Activation of NF-κB via the IκB Kinase Complex Is Both Essential and Sufficient for Proinflammatory Gene Expression in Primary Endothelial Cells*

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The activation of endothelial cells is the key process promoting initiation of inflammatory reactions. It is associated with a multistep cascade of events that results in the local recruitment of leukocytes to sites of inflammatory challenge. Selectins and members of the immunoglobulin superfamily of adhesion molecules are up-regulated by proinflammatory factors and sequentially mediate rolling, adhesion, and transmigration of leukocytes from the blood stream to underlying tissues (for review see Refs. 1 and 2). In addition, chemoattractant cytokines (chemokines) produced by and presented on endothelial cells deliver signals that further trigger firm adhesion of rolling blood cells and transmigration, e.g. by modulation of leukocyte integrin activity (3).

The process of endothelial activation underlies tight regulatory control mechanisms. Proinflammatory stimuli targeting the endothelium such as tumor necrosis factor-α (TNF-α), interleukin-1, or bacterial lipopolysaccharides elicit activation of intracellular signal transduction cascades. One of the most important pathways results in the activation of members of the nuclear factor-κB (NF-κB)/Rel family of transcription factors. NF-κB was suggested to be the major transcriptional regulator of endothelial adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) as well as of chemokines such as monocyte-chemoattractant protein-1 (MCP-1) or interleukin 8 (IL-8) (4).

Rel family members (p65/RelA, RelB, c-Rel, p50, and p52) form homo- or heterodimeric complexes with each other that constitute the NF-κB complex (5, 6). In resting cells, NF-κB is inactive because of association with inhibitor κB (IκB) proteins that mask the nuclear localization sequence of NF-κB, thereby retaining it in the cytoplasm and preventing DNA binding. Several IκB proteins are involved in the control of NF-κB activity, three of which, IκBα, IκBβ, and IκBe, act in a stimulus-dependent manner. Upon inflammatory activation, IκBα is phosphorylated in its N-terminal domain; subsequently it becomes ubiquitinylated and finally degraded by the proteasome. This allows nuclear translocation of NF-κB and binding to cognate DNA motifs in the promoter region of target genes, which subsequently initiates transcription. The critical step in NF-κB activation is the phosphorylation of IκB by a large multisubunit kinase complex consisting of IκB kinase (IKK) 1/α and 2/β as well as an additional essential protein, NF-κB essential modulator (NEMO)/IKKγ (reviewed in Ref. 6). NEMO represents the regulatory component of the IKK complex, whereas IKK1 and IKK2 act as catalytic subunits. Both IKKs can phosphorylate all three IκB proteins (α, β, and ε) to a similar extent. In endothelial cells all three IκB are degraded upon stimula-

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The abbreviations used are: TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemotactrant protein-1; IL-8, interleukin-8; IκB, inhibitor κB; IKK, IκB kinase; EGM, endothelial growth medium; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase; SEK, stress-activated protein kinase/ERK kinase; AP-1, activator protein-1.
tion with TNF-α but with slightly different kinetics (7–9). There have been some investigations into the role of IκBα for the function of endothelial cells (10–12). However, the relative contribution of the different IκB isoforms to cytokine-induced endothelial gene expression has not been studied in detail. Furthermore, although several studies reported a role for IKK2 in TNF-α-induced endothelial NF-κB activation (8, 13, 14), the relevance of IKK1 for this process has not been elucidated yet.

