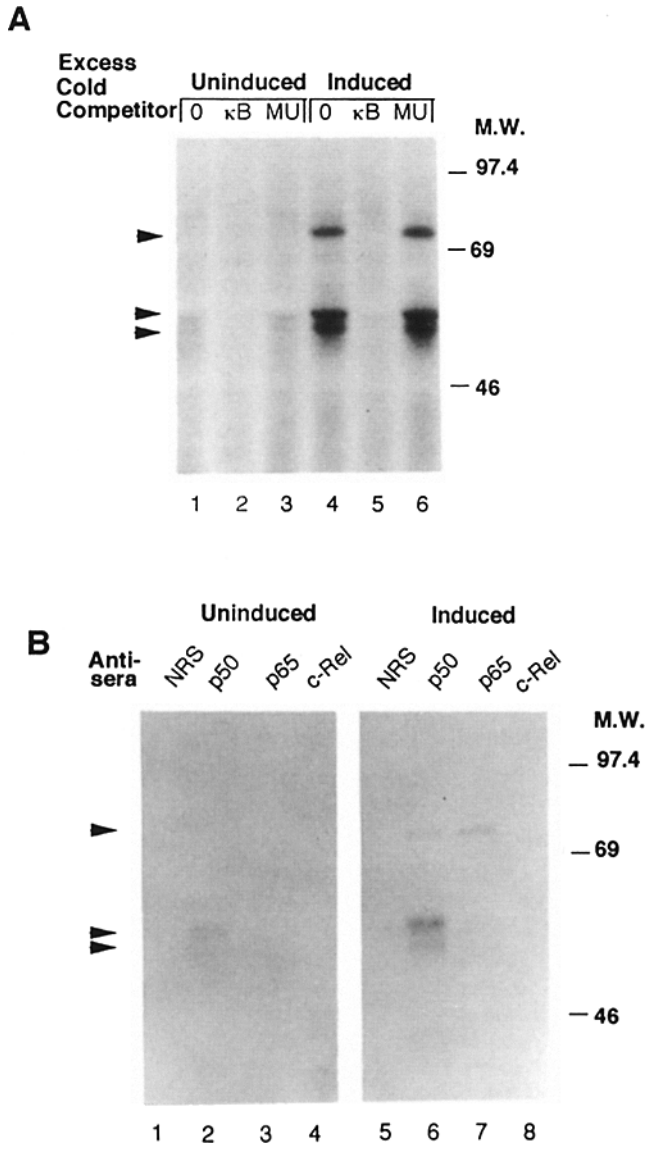

Figure 3. UV crosslinking analysis of DNA protein complexes formed with the E-selectin κB site. (*A*) Nuclear extracts from uninduced (lanes *1–3*) and TNF-α–treated (100 U/ml for 1 h, lanes *4–6*) endothelial cells were incubated with a photoreactive form of the ^{32}P-labeled κB probe in the presence or absence of 50-fold unlabeled wild-type (*κB*, lanes *2* and *5*) or mutant (*MU*, lanes *3* and *6*) oligonucleotide. After UV irradiation, protein DNA adducts were analyzed on 8% SDS-PAGE. (*B*) Immunoprecipitation of UV-crosslinked DNA protein complexes. Protein/DNA adducts as described in *A* were immunoprecipitated with antiserum to p50 (lanes *2* and *6*), p65 (lanes *3* and *7*), c-Rel (lanes *4* and *8*), or normal rabbit serum (*NRS*, lanes *1* and *5*). The resultant immune complexes were analyzed on 8% SDS-PAGE.

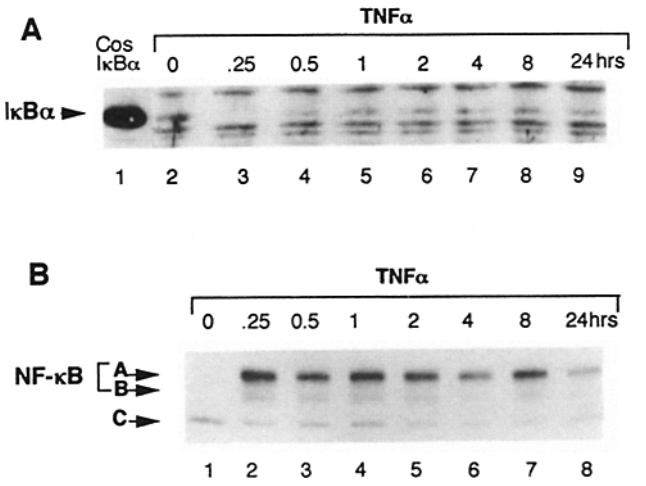

Figure 4. Degradation and resynthesis of IκB-α after endothelial activation by cytokine. Endothelial cells were stimulated with TNF-α (100 U/ml). (*A*) At the times indicated (lanes *2–9*), cells were collected for the preparation of cellular extracts and cytosols analyzed for IκB-α by Western blot analysis. Lane *1* contains extract from COS cells which were transfected with exogenous IκB-α. (*B*) Nuclear extracts from cells treated as described in *A* were analyzed for NF-κB DNA binding activity.

