A Sustained Reduction in IκB-β May Contribute to Persistent NF-κB Activation in Human Endothelial Cells*

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The responses of vascular endothelial cells (EC) to tumor necrosis factor-α (TNF), interleukin-1α (IL-1), and phorbol myristate acetate (PMA) were compared with respect to the kinetics of (i) NF-κB activation, (ii) IκB-α and IκB-β degradation, and (iii) NF-κB-dependent cell surface molecule expression. TNF rapidly (20 min) and persistently (>20 h) activates NF-κB; IL-1 rapidly activates NF-κB, but activity declines by 3 h and further by 20 h; PMA slowly and transiently activates NF-κB. Untreated EC contain the inhibitory proteins IκB-α and IκB-β. The onset of NF-κB activation correlates with degradation of IκB-α, but IκB-α reappears by 4 h without resequestration of NF-κB. TNF causes a rapid but partial (50%) reduction in IκB-β, which does not recover by 22 h; IL-1 and PMA cause slower and less sustained reductions in IκB-β. All three agonists induce de novo expression of E-selectin (CD62E) and vascular cell adhesion molecule-1 (CD106) and increase expression of intercellular adhesion molecule-1 (CD154) at 4 h. TNF induces sustained increases in vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and increases human leukocyte antigen class I molecules at 24 h. We conclude that TNF causes persistent activation of NF-κB in human EC and that this may result from sustained reductions in IκB-β levels.

Activation of the transcription factor NF-κB is required for the full expression of many TNF-inducible genes in vascular endothelial cells (EC), including E-selectin (CD62E), also known as endothelial-leukocyte adhesion molecule-1 (1), vascular cell adhesion molecule-1 (VCAM-1, CD106) (2), intercellular adhesion molecule-1 (ICAM-1, CD54) (3, 4), and human leukocyte antigen (HLA) class I (5). Activated NF-κB binds to 10-base pair recognition sequences (κB sites, consensus GGGRRHTYYCC (6)) found in the 5'-flanking regions of these genes and promotes transcription through direct interaction with the transcription initiation complex (7). Cytokine-induced expression of these molecules on the luminal surface of vascular endothelial cells is critical in recruiting leukocytes from the bloodstream into sites of inflammation (reviewed in Ref. 8).

NF-κB is composed of members of the Rel family of ubiquitous transcriptional activators that includes p50 (also called NFKB1), p52 (NFKB2), p65 (ReLa), c-Rel (Rel), and RelB (reviewed in Ref. 9). Within the Rel family, every member can form homo- and heterodimers except RelB, which is preferentially expressed in lymphoid cells and forms only heterodimers with p50 or p52 (10). Both p65 and c-Rel are transcriptional activators. The p50 homodimer can repress transcription from some promoters when expressed as a transgene in vitro (11), but it can activate transcription in vitro (12), and it is thought to mediate constitutive transcription of major histocompatibility complex class I heavy chain (13, 14) and light chain genes (15). Similarly, p50 homodimers stimulate transcription of human immunoglobulin κB-containing but not HIV κB-containing promoters (12). Variations within the κB consensus site may account for such differences, e.g., transcriptional activation mediated by the HIV κB (GGGACTTTCC) is strongly induced in vitro by NF-κB but the interferon-κB (GGGAAATTC, also called PRDII) is weakly induced (16). Sequences flanking within the κB core can also contribute to differential responses, e.g., the binding of a high mobility group-like protein adjacent to an NF-κB converts the NF-κB from a transcriptional activator to a repressor (17), and the binding of the high mobility group I(Y) protein to the AT-rich center of the IFN-κB has been shown to facilitate the binding or activity of NF-κB (18). The high mobility group I(Y) protein has been implicated in the transcriptional regulation of the endothelial-leukocyte adhesion molecule-1 (19) and VCAM-1 (20) genes by cytokines in EC.