We therefore initiated the present study to analyze the functional role of the IKKs and IκBs during activation of primary human endothelium. Human umbilical vein endothelial cells were infected with retroviruses that express transdominant mutants of IκBα, -β, and -ε or kinase-inactive versions of IKK1 and IKK2. We then studied TNF-α-induced expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) and chemokines (MCP-1, IL-8) in the infected cells. Whereas dominant negative IκBα and transdominant IκBα, -β, and -ε mutants completely inhibited expression of these molecules, dominant negative IKK1 blocked induction to a variable extent. Importantly, a constitutively active version of IKK2 was sufficient to yield maximal induction of the various target genes, demonstrating that NF-κB is not only essential but also sufficient for expression of activation markers on endothelial cells. Our data thus show that the IKK/IκB/NF-κB signal transduction cascade is the dominant control mechanism for inflammatory activation of endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Primary human endothelial cells derived from umbilical veins were obtained from Clonetics (via Cell Systems, St. Katharinien, Germany). They were cultured in endothelial basal medium (Clonetics) supplemented with 2% fetal bovine serum, 1.0 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 12 μg/ml bovine brain extract, 50 μg/ml gentamicin, and 50 μg/ml amphotericin B, which together constituted EGM. Experiments were performed with cells of passage 3. The dNX amphotropic retrovirus producer cells (a gift from G. Nolan, Stanford, CA) (15) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum (PAN Systems, Aidenbach, Germany), 100 units/ml penicillin (Life Technologies, Inc.), and 100 μg/ml streptomycin (Life Technologies, Inc.). Cells were stimulated with human recombinant TNF-α (R&D Systems, Wiesbaden, Germany) or human recombinant IFN-γ (Peprotech, London, UK) as indicated.

**Retroviral Infection of Endothelial Cells with Supernatant from dNX Producer Cells**—Four days before infection, endothelial cells were seeded in 6-well plates at a density of 5 × 10^4 cells/well in EGM medium. Twenty-four h before infection, the medium was changed from Dulbecco’s modified Eagle’s medium to EGM. The infection procedure was repeated 1 day later to increase the efficiency of infection, which was finally monitored by fluorescence microscopy or flow cytometry as described above. Infection efficiencies of endothelial cells ranged between 10 and 70%, depending on the retrovirus used.

**Western Blot Analysis and Electrophoretic Mobility Shift Assay (EMSA)**—Preparation of whole cell extracts was performed as described earlier (18). For Western blot analysis, 50 μg of protein extracts per lane were separated on 12.5% polyacrylamide gels and transferred onto polyvinylidenefluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 7.5% dry milk in PBS containing 0.2% Tween 20. For subsequent washes, 0.2% Tween 20 in PBS was used. Membranes were labeled with affinity-purified rabbit antisera against IκBα or JNK (Santa Cruz Biotechnology, Santa Cruz, CA) or phospho-JNK (New England Biolabs, Ipswich, MA) and incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Membranes were visualized by chemiluminescence (Pierce). Membrane bands were quantified with a densitometer (Model 890, Gel Doc, Bio-Rad, Hercules, CA).

**Flow Cytometry**—Cells were stained with fluorescent antibodies and analyzed by flow cytometry with a FACScalibur (Becton Dickinson, Heidelberg, Germany). Transfection efficiencies usually ranged between 70 and 80%. Twenty-four h after transfection, 1 mg/ml zeocin (Invitrogen, Groningen, The Netherlands) was added to the cells, which were then grown in the presence of this agent for another 2 weeks until all the cells were positive for GFP.
The Role of IKKs and IκB in Endothelial Activation

For the analysis of adhesion molecule expression on the cell surface, endothelial cells were stimulated with cytokines as indicated, incubated with 1% bovine serum albumin in PBS to block nonspecific binding, and immunostained with mouse monoclonal antibodies against E-selectin, VCAM-1 (both obtained from Dianova), or ICAM-1 (Immunootech, Marseille, France) diluted in 1% bovine serum albumin/2% normal goat serum/PBS. Thereafter, cells were successively stained with biotin-Sp-conjugated goat anti-mouse IgG (Dianova) and streptavidin-Cy-chrome (Becton Dickinson). Adhesion molecule expression of the GFP+ population as detected in the FL3 channel was determined with a FACScalibur. Nonviable cells were excluded from analysis by means of forward scatter and side scatter light parameters. Quantification of expression levels determined in the fluorescence-activated cell sorting was done by determining the mean value of the area covered by the curve. All flow cytometry experiments were repeated at least twice and revealed essentially similar results.