solic extracts from cells exposed to TNF-α for the times shown in Fig. 4 *A* (lanes *2–9*) were analyzed by Western blotting using antisera raised to an IκB-α peptide (16, 19). A 37-kD protein was detected in cytoplasmic extracts from unstimulated cells (Fig. 4 *A*, lane *2*) which comigrated with IκB-α present in extracts from COS cells transfected with an exogenous IκB-α cDNA expression vector (Fig. 4 *A*, lane *1*). In endothelial cells treated with TNF-α, cytoplasmic IκB-α completely disappeared within 15 min of stimulation. IκB-α gradually reappeared in the cytoplasm (Fig. 4 *A*). IκB-α did not accumulate in endothelial nuclear extracts (data not shown). The time course of the depletion of IκB-α after stimulation correlates with the nuclear appearance of NF-κB (Fig. 4 *B*). After 15 min of TNF-α exposure, NF-κB binding activity is present in endothelial nuclear extracts and IκB-α levels have fallen dramatically (Fig. 4 *B*, lane *2*). Although steady state levels of IκB-α mRNA were dramatically upregulated by TNF-α (Fig. 1 *C*), cytosolic IκB-α remained below baseline levels, perhaps indicating continuous degradation in response to cytokine. Nuclear NF-κB binding remains elevated (Fig. 4 *B*, lanes *6–8*) for 24 h, consistent with the failure of cytosolic IκB-α to return to baseline levels (Fig. 4 *A*, lanes *7–9*). At this time, there is no transcription from the E-selectin gene (4, and Fig. 1 *A*). Therefore, NF-κB cannot be entirely responsible for the temporally regulated pattern of cytokine-induced E-selectin gene expression in endothelial cells.

Immunoblotting analysis of the same nuclear and cytosolic extracts revealed that the loss of IκB-α in the cytoplasm correlated with the translocation of p50 and p65 from the cytoplasm into the nucleus. Cultured endothelial cells have a substantial pool of both p50 and p65 in a cytoplasmic reserve (Fig. 5). Consistent with the UV-crosslinking analysis (Fig. 3 *B*, lane *2*), p50 was present in small amounts in uninduced nuclear extracts, whereas p65 and c-Rel were not detected (Fig. 5, lane *9*). After 15 min of TNF-α stimulation, both NF-κB components p50 and p65 were markedly increased in the nucleus (Fig. 5, lane *10*), consistent with the kinetics of induction of κB binding (Fig. 4 *B*), as well as the supershift (Fig. 2 *B*) and UV crosslinking analyses (Fig. 3, *A* and *B*). Cytoplasmic pools of p50 and p65 were not depleted in

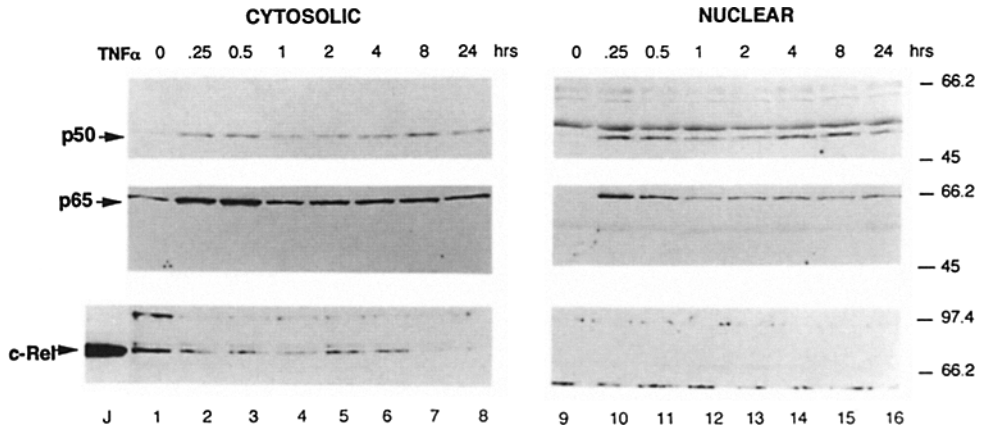

Figure 5. Subcellular location of NF-κB/Rel proteins in endothelial cells. Cytosolic (lanes *1–8*) and nuclear (lanes *9–16*) fractions prepared as in Fig. 4 were immunoblotted with antisera to p50, p65, and c-Rel. Lane *J* is cytosolic extract from PMA-treated Jurkat cells. (*Right*) Molecular weight markers.

