Temporal Control of NF-\(\kappa\)B Activation by ERK Differentially Regulates Interleukin-1\(\beta\)-induced Gene Expression*

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In cultured rat vascular smooth muscle cells, sustained activation of ERK is required for interleukin-1\(\beta\) to persistently activate NF-\(\kappa\)B. Without ERK activation, interleukin-1\(\beta\) induces only acute and transient NF-\(\kappa\)B activation. The present study examines whether the temporal control of NF-\(\kappa\)B activation by ERK could differentially regulate the expression of NF-\(\kappa\)B-dependent genes, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), vascular cell adhesion molecule-1 (VCAM-1), and manganese-containing superoxide dismutase (Mn-SOD). Treatment of vascular smooth muscle cells with interleukin-1\(\beta\) induced the expression of iNOS, COX-2, VCAM-1, and Mn-SOD in a time-dependent manner, but with different patterns. Either PD98059 or U0126, selective inhibitors of MEK, or overexpression of a dominant negative MEK-1 inhibited interleukin-1\(\beta\)-induced ERK activation and the expression of iNOS and COX-2 but had essentially no effect on the expression of VCAM-1 and Mn-SOD. The expression of these genes was inhibited when NF-\(\kappa\)B activation was down-regulated by MG132, a proteasome inhibitor, or by overexpression of an I-\(\kappa\)B\(\alpha\) mutant that prevented both the transient and the persistent activation of NF-\(\kappa\)B. Inhibition of ERK did not affect interleukin-1\(\beta\)-induced I-\(\kappa\)B phosphorylation and degradation but attenuated I-\(\kappa\)B\(\beta\) degradation. Thus, although NF-\(\kappa\)B activation was essential for interleukin-1\(\beta\) induction of each of the proteins studied, gene expression was differentially regulated by ERK and by the duration of NF-\(\kappa\)B activation. These results reveal a novel functional role for ERK as an important temporal regulator of NF-\(\kappa\)B activation and NF-\(\kappa\)B-dependent gene expression.

Nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) is a transcription factor that regulates the expression of numerous inducible genes involved in inflammation, cell differentiation, proliferation, and apoptosis (1–3). NF-\(\kappa\)B is normally sequestered in the cytoplasm by inhibitors of NF-\(\kappa\)B, designated I-\(\kappa\)B, that exist as several isoforms (e.g. I-\(\kappa\)B\(\alpha\) and I-\(\kappa\)B\(\beta\)). Cell activation by cytokines such as interleukin-1\(\beta\) (IL-1\(\beta\)) and tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) leads to the phosphorylation and ubiquitination-dependent degradation of I-\(\kappa\)B, accompanied by NF-\(\kappa\)B translocation to the nucleus, where it binds to consensus sequences in the promoter region of target genes and activates transcription (1–7). I-\(\kappa\)B\(\alpha\) is rapidly degraded after cytokine stimulation and rapidly resynthesized because of the presence of NF-\(\kappa\)B-binding motifs in the I-\(\kappa\)B\(\alpha\) gene promoter (8). This autoregulatory loop has been suggested to limit NF-\(\kappa\)B activation in a transient manner (8, 9). In contrast, I-\(\kappa\)B\(\beta\) slowly decreases after cytokine stimulation, a process that has been suggested to contribute to persistent activation of NF-\(\kappa\)B (9). Recently, the temporal control of NF-\(\kappa\)B activation by the coordinated degradation and synthesis of I-\(\kappa\)B proteins has been clearly revealed by using gene knock-out cell lines where NF-\(\kappa\)B activation was controlled by a single I-\(\kappa\)B isoform (10). TNF\(\alpha\) stimulation of fibroblasts that contained only the I-\(\kappa\)B\(\alpha\) isoform resulted in an oscillatory pattern of NF-\(\kappa\)B activation, whereas stimulation of cells harboring only I-\(\kappa\)B\(\beta\) resulted in sustained NF-\(\kappa\)B activation. Importantly, it was shown that the expression of NF-\(\kappa\)B-dependent genes may be differentially regulated by controlling the duration of NF-\(\kappa\)B activation (10). However, the mechanisms of differential control by which I-\(\kappa\)B and I-\(\kappa\)B\(\beta\) mediate NF-\(\kappa\)B activation following cytokine treatment remain unclear.