Latent forms of NF-κB are found in the cytoplasm where they are bound constitutively by members of a family of proteins called inhibitors of κB (IκB), which includes IκB-α (also called MAD-3, 37 kDa), IκB-β (46 kDa), IκB-γ, and Bcl-3 (reviewed in Ref. 21). Both IκB-α and IκB-β bind to the strongly transactivating Rel proteins p65 and c-Rel and inhibit their binding to DNA (16, 22). Neither IκB-α nor IκB-β bind to p50. The two additional characterized forms of IκB are specific for p50-containing dimers. IκB-γ corresponds to the carboxyl-terminal portion of the p50 precursor protein p105 and is the product of alternative splicing (23); however, it has only been detected in murine pre-B cells (24). Bcl-3 is found in only low amounts in a restricted set of tissues, including certain B cell leukemias, and preferentially inhibits the binding of p50 to DNA (25). Bcl-3 is also reported to act as a transcriptional activator in association with p52 homodimers (26), although this Bcl-3-mediated transactivation may be an indirect effect of sequestering the transcriptional repressors p50 and p52 (27).
Due to their low abundance and restricted tissue distribution, these IκB isoforms are thought to play only minor roles in regulating NF-κB in most cell types.

Activation of NF-κB DNA binding potential and movement of NF-κB to the nucleus results from the phosphorylation and subsequent proteolytic degradation of IκB (28, 29). IκB-α is rapidly degraded in many cell types, including cultured human umbilical vein EC, following treatment with cytokines that activate NF-κB (30, 31). The synthesis of IκB-α is itself induced by NF-κB and has been proposed to mediate eventual resequestration of NF-κB in the cytoplasm, forming a potential negative feedback loop (30). IκB-β, the second major IκB, has recently been cloned and characterized in B lymphocytes, where it was shown to be degraded upon treatment with IL-1 or lipopolysaccharide, which cause a prolonged activation of NF-κB in B cells, but not upon treatment with TNF or PMA, which cause only transient activation of NF-κB in B cells (22). In contrast to IκB-α, agonist-induced degradation of IκB-β occurs relatively slowly in these cells, appearing to decrease markedly only after 60 min of stimulation (at which time IκB-α has returned to normal levels), and remains low during the treatment period. In addition, IκB-β mRNA levels are not increased by activators of NF-κB in B cells (22).

In the present study we have examined the effects of TNF, IL-1, and PMA on NF-κB activation and IκB degradation in human EC. Our data show that TNF causes the most persistent NF-κB activation and the most persistent reduction in IκB-β levels and has the greatest effects on NF-κB regulated genes at later times.

EXPERIMENTAL PROCEDURES

Cells, Cytokine Treatments, and Harvest—Human umbilical vein EC were isolated by collagenase digestion of umbilical cords obtained from 2–4 donors, and cultured on collagen in 20% fetal calf serum/M199/glutamine/penicillin/streptomycin (all Life Technologies, Inc.) supplemented with endothelial cell growth factor (Collaborative Research, Bedford, MA) as described previously (32). Confluent cultures of human umbilical vein EC (passage 3 or 4) were adjusted to 8 ml on 10-cm plates and treated for the times indicated in the figures with recombinant human TNF (100 units, expressed in Escherichia coli; 2.5 × 10^9 cpm/mg, a gift of W. Fiers, State University of Ghent, Ghent, Belgium or 5 ng/ml, R&D Systems, Minneapolis, MN), PMA (100 ng/ml, Sigma), or recombinant human IL-1α (1 ng/ml, R&D Systems, Minneapolis, MN). Cells were harvested by rinsing twice with PBS (Mg2+- and Ca2+-free) and then incubating in 1 ml of 1 mM EDTA/PBS (10 min at room temperature). Cells were detached by pipetting and placed in microcentrifuge tubes on ice. Similar results were obtained when the cells were scrape-harvested into ice-cold PBS or harvested by trypsin treatment.