parallel with the nuclear translocation of these NF-κB components. After activation, nuclear p50/p65 levels remained high despite the appearance of newly synthesized IκB-α (Fig. 5 *A*). The mechanism by which NF-κB persists in the nucleus with continued cytokine stimulation is unclear. c-Rel was seen in endothelial cells which comigrated with the c-Rel present in cytoplasmic extracts from Jurkat cells (Fig. 5, lane *J*). Relative to the amount of c-Rel found in comparable numbers of Jurkat cells, the level of c-Rel in cytoplasmic extracts of cultured human endothelial cells was low. In striking contrast to the results with p50 and p65, c-Rel was not detected in the nucleus with cytokine stimulation. Thus, in endothelial cells, various Rel related proteins can coexist and respond independently to environmental signals.

Regulation of E-selectin Gene Expression by Recombinant NF-κB Components. These findings establish that p50 and p65 rapidly translocate into the nucleus of TNF-α–treated endothelial cells and are capable of binding to the E-selectin κB site. These observations, however, do not establish the functional significance of these NF-κB components in the transcriptional regulation of the E-selectin gene. Previous deletion analysis in endothelial cells revealed that the κB site in the E-selectin core promoter was necessary (but not sufficient) for the cytokine responsiveness of the gene (4). The NF-κB motif in the E-selectin promoter (GGGGATTTCC) is completely conserved in both the human (30) and the rabbit (36) promoters and is in agreement with a consensus sequence (GGRNTTTCC) for p65 binding (37). Little variation is