We have recently demonstrated in cultured rat vascular smooth muscle cells (VSMCs) that activation of extracellular signal-regulated kinases (ERK) was required for IL-1\(\beta\) to induce persistent activation of NF-\(\kappa\)B that in turn was required for the subsequent expression of inducible nitric oxide synthase (iNOS) (11, 12). Inhibition of ERK activation, either by the selective chemical inhibitors, PD98059 or U0126, or by antisense phosphorothioate-modified oligodeoxynucleotides that down-regulated ERK synthesis, selectively inhibited IL-1\(\beta\)-induced prolonged activation of NF-\(\kappa\)B and iNOS expression. Interestingly, inhibition of ERK activation had no effect on early transient NF-\(\kappa\)B activation induced by IL-1\(\beta\). This suggests an important ERK-dependent mechanism may temporally control the activation of NF-\(\kappa\)B and NF-\(\kappa\)B-dependent gene expression. The present study was performed to test this hypothesis, using iNOS, cyclooxygenase-2 (COX-2), vascular cell adhesion molecule-1 (VCAM-1), and manganese-containing superoxide dismutase (Mn-SOD) as gene products representative of those controlled by either persistent or acute activation of NF-\(\kappa\)B. The results indicate that inhibition of ERK activation differentially regulates the expression of iNOS, cyclooxygenase synthetic, SOD, superoxide dismutase; TNF\(\alpha\), tumor necrosis factor-\(\alpha\); DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMISA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; IFN\(\gamma\), interferon-\(\gamma\); RT, reverse transcription.

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Fig. 1. ERK activation is required for iNOS and COX-2 induction, but not for VCAM-1 and Mn-SOD induction. In A and B, rat VSMCs were treated with IL-1β (3 ng/ml) for the indicated periods in the absence or in the presence of either PD98059 (20 μM) or U0126 (20 μM) that was added 1 h before IL-1β. Whole cell lysates (20 μg of proteins/lane) were used for Western blot analysis of indicated proteins. p-ERK, phosphorylated ERK. In C, VSMCs were treated with IL-1β (3 ng/ml) for the indicated periods in the absence or in the presence of PD98059 (20 μM). Total RNA was extracted and used for RT-PCR to determine mRNA levels of iNOS, COX-2, VCAM-1, Mn-SOD, and GAPDH. Relative intensity ratios to GAPDH mRNA are shown in the bottom graph. The results shown in A–C are representative of three separate experiments each.
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EXPERIMENTAL PROCEDURES

Materials—DMEM/Ham’s F12 medium (DMEM/F12) and fetal calf serum were purchased from Invitrogen. Recombinant human IL-1β (specific activity: 1.9 × 10^7 units/mg) was kindly provided by Dr. Aurigemma, the Biological Resources Branch Preclinical Repository, National Cancer Institute. Recombinant rat interferon-γ (IFNγ) was purchased from R&D Systems. Recombinant human TNFα, PD98059, U0126, and MG132 were from Calbiochem. Bovine serum albumin (BSA) was from Sigma. Monoclonal antibodies against iNOS and COX-2 were from Transduction Laboratories. Antibody against Mn-SOD was from Santa Cruz Biotechnologies. Antibodies against phospho-p44/42 MAPK (Thr-202/Tyr-204), p44/42 MAPK, phospho-IκBα (Ser-32/Ser-36), and IκBα were from Cell Signaling. Antibodies against VCAM-1, IκBα, and NF-κB p65 were from Santa Cruz Biotechnology. NF-κB consensus oligonucleotide was from Promega. [-32P]ATP was from PerkinElmer Life Sciences. All other materials used were commercial products of the highest grade available.

Cell Culture—Rat VSMCs were isolated from the thoracic aorta and cultured as described previously (13). Cells were used between passages 5 and 9. When confluent, the cells were washed with serum-free medium and then maintained in DMEM/F12 with 0.1% BSA for 24 h. Whole cell lysates (20 μg of proteins/lane) were used for Western blot analysis. The overexpression of MEK1dn was confirmed by immunoblotting with antibody against MEK-1 (lower panel). Results shown are representative of three separate experiments. p-ERK, phosphorylated ERK.

Coexpression of these NF-κB-dependent genes in response to IL-1β stimulation by specifically down-regulating the persistent activation of NF-κB.