Cytoplastic and Nuclear Extractions—Cytoplastic and nuclear extracts were prepared by a modified mini-extraction protocol (33). Cells were pelleted (approximately 500 × g, 4 min, 4°C) and resuspended in hypotonic buffer A (0.2 ml, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2) supplemented with protease inhibitors (leupeptin and aprotinin each 1 μg/ml, phenylmethylsulfonyl fluoride 0.5 μM) and incubated for 15 min on ice. Cells were lysed by the addition of 25 μl 2.5% Nonidet P-40 buffer A and mixed by inversion, and the nuclei were pelleted (500 × g, 4 min, 4°C). Supernatant was recovered (200 μl of cytoplasmic extract), placed into microcentrifuge tubes, and frozen by immersion in liquid nitrogen. The nuclear pellet was resuspended in extraction buffer C (20 ml HEPES, pH 7.9, 0.45 NaCl, 1 mM EDTA supplemented with the protease inhibitors) and incubated for 15 min at 4°C on a rocking platform, and then the tubes were centrifuged (10 min at 14,000 × g, 4°C). Extracts were diluted 1:1 in buffer D (20 ml HEPES, pH 7.9, 0.1 mM KCl, 10% glycerol (34)); binding 1.5–2.0 μg/ml against a bovine serum albumin standard in a modified Bradford protein assay (Bio-Rad), frozen in liquid nitrogen, and stored at −20°C.

DNA Binding Assay (Electrophoretic Mobility Shift Assay)—Consensus sequence κB DNA was purchased (5'-AGTTGAGGAGGAGTTCC-CCAGGC, Promega). The core κB sequences that match HIV and mouse Ig κB is underlined (35). HLA class II κB sequence oligos containing a symmetrical κB sequence (5'-CGTTGGGAGTTCCCTC) and HLA interferon consensus sequence (CCACAGTTCACCTGT-CACCT (5)) oligos were synthesized (Critical Technologies Program, Yale Medical School) and annealed. Both (blunt-end) probe DNAs were labeled with [32P]-ATP with polynucleotide kinase (New England Biolabs, Beverly, MA) and separated from unincorporated nucleotides over a Sephadex G-25 (Pharmacia Biotech Inc.) spun column. To 1 μl of double-stranded nonspecific DNA (1 μg polyclonal antibody), Pharmacia) was added 4 μl of nuclear extract (6–8 μg/ml) and 5 μl of diluted probe DNA. The reaction was incubated by gentle rocking and incubated at room temperature for 20 min, and then 5 μl was loaded on a 15% polyacrylamide gel/0.25 × TBE buffer (Tris, borate, EDTA) and separated at 220 V/18 cm for 2 h. Gels were dried under vacuum and exposed to a storage phosphor screen for quantification and documentation (PhosphorImager, Molecular Dynamics, Sunnyvale, CA). Competition experiments were performed as above except that 100-fold excess competitor DNA was added to the incubations in 1 μl immediately prior to the addition of probe DNA in 4 μl.

Antibody EMSA—To 1 μl of nonspecific DNA (1 μg polyclonal antibody) was added 4 μl (6–8 μg/ml of protein) of nuclear extract, 2 μl of water, and 1 μl of affinity-purified rabbit polyclonal antiserum for RelA (sc-109), p50 (sc-114), or dRel (sc-71; all antisera were from Santa Cruz Biotechnology, Santa Cruz, CA). After incubated on ice for 1 h, 2 μl (2 fmol) of probe DNA was added, and the incubation was continued at room temperature for 20 min; then 5 μl was loaded on a gel and analyzed as described above.

Western Blotting of IκB-α and IκB-β—Cytoplastic extracts that had been treated in liquid nitrogen were thawed and diluted 1:40 at room temperature. 300,000 × g for 30 min at 4°C (90,000 rpm, TL100 rotor, Bedman). The protein was quantified in a Biorad protein assay with bovine serum albumin standard (Pierce). Extracts (25 μg) were resolved by SDS-polyacrylamide gel electrophoresis, electroblotted onto a polyvinylidene difluoride membrane (Immobilon, Millipore), stained by antibodies against IκB-α (Santa Cruz) or IκB-β (rabbit antiserum raised against the IκB-β protein (22)) and a horseradish peroxidase-conjugated secondary antibody (ECL, Amersham Corp.), and detected on film. Molecular weight standards were run in an adjacent lane (Rainbow markers, Amersham Corp.). Autoradiographs were quantitated by densitometry (Molecular Dynamics), and the level of significance was determined by a one-tailed Student's t test with unequal variance (36).