RESULTS

Retroviral Transduction of Endothelial Cells with Dominant-interfering Mutants of the IKK/NF-κB Pathway—To study the contribution of the IKK/NF-κB signaling module to endothelial gene expression after inflammatory activation, we used retroviral gene transfer to express dominant interfering mutants of this pathway. Negative modulation of NF-κB signaling was achieved by using transdominant mutants of different IκB proteins as well as kinase-inactive mutants of IKK1 and IKK2. For selective activation of the IKK/NF-κB module, we used an active mutant of IKK2 to investigate whether constitutive NF-κB activity is sufficient to drive expression of the examined target genes. Genes for the interfering mutants were transduced by a retroviral infection approach (Fig. 1c), which results in DNA integration and stable chromatin-embedded expression of the mutant proteins. The long terminal repeat-mediated expression of the inserted gene is coupled to the expression of a GFP-zeocin resistance fusion gene through an internal ribosome entry site (16). This allowed us to distinguish between infected and noninfected cells by flow cytometric analysis of GFP expression. The percentage of GFP-expressing cells was used as an indicator for transduction efficiencies. Furthermore, this method permitted us to compare equally treated uninfected and infected cells in the same dish regarding the effects of mutant proteins on expression of endothelial adhesion molecules and chemokines.

Expression of Transdominant Mutants of the IκB Proteins Results in a Blockade of Proinflammatory NF-κB Activation in Endothelial Cells—To analyze whether the transdominant IκB mutants are functional in inhibiting NF-κB activation, human umbilical vein endothelial cells were infected with different retroviruses and stimulated with TNF-α for various time intervals. Subsequently, the expression of endogenous (wild-type) and mutant IκB proteins was studied by Western blot analysis. Endogenous IκBα was degraded within 5 min of TNF-α exposure, and the protein completely disappeared after 30 min (Fig. 1b). In contrast, the mutant protein remained stable over the whole observation period (Fig. 1c). Endothelial cells transduced with the empty vector showed maximal NF-κB DNA-binding activity after 5 min and remained at this level over the period investigated (Fig. 1b). Supershift analysis revealed that these DNA-binding complexes predominantly consisted of p50/RelA heterodimers (data not shown). Endothelial cells expressing transdominant IκBα displayed almost no NF-κB DNA-binding activity in response to TNF-α (Fig. 1c). The weak residual activity is most likely contributed by the uninfected cells. The same results were obtained when transdominant IκBβ and IκBε mutants were studied (data not shown). Thus, the expression of transdominant IκBα proteins results in an efficient inhibition of induced NF-κB activation in human primary endothelial cells.

Expression of Transdominant IκB Mutants Completely Abolishes TNF-α-induced Up-regulation of Endothelial Adhesion Molecules and Chemokines—To examine the effect of mutant IκB protein expression on proinflamatory induction of endothelial proteins, endothelial cells were infected with retroviruses expressing transdominant IκBα or the parental vector as

![Image](http://www.jbc.org/)

Fig. 2. Expression of transdominant IκBα inhibits TNF-α-induced up-regulation of VCAM-1. Endothelial cells infected with parental vector as control (left panels) or a retrovirus carrying a transdominant (TD) mutant of IκBα (right panels) were exposed to 2 ng/ml TNF-α 24 h after infection for another 16 h. FSC, forward scatter light. Cells were then harvested, immunostained for VCAM-1 surface expression, and processed for flow cytometry as described under “Experimental Procedures.” Successfully infected cells, i.e. cells expressing the IκBα mutant, are labeled by co-expressed green fluorescent protein (top panel). The flow cytometric profiles in the middle and bottom panels show endothelial expression of VCAM-1 after exposure to TNF-α (green line) or to medium as control (black line). The middle panel shows VCAM-1 expression in the uninfected GFP+ population, and the bottom panel shows VCAM-1 expression in the infected GFP+ population.

Flow Cytometry Analysis—The chemokines MCP-1 and IL-8 were detected by an intracellular flow cytometry staining procedure as described before (13). Endothelial cells were stained with cytokine (or medium as control) for the time intervals indicated in the presence of 2 μM monensin to avoid secretion via the Golgi pathway. Cells were subsequently harvested, washed, and fixed with 4% paraformaldehyde at 4 °C for 20 min. Endothelial cells were subsequently incubated with mouse monoclonal antibodies against MCP-1, IL-8, or isotype control antibodies (all obtained from Becton Dickinson), which had been diluted in permeabilization buffer (0.1% saponin/1% fetal calf serum/PBS). Thereafter, cells were successively stained with biotin-Sp-conjugated goat anti-mouse IgG (Dianova) and streptavidin-Cy-chrome (Becton Dickinson), and fluorescence of induced proteins was determined in the FL3 channel using a FACScalibur (Becton Dickinson).