FIG. 2. Overexpression of a dominant negative MEK-1 selectively inhibits iNOS and COX-2 but not VCAM-1 and Mn-SOD expression induced by IL-1β. VSMCs were cultured for 24 h in DMEM/F12 that contained 0.1% BSA without adenovirus (lanes 1 and 2) or with AdvMEK1dn (5.4 or 2.7 × 10^11 viral particles, lanes 3 and 4) or Adv-LacZ (5.4 × 10^11 viral particles, lane 5) and then untreated or treated with IL-1β for 24 h. Whole cell lysates (20 μg of proteins/lane) were used for Western blot analysis. The overexpression of MEK1dn was confirmed by immunoblotting with antibody against MEK-1 (lower panel). Results shown are representative of three separate experiments. p-ERK, phosphorylated ERK.

FIG. 3. Inhibition of ERK activation selectively inhibits IL-1β-induced prolonged NF-κB activation. VSMCs were treated with IL-1β (5 ng/ml) for 30 min or 8 h as indicated in the absence or presence of either PD98059 (20 μM) or MG132 (1 μM). Results shown represent two separate experiments.

gene) with the primer pairs 5’-GCT CAT CGA CGC CAT GGC CAA CCG CTT CGT GGG-3’ and 5’-CCC ACG AAG GCG TTG GCC ATG GCG TCG ATG AGC-3’ (the underlining indicates the mutation from T to G or from A to C), which made the mutation of MEK-1 Ser-218 and Ser-222 to Ala-218 and Ala-222. MEK1dn cDNA (Xhol/HindIII) from the pCMV-MEK1dn was then inserted into pBghuttle-CMV vector. The adenovirus was generated by the same method as mentioned above. Adenoviruses were purified through two steps of ultracentrifugation, with discontinuous and continuous CsCl gradients, respectively. After dialysis against 10 mM Tris-HCl, pH 8.0, containing 2 mM MgCl2 and 4% sucrose, viral particles in solution were evaluated based on DNA content. DNA was determined by absorbance at 280 nm assuming an extinction coefficient of 1.1 × 10^12 viral particles/OD unit.

Western Blot Analysis—Whole cell lysates were prepared, and Western blot analyses were performed as described previously (14). Protein content of the cell lysates was determined with BCA protein assay reagent (Pierce), with BSA used as a standard. The images were obtained and analyzed by using Model GS-700 imaging densitometer (Bio-Rad).

Reverse Transcription (RT)-PCR—Total RNA was extracted from cells by using TRIzol reagent as described previously (14). The first strand cDNA was synthesized from 1 μg of total RNA using oligo(dT)20-M4 adaptor primers and avian myeloblastosis virus reverse transcriptase (Takara Shuzo Co.). Synthetic gene-specific primer sets used in PCR were: 1) iNOS forward 20-mer, 5′-GCT ACT CTT CCA ACC CAG CA A-3′, and reverse 20-mer, 5′-TGG GTG GGA GGG GTA GTG AT-3′, which amplified a 430-bp sequence between +2081 and +2510 of rat iNOS cDNA; 2) COX-2 forward 22-mer, 5′-TGG GTG GGA GGG GTA GTG AT-3′, which amplified a 388-bp sequence between +388 and +743 of rat COX-2 cDNA; 3) VCAM-1 forward 21-mer, 5′-ACA CCT CCC CCA AGA ATA CAG-3′, and reverse 21-mer, 5′-GCT CAT CCT CAA CAC CAG G-3′, which amplified a 477-bp sequence between +659 and +1135 of rat VCAM-1 cDNA; 4) Mn-SOD forward 21-mer, 5′-TGC CTT GCC TTA CGA GTA TGG-3′, and reverse 20-mer, 5′-GCG ACC TGG TCC CTT ATT GA-3′, which amplified a 388-bp sequence between +87 and +474 of rat Mn-SOD cDNA; and 5) GAPDH forward 20-mer, 5′-GTC CTT AAC CAC GCC TCC TTC AT-3′, and reverse 20-mer, 5′-CGC CTT CCA CAT CAC ATC CT-3′, which amplified a 702-bp sequence between +88 and +189 of rat GAPDH cDNA. PCR was
but temporal differences were observed. After IL-1β/H9252, COX-2, VCAM-1, and Mn-SOD.