Cell Surface Molecular Expression (Fluorescence Flow Cytometry)— Approximately 5 × 10^7 cells were treated with saturating amounts of mouse monoclonal antibodies specific for HLA class I (W6/32), VCAM-1 (E1/6), endothelial-leukocyte adhesion molecule-1 (H4/18), or ICAM-1 (RI1/1) diluted 1:100 in 50 μl of 2% fetal calf serum/0.01% NaN3/PBS for 45 min, washed two times, and then stained with saturating amounts of the fluoresceinated secondary F(ab')2 goat-anti-mouse IgG (H + L) (1:100 in 50 μl, Boehringer Mannheim) for 45 min After washing two times and fixation in 1% paraformaldehyde/PBS, 2000 cells were measured on FACSsort (Becton Dickson, Sunnyvale, CA). The values are expressed as the mean fluorescence intensity of the entire population of cells, which were gated on forward and side light scatter parameters.

RESULTS

The time course of NF-κB activation in EC after treatment with TNF, IL-1, or PMA was examined. Nuclear extracts prepared from EC were assayed for activated NF-κB in an EMSA of DNA binding factors using a radiolabeled consensus sequence κB probe containing the core κB site, GGAGCTTTCC, that matches the HIV-1 and the murine Ig gene κB sequences. Nuclear extracts from control cells form two complexes, a faster migrating lower complex (Fig. 1A, LC) and an upper complex (Fig. 1A, UC). The time courses of NF-κB activation by the agonists TNF, IL-1α, or PMA in endothelial cells were determined by testing nuclear extracts prepared from replicate cultures of EC treated for 20 min, 3 h, or 20 h. Different kinetics of NF-κB activation were observed with each of the three agonists, TNF, IL-1, and PMA, varying in the rate of onset and persistence of NF-κB activation (Fig. 1, a and b). TNF and IL-1α rapidly (∼20 min) activate NF-κB (Fig. 1A, lanes 3 and 9), which remains nearly constant for >20 h in the case of TNF and declines by 3 h in the case of IL-1α. PMA activates NF-κB more slowly and transiently, with little effect at 20 min but significant activation at the 3 h time point (Fig. 1A, lane 7) and declining to nearly control levels by 20 h. Similar results were obtained in seven independent experiments with TNF and...
PMA and five experiments with IL-1α using as probes either the IgκB or the HLA class I κB. The amount of NF-κB activated in two independent experiments was quantified, and the mean and standard deviations were determined (Fig. 1b).

To test the specificity of the DNA binding activity, competition experiments were performed with unlabeled κB and an unrelated interferon consensus sequence DNA (Fig. 1c). Both the upper and lower complexes formed by TNF-activated NF-κB binding to an HLA class I κB probe DNA were completely competed by 100-fold excess unlabeled HLA class I κB (Fig. 1c, lane 4) and IgκB (Fig. 1c, lane 5) but not by the unrelated interferon consensus sequence DNA (Fig. 1c, lane 6).

The doses of agonists used here were established as optimal in pilot experiments. Nevertheless, we considered the possibility that the decline in IL-1α-activated NF-κB at later times of treatment was the result of cytokine depletion. The addition of fresh IL-1α for 30 min to cultures that had been treated with IL-1α for 20 h (when activated NF-κB has declined to approximately 65% of the maximum levels) resulted in the activation of only a small amount of additional NF-κB (increasing to approximately 75% of maximal levels, data not shown). Thus, the decline of activated NF-κB in IL-1-treated cells cannot be attributed to cytokine depletion.