For the analysis of mRNA expression on the cell surface, endothelial cells were stimulated with cytokines as indicated, incubated with 1% bovine serum albumin in PBS to block nonspecific binding, and immunostained with mouse monoclonal antibodies against E-selectin, VCAM-1 (both obtained from Dianova), or ICAM-1 (Immunootech, Marseille, France) diluted in 1% bovine serum albumin/2% normal goat serum/PBS. Thereafter, cells were successively stained with biotin-Sp-conjugated goat anti-mouse IgG (Dianova) and streptavidin-Cy-chrome (Becton Dickinson). Adhesion molecule expression of the GFP+ population as detected in the FL3 channel was determined with a FACScalibur. Nonviable cells were excluded from analysis by means of forward scatter and side scatter light parameters. Quantification of expression levels determined in the fluorescence-activated cell sorting was done by determining the mean value of the area covered by the curve. All flow cytometry experiments were repeated at least twice and revealed essentially similar results.

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a control. Cells were then exposed to TNF-α, and expression of chemokines and adhesion molecules were analyzed by flow cytometry using specific antibodies.

In the representative experiment depicted in Fig. 2, about 70% of the cells expressed GFP, indicating successful transduction of the transgenes. GFP+ and GFP− populations of the same sample were analyzed for TNF-α-induced adhesion molecule (Fig. 2, VCAM-1) and chemokine expression (see below). Cells transduced with the empty vector showed a strong up-regulation of VCAM-1 in both the GFP+ and GFP− populations. A comparable induction of VCAM-1 was detected in the GFP− population of endothelial cells that had been treated with the transdominant IκBα virus (Fig. 2). However, there is also a population that does not efficiently up-regulate VCAM-1 in response to TNF-α. These are most likely cells that had been infected with the virus but show only low GFP levels and therefore fall in the lower gate. In contrast, VCAM-1 was not inducible in GFP+ cells, i.e. in the successfully infected subpopulations expressing the transdominant IκBα protein. Iso-type controls have been performed for this and all the following experiments and showed that the observed effects were specific for the mutants used. For the sake of clarity, these isotype controls have been omitted from the figures. Similar experiments were performed regarding TNF-α-induced up-regulation of the adhesion molecules ICAM-1 and E-selectin and chemokines MCP-1 and IL-8. Expression of transdominant IκBα virtually completely abolished inducibility of these molecules by TNF-α (Fig. 3a). Furthermore, transdominant mutants of IκBβ and IκBε were capable of inhibiting induction of these adhesion molecules and chemokines as well (Fig. 3a). Our data indicate that activation of NF-κB is essential for the expression of all these proteins investigated. Moreover, all three IκB mutant proteins act as equally competent inhibitors of adhesion molecule and chemokine expression in endothelial cells (Fig. 3a), indicating that each of the IκBs may critically contribute to the regulation of NF-κB-mediated proinflammatory activation of endothelium.

To prove specificity of transdominant IκB-mediated inhibi-
tion of endothelial gene expression for the NF-κB pathway, infected endothelial cells were also stimulated with IFN-γ, a known inducer of ICAM-1 expression in endothelial cells. IFN-γ employs the Janus kinase/signal transducer and activator of transcription rather than the NF-κB signalosome. None of the mutant IκBs was capable of inhibiting IFN-γ-induced expression of ICAM-1 (Fig. 3b). In addition, IFN-γ-induced up-regulation of endothelial HLA-DR was not blocked by transdominant IκB mutants either (data not shown). These observations clearly demonstrate that there is no general signaling defect in the stably transduced cells. Retrovirally infected endothelial cells expressing mutant IκB proteins rather show a selective blockade of the NF-κB signaling cascade.