Inhibitor MG132 (1/H9262) were maintained between 6 and 24 h (4.9-, 12.4-, and 13.9-fold)
treatment, VCAM-1 was clearly increased by 3 h, and high levels
were detectable without cytokine treatment whereas iNOS
expression by the chemical inhibitors was due to the specific
expression of MEK1dn was determined. Consistent with the
overexpression of MEK1dn induced nuclear translocation of
NF-κB p65. VSMCs were cultured for 24 h in DMEM/F12 that con-
tained 0.1% BSA with or without AdvIκBαM (2.6 × 106 viral particles/ml) and then
untreated or treated with IL-1β (3 ng/ml) for 24 h. Whole cell lysates (20 μg of proteins/lane) were used for Western blot analysis to detect
the indicated proteins. The overexpression of IκBαM was confirmed by
immunoblotting with antibody against IκBαs (lower panel). Results
showed are representative of two separate experiments. In B, overex-
pression of the IκBαM inhibits IL-1β-induced nuclear translocation of
NF-κB p65. VSMCs were cultured for 24 h in DMEM/F12 that con-
tained 0.1% BSA with or without AdvIκBαM (2.6 × 106 viral particles/
ml) and then untreated or treated with IL-1β (3 ng/ml) for 1 or 6 h as
indicated. Immunofluorescent staining of p65 was performed as de-
scribed under “Experimental Procedures.”

**RESULTS**

**ERK Activation Is Required for iNOS and COX-2 Induction, but Not for VCAM-1 and Mn-SOD Induction**—In cultured rat
VSMCs, there was basal expression of VCAM-1 and Mn-SOD that
could be detected by Western blot analysis, whereas iNOS
and COX-2 were not detectable without cytokine treatment
(Fig. 1, A and B). IL-1β increased the expression of these genes, but
temporal differences were observed. After IL-1β stimulation,
VCAM-1 was clearly increased by 3 h, and high levels
were maintained between 6 and 24 h (4.9-, 12.4-, and 13.9-fold
greater than basal levels, respectively). In contrast, iNOS protein
was undetectable at 3 and 6 h but readily detectable 16 h
after IL-1β addition. The induction of COX-2 and Mn-SOD by
IL-1β occurred in a temporal pattern similar to that of iNOS,
expression being delayed as compared with that of VCAM-1.
Treatment of the cells with IL-1β also caused a sustained
increase of ERK phosphorylation. Notably, inhibition of ERK
phosphorylation by either PD98059 or U0126, specific inhibi-
tors of ERK kinases, dramatically reduced the expression of
both iNOS and COX-2 but did not prevent the induction of
VCAM-1 as well as Mn-SOD by IL-1β (Fig. 1, A and B). RT-PCR
(Fig. 1C) showed that the inhibition of ERK phosphorylation by
PD98059 influenced IL-1β induction of iNOS and COX-2 at the
transcriptional level. At 6 and 9 h after IL-1β addition, iNOS
mRNA was detectable in the absence of PD98059 but was not
detectable in the presence of PD98059. COX-2 mRNA showed
a biphasic increase during IL-1β treatment, with an increase at
1 h followed by a reduction at 3 h and then another increase
that reached the maximum between 6 and 9 h. The reduction of
COX-2 mRNA at 3 h was not due to artifactual RNA degra-
dation during RNA preparation or the reverse transcription re-
tection because the result was reproducible, and there was no
decrease in GAPDH mRNA levels. In the presence of PD98059,
IL-1β still induced an increase in COX-2 mRNA at 1 h but
failed to induce the second phase increase at 6 and 9 h. Both
VCAM-1 mRNA and Mn-SOD mRNA gradually increased dur-
ing the IL-1β treatment and remained at high levels for up to
6 and 9 h. PD98059 had no effect on the enhancement of
VCAM-1 and Mn-SOD mRNA levels induced by IL-1β.

To further confirm that the inhibition of iNOS and COX-2
expression by the chemical inhibitors was due to the specific
inhibition of ERK signaling, the effect of adenoviral mediated
expression of MEK1dn was determined. Consistent with the
results obtained using chemical inhibitors, Western blot anal-
ysis showed that overexpression of MEK1dn reduced IL-1β-
induced ERK phosphorylation and selectively reduced the induction of iNOS and COX-2 without significantly influencing the induction of VCAM-1 or Mn-SOD by IL-1β (Fig. 2). The control adenovirus expressing LacZ showed no effect on these processes.