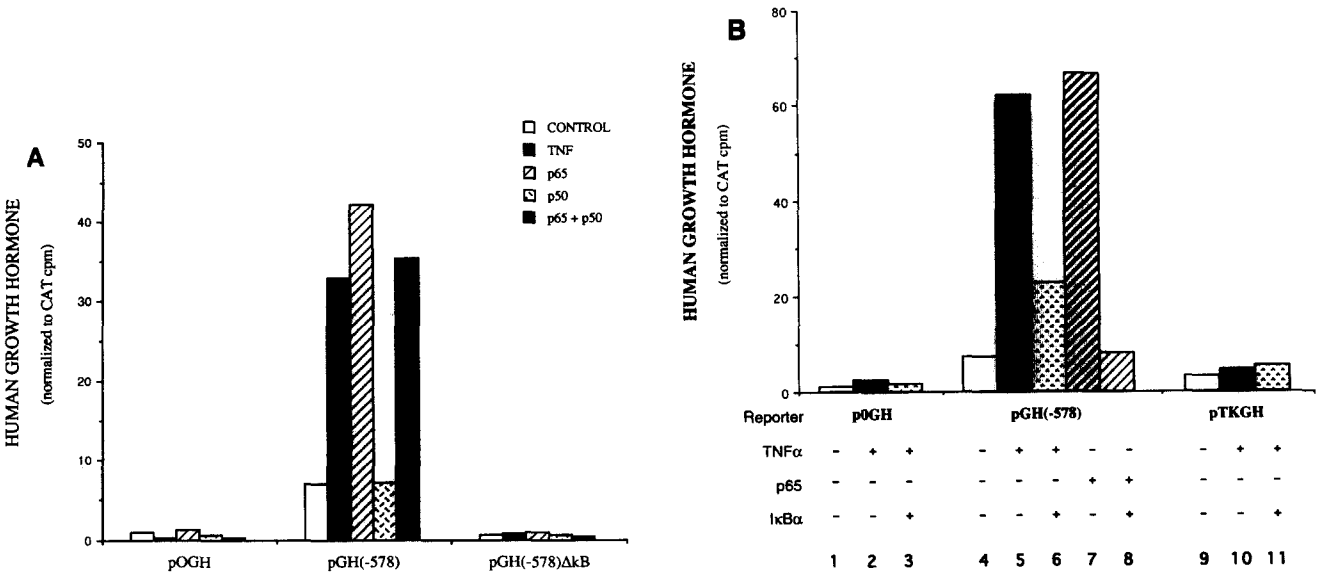

Figure 6. Regulation of E-selectin gene expression by NF-κB and IκB-α. (*A*) E-selectin gene expression induced by NF-κB components. Human umbilical vein endothelial cells were cotransfected with 1 μg of the NF-κB component expression vectors, 20 μg of an E-selectin–GH reporter plasmid, and 5 μg of pCATcontrol vector. Transfection efficiencies were normalized by measuring CAT activity. (*B*) IκB-α–mediated inhibition of E-selectin gene expression induced by recombinant p65 or TNF-α. BAEC were cotransfected with 10 μg of an E-selectin–GH reporter plasmid, 10 μg IκB-α expression vector, and 2.5 μg of the control plasmid. Reporter construct activity is expressed as ng/ml growth hormone (*GH*), divided by cpm of CAT activity. The value of control pOGH is then set to 1.0.

tolerated in the 3′ half-site of p65 binding sites (37), and interestingly, in the mouse E-selectin gene, only the 3′ half site of the functional NF-κB promoter element is conserved (38). The structure of the E-selectin NF-κB site suggests that the p65 component of NF-κB could play an important role in the induced expression of the E-selectin gene.

The potential function of the κB site in the E-selectin gene was analyzed by cotransfection experiments with an E-selectin promoter reporter construct and various NF-κB expression constructs. p65, or mixtures of p50 and p65, stimulated expression of the chimeric reporter gene. The responsiveness of the E-selectin promoter to stimulation was dependent upon the κB regulatory element. Responses were similar in either human umbilical vein endothelial cells (Fig. 6 *A*), or BAEC (data not shown). Similar results were obtained with p65 expression constructs and a *VCAM-1* promoter–reporter construct (39). These results are consistent with the finding of κB binding proteins observed in nuclear extracts from cytokine-stimulated endothelial cells and suggest that p65, either alone or in combination with p50, can induce expression of at least two NF-κB–dependent genes in endothelial cells.

IκB-α Inhibits p65 and TNF-α–stimulated E-Selectin-Reporter Gene. To determine whether induced E-selectin gene expression by NF-κB could be regulated by IκB-α, expression vectors for different NF-κB subunits were cotransfected with an expression vector for IκB-α and an E-selectin–reporter gene. With an E-selectin–GH reporter, transfection of IκB-α almost completely abolished marker gene expression stimulated by p65 in bovine endothelial cells (Fig. 6 *B*, lanes 7 and *8*), as well as in human umbilical vein endothelial cells and COS cells (data not shown). These findings demonstrate that the transcriptional activation of the E-selectin gene induced by NF-κB can be regulated by IκB-α. Similar results were obtained with p65, IκB-α, and a VCAM-1 promoter–reporter construct (39), raising the possibility that IκB-α may regulate expression of multiple NF-κB–dependent genes in endothelial cells.

These findings are consistent with the inhibition of p65-mediated transcription by transfected IκB-α reported earlier with other cell types and different reporter genes (e.g., 16, 17). To extend these observations, we determined whether IκB-α could block E-selectin reporter gene expression induced by TNF-α in endothelial cells (Fig. 6 *B*). Endothelial cells were transfected with an E-selectin–GH reporter, the IκB-α expression vector, and then challenged with human TNF-α. Transfection of the IκB-α expression vector blocked the TNF-α–induced expression of the E-selectin reporter gene (Fig. 6 *B*, lanes *5* and *6*). Suppression of the E-selectin-GH expression by IκB-α was dose dependent (data not shown). In contrast, a constitutively expressed reporter gene (thymidine kinase–growth hormone, pTKGH), was not affected by expression of IκB-α (Fig. 6 *B*, lanes *9–11*), suggesting that the suppressive effect was specific for genes that are dependent upon NF-κB. These studies demonstrate that IκB-α can suppress TNF-α–induced expression of an E-selectin reporter gene in cultured endothelial cells.

Discussion

These studies demonstrate that human endothelial cells express multiple members of the Rel family transcription factors, and that stimulation by TNF-α results in translocation of p50 and p65 to the nucleus. The functional significance of two of these factors in endothelial gene regulation was demonstrated by transient transfection analysis, where cotransfection of p65 or p65/p50 resulted in increased levels of expression of an E-selectin–reporter construct. Endothelial cells also express an inhibitor of NF-κB activation, IκB-α, and can dramatically modulate the level of this inhibitor in parallel with the activation process. IκB-α is functionally relevant in cytokine-induced expression of E-selectin, in that overexpression of this protein in endothelial cells blocks TNF-α and p65-mediated expression of an E-selectin promoter–reporter gene. These data suggest that NF-κB and IκB-α are involved in cytokine-mediated signaling and may play an important role in regulation of gene expression during endothelial activation.