A Kinase-deficient Mutant of SEK Attenuates TNF-α-induced Up-regulation of Specific Adhesion Molecules—Promoter regions of many proinflammatory cytokine-inducible genes carry binding motifs not only for NF-κB but also for transcription factors of the activator protein-1 (AP-1) family. The activity of several AP-1 factors such as c-Jun, ATF-2, and JunD is regulated via phosphorylation by JNKs. Similar to NF-κB, JNK is activated by TNF-α/TNF receptor signaling, which activates the upstream kinase SEK, which then in turn phosphorylates JNK. To investigate the extent of SEK/JNK/AP-1 involvement in the regulation of endothelial adhesion molecules and chemokines, endothelial cells were retrovirally transduced with a kinase-deficient mutant of SEK. To prove the functionality of the mutant, we first monitored the phosphorylation of JNK in response to TNF-α treatment in the stably transfected producer cell line. In cells stably transfected with the parental vector, JNK was phosphorylated after 10 min of TNF-α treatment. In contrast, cells expressing the SEK mutant did not show measurable JNK phosphorylation in response to TNF-α (Fig. 4a). Endothelial cells expressing the kinase-deficient SEK mutant were exposed to TNF-α, and the expression of MCP-1, IL-8, ICAM-1, and VCAM-1, and the expression of chemokines and adhesion molecules was evaluated by flow cytometry as described under “Experimental Procedures.” Only the GFP+ population is considered for analysis. Quantitative evaluation of the expression levels was performed by analyzing the mean of the area covered by the curve (i.e. geo mean in CellQuest software).

![Fig. 4. A kinase-deficient mutant of SEK inhibits agonist-induced phosphorylation of JNK but fails to block up-regulation of endothelial chemokines and adhesion molecules.](http://www.jbc.org)
IKK1 and IKK2 Differentially Contribute to TNF-α-induced Expression of Endothelial Chemokines and Adhesion Molecules—IKK1 and IKK2, kinases present in the IκB kinase complex, are both capable of phosphorylating IκB proteins in response to extracellular stimulation. Thus far, the relative contribution of these to TNF-α-induced endothelial gene expression remained elusive. To address this aspect, endothelial cells were retrovirally transduced with kinase-inactive mutants of IKK1 or IKK2 and subsequently analyzed for TNF-α-mediated chemokine and adhesion molecule expression as described above.

Kinase-inactive IKK2 virtually completely inhibited TNF-α-induced expression of all these endothelial molecules (Fig. 5a). In contrast, kinase-inactive IKK1 showed a more selective effect; although the induction of IL-8 and VCAM-1 was blocked by both mutant kinases to an almost similar extent, dominant negative IKK1 only partially inhibited the synthesis of MCP-1, ICAM-1, and E-selectin (see “Discussion”). Paralleling the results obtained with the transdominant IκB proteins, kinase-deficient IKK1 and IKK2 mutants did not interfere with IFN-γ-induced ICAM-1 expression (data not shown). These data indicate that the kinase activity of IKK2 is absolutely required for induction of all the proteins investigated, whereas IKK1 seems essential for only a subset of endothelial NF-κB target genes.

A Constitutively Active IKK2 Mutant Is Sufficient to Drive the Expression of Chemokines and Adhesion Molecules in Primary Endothelial Cells—Because IKK2 obviously plays an important role in regulating endothelial gene expression, we next addressed the question of whether constitutively active IKK2 by itself is sufficient to induce the expression of target genes. Expression of an active IKK2 mutant, which is characterized by the replacement of two serines of the activation loop by glutamic acid (19, 20), in the producer cell line 6NX resulted in a strong constitutive DNA binding of NF-κB (Fig. 6a). Although infection efficiencies of endothelial cells infected with a retrovirus containing active IKK2 only reached ~10%, the transduced subset within the pool of cells analyzed by EMSA still gave rise to a measurable increase in NF-κB DNA-binding activity in the absence of TNF-α (Fig. 6a).