The Duration of NF-κB Activation Is Responsible for the Differential Regulation of IL-1β-induced Gene Expression—As shown using EMSA (Fig. 3), at 30 min after IL-1β addition, the major DNA-bound form of NF-κB was the p65/p50 heterodimer, whereas the p50/p50 homodimer showed a relatively weak signal. However, at 6 h, when p65/p50 DNA binding was still obvious, the DNA binding of the p50/p50 homodimer was increased. Inhibition of ERK by PD98059 reduced the prolonged activation of NF-κB as evidenced by reduced binding of both p65/p50 and p50/p50 at 6 h, without having a significant effect on the early activation as evidenced by p65/p50 binding at 30 min (Fig. 3) (11, 12). Because inhibition of ERK activation selectively inhibits prolonged NF-κB activation, we tested the possibility that the expression of VCAM-1 and Mn-SOD induced by IL-1β might be dependent solely on the earlier and transient NF-κB activation. We therefore treated the cells with MG132, a proteasome inhibitor that inhibits IκB degradation. In contrast to PD98059, pretreatment of the cells with MG132 reduced both early and prolonged NF-κB activation induced by IL-1β, decreasing p65/p50 DNA binding to 56 ± 5% at 30 min and to 9 ± 1% at 6 h, as compared with those without MG132 treatment. In addition, MG132 reduced p50/p50 DNA binding at 6 h.

Western blot analysis (Fig. 4A) showed that phosphorylated IκBα was detected after IL-1β addition, and the abundance increased in the cells pretreated with MG132. Phosphorylated IκBα was not detectable in untreated cells but was observed in the cells treated with MG132 alone (lane 3), suggesting an accumulation of basal levels due to reduced degradation. The expression of iNOS, COX-2, VCAM-1, and Mn-SOD induced by IL-1β was obviously inhibited in cells pretreated with MG132, indicating the involvement of NF-κB in the expression of all four proteins. MG132 had no effect on the sustained phosphorylation of ERK that was observed after IL-1β addition. The ability of MG132 to prevent iNOS and COX-2 induction was apparent if the inhibitor was added at any time from 1 h preceding IL-1β addition up to 6 h after the cytokine was added (Fig. 4B). However, MG132 did show a lesser effect on inhibition of VCAM-1 and Mn-SOD induction if the inhibitor was added at 6 h after IL-1β addition.

To confirm that the inhibitory effect of MG132 on IL-1β-induced gene expression was due to its influence on components of NF-κB, the cells were infected with AdvIkBaM (S32A/S36A). Similar to MG132, the adenoviral-mediated overexpression of the degradation-resistant IkBa mutant (S32A/S36A) prevented the IL-1β-induced increase in iNOS, COX-2, VCAM-1, and Mn-SOD seen after treatment for 24 h (Fig. 5A). Immunofluorescent staining of the NF-κB p65 subunit was performed to determine whether the IκBα mutant prevented the nuclear translocation of the predominant heterodimeric form of NF-κB. The images (Fig. 5B) show clearly that IL-1β induced nuclear translocation of p65 by 1 h and that this persisted up to 16 h. Overexpression of the IκBα mutant inhibited IL-1β-induced nuclear translocation of NF-κB p65 at both time points, consistent with the inhibitory effect of the IκBα mutant on IL-1β-induced gene expression shown in Fig. 5A.

Inhibition of ERK Activation Attenuates IL-1β-induced IκBβ Degradation—We further determined whether the ability of the ERKs signaling cascade to regulate the persistent activation of NF-κB might relate to temporal changes in IκB levels. As shown in Fig. 6, A and B, IL-1β induced IκBα phosphorylation during the entire period observed. The total IκBα levels were dramatically reduced at 30 min and then gradually approached basal levels thereafter. Inhibition of ERK activation by either PD98059 (Fig. 6A) or overexpression of MEK1dn (Fig. 6B) did not prevent IL-1β-induced IκBα phosphorylation or degradation at 30 min. IL-1β stimulation also reduced IκBβ levels (Fig. 6, C and D). The reduction of IκBβ was not as pronounced as that of IκBα at 30 min, but the reduction of IκBβ at 6 h was obvious. Inhibition of ERK activation by PD98059 attenuated the reduction in IκBβ induced by IL-1β, although the effect of PD98059 on IκBβ degradation was less marked than that of MG132, suggesting that ERK might be involved in the regulation of IκBβ degradation.