The composition of TNF-activated NF-κB could be expected to change over time because TNF treatment of EC has been shown to greatly increase the level of IκB-α and p105 (p50 precursor) mRNAs and transiently induce cRel mRNA, whereas p65 mRNA levels are induced only slightly (31, 30). Because different constituents of NF-κB provide different transcriptional activities, such a change could contribute to the effects of persistent activators of NF-κB. The composition of activated NF-κB was investigated by antibody EMSA (supershifts) with nuclear extracts of cells treated with TNF for 30 min or 22 h (Fig. 2a) or treated with TNF, PMA, or IL-1 for 3 h (Fig. 2b). At early or late times of TNF treatment or after treatment with TNF, PMA, or IL-1 for 3 h, the upper complexes contain p65, the lower complexes contain p50, and fractions of both the upper and lower complexes contain cRel (Fig. 2). Therefore, no change was detected in the subunit composition of NF-κB in EC during the first 24 h of TNF treatment, nor were differences detected in the subunit composition of NF-κB activated by TNF, IL-1, or PMA at 3 h of treatment.

All known activators of NF-κB (e.g., IL-1, lipopolysaccharide, TNF, and PMA) induce the degradation of IκB-κ in B cells, but only persistent activators (e.g., IL-1 and lipopolysaccharide) have been shown to induce the degradation of IκB-β. To compare the levels of IκB-α and IκB-β in agonist-treated EC, cytoplasmic extracts from treated cells were Western blotted with IκB-isoform-specific antisera. Consistent with previous reports (31, 29), TNF and IL-1 rapidly induce nearly complete proteolysis of IκB-α (Fig. 3a). In parallel with the slower activation of NF-κB by PMA, proteolysis of IκB-α induced by PMA is also somewhat delayed and reaches a maximum between 30 and 60 min (data not shown). IκB-β is detected in the cytoplasm of untreated EC (Fig. 3b, lane 1), and all three agonists induce significant reductions in IκB-β (Fig. 3, b and c). Similar results were obtained in five independent experiments. (The additional lower unmarked bands on the IκB-β blot are not found consistently; these bands may correspond to degradation products, or they may result from cross-reactivity of the polyclonal antisera.) To assist in evaluating the quantity of proteins in the extracts, one lane was intentionally loaded with one-half the normal protein load (Fig. 3, lane 2, in a and b, labeled half).

To obtain a measure of the extent of IκB-β reduction, densitometry was performed on the autoradiograph shown in Fig. 3b and on autoradiographs from two additional independent ex-
experiments. All values were normalized by subtraction of background counts and division by the counts in the untreated lane. The time of treatment varied slightly among experiments, so for the purpose of data analysis, treatments of 20–30 min, 3–4 h, and 20–26 h were pooled, and the means and standard deviations were determined (Fig. 3c). TNF rapidly induces the reduction to approximately 25% of control IκB-β levels, and levels of IκB-β remain low throughout the period of treatment. IL-1 and PMA more slowly induce the reduction to approximately 40% of control IκB-β levels, which return to control levels by 20 h of IL-1 treatment but remain low for up to 20 h after PMA treatment.

Newly synthesized IκB-α has been reported to be localized in the nucleus of HeLa S3 cells treated briefly with TNF (37). Nuclear extracts from IL-1α-treated EC were tested for the presence of IκB isoforms. Although immunoreactive species were detected, only trace amounts of proteins of the same size as the cytoplasmic proteins were observed in both untreated or IL-1-treated nuclear extracts (data not shown), suggesting that nuclear IκB is not a major cause of the reduced NF-κB binding activity in at later times of IL-1 treatment.