Endothelial cells expressing constitutively active IKK2 showed a pronounced up-regulation of ICAM-1, VCAM-1, E-selectin, MCP-1, and IL-8 already in the absence of a proinflammatory stimulus (Fig. 6b). The protein levels induced were even higher than those observed upon exposure to TNF-α. The active IKK mutant acts specifically on NF-κB, because endothelial HLA-DR expression, which does not depend on NF-κB activity, was not affected (data not shown). These observations indicate that the IKK mutant is not a general activator of endothelial cells but selectively targets genes that are NF-κB-dependent. Interestingly, when cells expressing the active IKK mutant were additionally exposed to TNF-α, protein synthesis of the above-mentioned read-out molecules was not further enhanced but down-regulated to the level of TNF-α-stimulated empty vector-infected control cells. Taken together, our data indicate that expression of a constitutively active IKK2 mutant widely mimics the proinflammatory activation of endothelial cells, because it is sufficient to drive full expression of endothelial chemokines and adhesion molecules.
The activation of endothelium is a critical step in the onset of inflammatory responses and is associated with the up-regulation of a variety of chemokines and adhesion molecules. Here we have analyzed the contribution of the IKK/IκB/NF-κB signaling pathway to endothelial protein expression upon inflammatory stimulation. We used a retroviral transduction approach that allowed the expression of dominant interfering mutants of components of the NF-κB signaling pathway. The consequences of this modulation of NF-κB activity on the expression of endogenous genes in endothelial cells was analyzed. With this approach we were able to demonstrate that the inhibition of NF-κB signaling by expression of transdominant mutants of IκBα, -β, and -ε or a kinase-deficient form of IKK2 results in a complete blockade of TNF-α-induced expression of the chemokines MCP-1 and IL-8 and adhesion molecules ICAM-1, VCAM-1, and E-selectin. In contrast, a constitutively active version of IKK2 by itself was sufficient to induce maximal expression of these genes. A dominant-negative SEK mutant that interfered with the activation of the AP-1 transcription factor had only marginal effects on some of the investigated molecules.

Each isoform of the IκB proteins inhibited the expression of endothelial activation markers to a similar extent. The main dimer induced upon TNF-α treatment of endothelial cells is the p50/RelA heterodimer (Refs. 21–23 and data not shown), for which it was reported before that it can be efficiently inhibited by IκBα and IκBβ (24, 25). Interestingly, the publications describing the inhibition profile of IκBε were somehow contradictory with respect to the inhibition of p50/RelA-containing complexes, and it has been suggested that IκBε may play a special role in regulating c-Rel-containing complexes (9, 26–28). On the basis of inhibition of c-Rel-containing complexes, a selective role for IκBε in ICAM-1 regulation was proposed (9). Here we could show that IκBε was able to inhibit TNF-α-induced gene expression as effectively as IκBα and IκBβ, and in other experiments we could demonstrate that IκBε indeed inhibits p50/RelA-activated transcription (data not shown). From our results we conclude that the expression of ICAM-1 is not exclusively regulated by NF-κB complexes bound to IκBε but also by complexes controlled by IκBα and IκBβ.

Our results demonstrate that the activation of NF-κB is essential for the expression of various target genes in endothelial cells. Furthermore, they show that the signal transduction cascade critically involves the IκB-kinase complex, most notably the IKK2 kinase. During the preparation of this paper, Oitzinger et al. (14) described the effect of a dominant negative IKK2 mutant on inflammatory marker genes. Consistent with our results, they found that adenoviral expression of dominant negative IKK2 completely inhibits the activation of endothelial cells in response to proinflammatory cytokines. Although IKK2 seems to play a very prominent role in NF-κB