Effects of ERK Signaling on NF-κB-dependent Gene Expression Induced by a Combination of Cytokines—Although treatment of rat VSMCs with IFNg or TNFa alone does not induce iNOS, either IFNg or TNFa does enhance iNOS expression...
induced by IL-1β (14, 15). We therefore examined whether or not ERK signaling could similarly regulate IL-1β-induced NF-κB-dependent gene expression in the presence of IFNγ or TNFα. As shown in Fig. 7A, both IFNγ and TNFα enhanced IL-1β-induced iNOS, COX-2, and VCAM-1, but not Mn-SOD expression, although the synergistic effect was more pronounced when TNFα was added with IL-1β. Inhibition of ERK activation by PD98059 dramatically reduced iNOS and COX-2 expression but not that of VCAM-1 and Mn-SOD induced by IL-1β alone or in combination with IFNγ or TNFα. In addition, although TNFα alone did not induce iNOS and COX-2 expression, it did increase VCAM-1 and Mn-SOD levels, and these were by a mechanism not inhibited by PD98059. To examine whether TNFα could activate ERK and NF-κB in a manner similar to IL-1β, we determined the changes in both I-κB levels and the phosphorylation of ERK at different time points after TNFα addition (Fig. 7B). Upon TNFα stimulation, I-κBα levels rapidly decreased by 30 min and then returned to basal levels by 3 h, whereas I-κBβ decreased gradually and remained at a lower level at later time points (3 and 9 h). TNFα activated ERK with a strong phosphorylation observed at 15 min and then returned to basal levels at 1 h. There was a second phase of ERK phosphorylation persisting from 3 to 9 h after TNFα addition. These effects of TNFα on I-κB degradation and ERK phosphorylation were similar to those observed with IL-1β (11).

We further tested whether ERK activation was still required for IL-1β to induce persistent activation of NF-κB in the presence of TNFα. As shown by EMSA (Fig. 7C), although PD98059 had essentially no effect on NF-κB activation at 1 h after cytokine addition, it dramatically reduced the prolonged activation of NF-κB induced by either IL-1β alone or IL-1β plus TNFα as documented by the data shown at the 16-h treatment time. The prolonged NF-κB activation induced by TNFα alone, like that caused by IL-1β alone, was also reduced by inhibition of ERK with PD98059.

**DISCUSSION**

In many cell types, NF-κB activation is essential for the expression of numerous genes such as iNOS, COX-2, VCAM-1, and Mn-SOD in response to inflammatory stimuli, including IL-1β (16–20). The present studies clearly show that IL-1β induces all of the genes mentioned above in cultured rat VSMCs but that the expression of these genes occurs in a temporally distinct manner. This temporal control is dependent upon the temporal activation of both ERK and NF-κB, and ERK phosphorylation is a requirement for the persistent activation of NF-κB.

Neither iNOS nor COX-2 mRNA or protein was detectable in the cells without IL-1β stimulation. After IL-1β stimulation, iNOS and COX-2 expression was delayed in comparison with changes in VCAM-1 or Mn-SOD. Unlike VCAM-1 or Mn-SOD, iNOS and COX-2 accumulation was entirely dependent on the prolonged activation of both ERK and NF-κB. The decrease in p65/p50 caused by ERK inhibition likely was I-κBα-independent as shown by persistent I-κBα phosphorylation and early I-κBα degradation that was unchanged by PD98059. Rather, as suggested previously, prolonged NF-κB activation may be mediated by I-κBβ (9, 21). This possibility is also supported by the analysis of the indicated proteins. Results shown in A and B are representative of two separate experiments each. p-ERK, phosphorylated ERK. In C, VSMCs were treated with IL-1β (3 ng/ml) or TNFα (10 ng/ml) or a combination of both for 1 or 16 h in the absence or in the presence of PD98059 (20 μM) that was added 1 h before IL-1β. Nuclear proteins were extracted and used for EMSA. Relative intensities of the NF-κB-DNA complex are shown in the bottom bar graph, with the intensities from the complex without PD98059 as 100%.
fact that in this study, inhibition of ERK attenuated IL-1β-induced IκBβ degradation. It is known that IκB kinases (IKKs) can phosphorylate IκBβ at Ser-19 and Ser-23, two phosphorylation sites similar to those in IκBα and responsible for its subsequent degradation (22, 23). Because activation of ERK alone does not induce NF-κB activation, as we reported previously (12), and inhibition of ERK does not affect IκBα phosphorylation induced by IL-1β, the influence of the ERK signaling cascade on IL-1β-induced IκBβ degradation may be mediated by mechanisms other than influencing IKK activity. Further studies are required to elucidate whether or not the ERK signaling cascade influences the susceptibility of IκBβ to phosphorylation by IKKs or to degradation by the proteasome. Although the requirement of ERK activation for iNOS and COX-2 induction might relate to multiple mechanisms, one role for ERK signaling apparently is to maintain the persistent activation of NF-κB.