NF-κB is an important transcriptional regulator of the inducible endothelial cell surface molecules E-selectin, VCAM-1, ICAM-1, and HLA class I. To compare the activation of NF-κB with the induction of these molecules, untreated EC and EC treated with TNF, PMA, or IL-1 were stained by indirect immunofluorescence and measured by fluorescence flow cytometry. All three agonists effectively induce expression of ICAM-1, VCAM-1, and especially E-selectin at 4 h, but TNF is much more effective than PMA or IL-1 in inducing ICAM-1 and VCAM-1 expression at later times (Fig. 4). TNF alone significantly induces expression of HLA class I molecules, which begin to increase only at later times of treatment. Therefore, the strength of induction of these persistent surface molecules correlates with the strength and persistence of NF-κB activation.

**DISCUSSION**

The agonists TNF, IL-1, and PMA activate NF-κB with different kinetics in EC. TNF activates NF-κB rapidly and persistently, IL-1 activates NF-κB rapidly but with less persistence, and PMA activates NF-κB slowly and transiently. In contrast to these results obtained from endothelial cells, in B cells IL-1 activates NF-κB for over 24 h, and TNF activates NF-κB only transiently (22), whereas in a murine T cell lym-
phoma TNF, IL-1, and PMA all activate NF-κB with similar kinetics and only transiently (maximal between 15 and 240 min) (38). These contrasting results demonstrate a strong dependence on cell type for the behavior of agonists that activate NF-κB.

No differences in subunit composition were detected in the NF-κB activated by TNF at 30 min, 3 h, or 22 h of treatment or by PMA or IL-1 at 3 h (Fig. 2). In each case, the predominant upper complex contains p65, and the lower complex contains p50 and c-Rel. Similarly, no differences in Rel proteins activated by TNF, PMA, or IL-1 were noted in a mouse T cell line (38). Therefore, the increases in Rel and NF-κB mRNA may not be directly reflected in increased protein expression. Such post-transcriptional control of IκBα expression has been observed in primary human monocytes (39). Alternatively, NF-κB-induced transcription of the genes encoding IκBα and the p50 precursor (p105) might fail to produce changes in NF-κB subunit composition because of compensating effects of IκBα induction and degradation together with the IκB function of the p105 protein (IκBγ).

The persistence of NF-κB activation by different agonists may differ depending upon the duration of the intracellular signals initiated by the agonist. For example, activation of NF-κB in HeLa cells requires the continued presence of TNF; when TNF is washed away from the cultured cells, κB binding activity rapidly disappears from the nucleus (data not shown). It seems likely, therefore, that TNF continues to induce the proteolysis of IκBα and release of NF-κB, establishing a new balance through the concomitant NF-κB-driven increased synthesis of IκBα and p105. In contrast, PMA activation of PKC in EC is followed by down-regulation of PKC and termination of the signal even in the continued presence of the PMA agonist (40).

The rapid depletion of IκB-β in EC is the principal difference in the behavior of IκB-β between EC and B cells in response to chronic activators of NF-κB (22). IκB-β mRNA is not increased by activators of NF-κB (22), which may contribute to the failure of IκB-β to return to resting levels in EC. TNF may also cause the persistent degradation of IκB-α, which is countered by concomitant NF-κB-mediated increase in IκB-α synthesis (31). PMA activation of NF-κB is maximal around 3–4 h, which coincides with the maximum depletion of IκB-β (Figs. 1 and 3). Thus, NF-κB release from IκB-β may account for the majority of the PMA-activated NF-κB. IL-1 activation of NF-κB is rapid, which probably results from the rapid depletion of IκB-α, but not as strong as TNF activation, perhaps because IL-1 induces the degradation of IκB-β more slowly. IL-1 activation of NF-κB is also less sustained than TNF activation, which correlates with the less sustained reduction of IκB-β induced by IL-1.

The results of these experiments support the hypothesis that persistent activators of NF-κB reduce IκB-β levels and further suggest that a rapid reduction in IκB-β levels observed in EC may also contribute to initial NF-κB activation. Correlations between NF-κB and IκB-α and -β will have to be sought in additional cell types and with additional agonists in order to test the generality of the model wherein both IκB-α and IκB-β contribute regulating nuclear translocation of NF-κB.

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