Regulation of the NF-κB/IκB-α system in endothelial cells may be a tightly controlled process that ensures that the endothelium can make appropriate responses to environmental stimuli. The presence of cytoplasmic pools of p50 and p65 in endothelial cells suggests that the cells are primed for activation. If endothelial cells are similar to other cell types, the NF-κB heterodimers are maintained in the endothelial cytoplasm by IκB-α (for reviews see references 12–15). Here we demonstrate that activation-induced IκB-α degradation in endothelial cells is coupled to NF-κB nuclear translocation. Specific signals, such as cytokines or LPS, release p50/p65 from the IκB-α, resulting in rapid translocation of transcriptionally active heterodimers to the endothelial nucleus. The exact signal transduction pathways for activating NF-κB in endothelial cells are not understood, although TNF-α–induced cell adhesion to human endothelial cells is mediated through the 55-kD TNF-α receptor (40). In other cell types, TNF-α has been shown to activate NF-κB through generation of ceramide, which is released from sphingomyelin by enzymatic hydrolysis (41). Other Rel-related factors in endothelial cells, specifically, c-Rel, may be activated by distinct, as yet undefined stimuli.

The full complement of physiologically relevant target genes for NF-κB in endothelial cells is not clear. The nature of the responsive target genes probably depends upon the activation of specific Rel-related protein complexes and the structure of the κB sites (37). Here we demonstrate that p50/p65 act on the E-selectin promoter, and in parallel studies suggest that the VCAM-1 κB sites are also a target of NF-κB activation (39). Additionally, we show here that the genes that code for p65, p105, and IκB-α are highly inducible in endothelial cells in response to the stimuli that activate NF-κB. These results are consistent with recent observations that the promoter of the IκB-α gene contains κB binding sites (42) and that p65 stimulates IκB-α gene expression (42–45). Thus p65 and IκB-α have been directly linked in an autoregulatory loop. Additionally, p65 stabilizes IκB-α protein (43, 45),

which may help to maintain cytoplasmic levels of the inhibitor. The significance of the autoregulatory loop for NF-κB and IκB-α is that it allows a continuous maintenance of the cytoplasmic reservoirs of NK-κB complexes for stimulation in an acute response, and also prepares the endothelial cell to return NF-κB to its uninduced condition.

The NF-κB and IκB-α mechanism insures that the induction of NF-κB is transient and that the activated endothelial cell returns to a quiescent state within a relatively short period. The rapid cytokine-induced activation of NF-κB in endothelial cells is probably similar to that seen in other cell types; however, the recovery kinetics of the NF-κB/IκB-α response in endothelium may be different. The data presented here suggest that NF-κB remains activated and IκB-α levels remain below basal levels for longer periods of time than seen in cells programmed for rapid responses, such as macrophages. It is possible that cytokines released by activated endothelial cells result in an autocrine activation of NF-κB and continued degradation of IκB-α. Such prolonged endothelial activation may play a role in recruiting circulating cells to sites of ongoing inflammatory or immune processes.

The regulation of NF-κB activation may be important in the initial onset of multiple forms of vascular pathobiology. Situated between the circulating blood elements and the surrounding tissues, vascular endothelium is in a privileged anatomic setting. Endothelial cells at sites of inflammatory responses are exposed to activating cytokines and various forms of reactive oxygen species. Many of these agents may have in common the ability to generate oxidant stress and activate NF-κB (46). Since multiple genes relevant to the pathobiology of endothelial activation have potentially functional NF-κB sites, activation of the pleiotropic mediator NF-κB in endothelial cells could coordinate the expression of numerous endothelial products which are important in endothelial activation, including some cell surface adhesion proteins, cytokines, growth factors, and components of the coagulation system. These endothelial cell products may act cooperatively to facilitate inflammatory or immune responses. Increased expression of inhibitors of NF-κB activation might decrease NF-κB activation and diminish expression of NF-κB–dependent genes. This regulatory mechanism insures that the induction of NF-κB is transient and that the activated endothelial cell returns to a quiescent state. This dynamic balance might be offset during the initial onset of vascular pathology, such as in the initiation of the atherosclerotic lesion (47).

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