**FIG. 6.** Expression of a constitutively active mutant of IKK2 is sufficient to activate NF-κB and drive the expression of chemokines and adhesion molecules in endothelial cells. Endothelial cells were infected with a retrovirus carrying a constitutively active IKK2 mutant parental vector. Subsequently, endothelial cells were exposed to 2 ng/ml TNF-α (φNX producer cells at 80 ng/ml TNF-α) or medium as control for 15 min (a). IKK2-EE, constitutively active IKK2 (two serine residues mutated to glutamic acid). Whole-cell lysates were obtained and studied for NF-κB DNA-binding activity by EMSA as described under “Experimental Procedures.” NF-κB complexes are indicated. Unspecific bands are indicated by an asterisk. b, endothelial cells expressing constitutively active IKK2 or controls were exposed to 2 ng/ml TNF-α (green profile lines) or medium as control (black profile lines) and evaluated for expression of chemokines and adhesion molecules by flow cytometry as described under “Experimental Procedures.” Only the GFP population is considered for analysis.
activation and gene expression in endothelial cells, the contribution of IKK1 is less pronounced and differs for the target molecules analyzed. Nevertheless, this observation is intriguing because it had been suggested earlier from gene disruption studies and transient transfections that IKK1 is not required for cytokine-induced NF-κB activation at all (20, 29, 30). Our data show that at least in endothelial cells IKK1 contributes to the TNF-α-induced expression of some NF-κB target genes. This might be because of the fact that IKK1 is able to phosphorylate and thereby activate IKK2 both in unstimulated and in TNF-α-stimulated cells (31, 32). A strong overexpression of kinase-deficient IKK1 might therefore tivate the endogenous wild-type IKK1 away from the IκB-kinase complex and thereby inhibit this phosphorylation. The difference among the various NF-κB target genes might be caused by different dependencies of these molecules on IKK1 kinase activity.

An important outcome of our experiments was the finding that the activation of NF-κB alone (by means of the constitutively active IKK2) was sufficient for full induction of endothelial effector proteins in the absence of additional proinflammatory stimuli. Thus, the activation of NF-κB via IKK2 is both essential and sufficient for endothelial chemokine and adhesion molecule expression. Interestingly, expression of the various target genes was even higher in cells expressing the constitutively active IKK2 protein as compared with TNF-α-induced cells. When cells expressing constitutively active IKK2 were further stimulated with TNF-α, the expression of chemokines and adhesion molecules was attenuated. A potential explanation for this observation is that TNF-α induces some modulators of the transcriptional activity of NF-κB in addition to stimulating NF-κB activity. One candidate for such a modulator is A20, an antiapoptotic protein that is induced by TNF-α, the expression of which depends on NF-κB (33–35). Furthermore, it was shown that A20 can inhibit NF-κB and thereby also endothelial activation (36, 37). The exact mechanism of A20 function is not clear yet. Recently it was shown that A20 interacts with the IKK complex upon TNF receptor stimulation (38). Thereby it could act directly on the IκB kinases. In addition to A20, other antiapoptotic proteins like A1, Bcl-2, and Bcl-XL have been described as inhibitors of NF-κB and endothelial activation (39, 40). TNF-α, by stimulating pathways in addition to NF-κB, may increase the expression level of these proteins and thereby attenuate the NF-κB response. Such a desensitizing signal may help to protect the cell from a signaling overflow and may be part of a switch-off mechanism once the positive signal has fulfilled its task. Another possibility for down-regulation of target gene expression by TNF-α could be that TNF-α induces other transcription factors in addition to NF-κB that titrate away cofactors that are necessary for NF-κB transcriptional activation. Such a competitive interaction was shown recently for NF-κB and Smad proteins activated by transforming growth factor-β. When endothelial cells were treated with transforming growth factor-β or transfected with Smad expression vectors in addition to interleukin-1α stimulation, E-selectin gene expression was inhibited, because the coactivator CREB-binding protein was titrated away from the NF-κB dimers (41). A similar mechanism could also be initiated by TNF-α in a situation where NF-κB activation is already maximal.

It was reported earlier that signal transduction cascades apart from the NF-κB pathway such as the JNK/stress-activated protein kinase mitogen-activated protein kinase pathway initiated by TNF-α are important for the induction of endothelial genes, e.g., for the expression of E-selectin (42, 43). By expressing a dominant negative mutant of SEK, the upstream activator of JNK, we observed only marginal changes in induced expression of E-selectin and VCAM-1 and no reproducible effects on ICAM-1, MCP-1, or IL-8, although the kinase-deficient mutant efficiently blocked TNF-α-induced JNK activation. Thus, the JNK pathway seems to play a modulating rather than an essential role in regulating endothelial gene expression upon inflammatory activation.

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