TNFα alone was unable to induce iNOS expression (14, 15), although TNFα induced ERK activation and NF-κB activation in a pattern similar to that induced by IL-1β, suggesting the involvement of other signaling pathway(s) that may either induce or suppress iNOS expression. IFNγ alone did not activate NF-κB and was unable to induce iNOS expression in VSMCs (14, 15). However, it is known that both TNFα and IFNγ potentiate IL-1β-induced gene expression, probably due to the involvement of other signaling pathways. For example, signal transducer and activator of transcription (STAT)-1 is activated by IFNγ (14, 15) and this transcription factor has been shown to promote iNOS expression (24). Although IL-1β-induced iNOS and COX-2 expression was enhanced by either TNFα or IFNγ, the expression of these two genes was dramatically suppressed by inhibition of ERK activation, whereas the induction of VCAM-1 and Mn-SOD by the combination of cytokines was not affected. These data further demonstrated that the ERK signaling pathway has a major role in regulating the expression of certain NF-κB-dependent genes even when multiple cytokines coexist.

VCAM-1 and Mn-SOD were constitutively expressed at relatively low levels but were detectable by either Western blot for the proteins or RT-PCR for mRNA in rat VSMCs under basal conditions. A low basal turnover of IκBα proteins is supported by evidence obtained from treating cells for 25 h with MG132, a proteasome inhibitor, which inhibited IκBα degradation in the absence of added cytokine and led to an accumulation of phosphorylated IκBα. The IκBα-mediated NF-κB activation was acutely and dramatically enhanced by IL-1β stimulation, which was accompanied by increased expression of VCAM-1. Consistent with previous reports by others, one of the NF-κB-dependent genes is IκBα itself (8), which is activated in a similar time frame to VCAM-1 (protein levels increased within 3 h after IL-1β addition). Interestingly, the newly synthesized IκBα could not prevent the activation of NF-κB by IL-1β, possibly due to the fact that newly synthesized IκBα underwent constitutive and rapid phosphorylation and degradation during IL-1β stimulation. Because inhibition of IκBα degradation by MG132 reduced NF-κB activation without influencing IκBα phosphorylation, the activation of VCAM-1 gene transcription may be dependent on the turnover rate of IκBα. Neither PD98059 nor MEK1dn affected IL-1β-induced IκBα phosphorylation at Ser-32/Ser-36. Considering that inhibition of ERK showed no effect on IκBα phosphorylation and degradation, and also had no obvious effect on VCAM-1 expression, it is apparent that IκBα-mediated NF-κB activation in response to IL-1β is ERK-independent and responsible for triggering VCAM-1 gene expression. The regulation of Mn-SOD gene expression appears to be similar to that of VCAM-1, with respect to not requiring the persistent activation of both ERK and NF-κB.

The differential regulation of NF-κB-dependent gene expression could be important in determining the nature of the inflammatory response. The duration of NF-κB activation might determine the course of the inflammatory response with regard to the local reactions and resulting morphologic changes, the destruction or removal of the injurious material, and the responses that lead to repair and healing (1–3, 25). A potential role for NF-κB in the resolution of inflammation has been reported recently (26). Because activation of NF-κB may regulate the expression of numerous genes encoding either proinflammatory or anti-inflammatory mediators, including cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as iNOS, COX-2, and Mn-SOD, each of which may play critical roles during different stages of the inflammation, our findings demonstrating a role for ERK in the temporal control of NF-κB activation and NF-κB-dependent gene expression provides new insight into the mechanisms regulating NF-κB activation.

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