Stimulation of Interleukin-8 Production by Okadaic Acid and Vanadate in a Human Promyelocyte Cell Line, an HL-60 Subline

POSSIBLE ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE ON THE OKADAIC ACID-INDUCED NF-κB ACTIVATION§

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Most types of cells can produce interleukin (IL)-8 in response to various inflammatory stimuli. To study the role of protein phosphatases in the signal transduction leading to IL-8 production, a subline of HL-60 (C-15) was treated with okadaic acid (OA) and sodium orthovanadate (VA), inhibitors of phosphoserine/phosphothreonine phosphatase and phosphotyrosine phosphatase, respectively. Both OA and VA dramatically increased IL-8 secretion up to 200-fold in the HL-60 cells. OA and VA stimulation was accompanied by a marked increase in IL-8 mRNA expression and also by activation of a transcription factor, NF-κB. In addition, an essential role of the NF-κB site in the IL-8 gene activation was confirmed by the chloramphenicol acetyltransferase assay. IL-8 production by OA or VA was inhibited by protein kinase inhibitors, including staurosporine, H-7, K252a, herbimycin A, and genistein.

Both OA and VA induced significant tyrosine phosphorylation of p44, which was presumed to be Erk1, a member of the mitogen-activated protein kinase family, with concomitant activation of the mitogen-activated protein kinase activity. In parallel, rapid degradation of IκB-α, an inhibitory component of NF-κB, was observed. Since OA-activated Erk1 phosphorylated recombinant IκB-α in vitro, we assumed that Erk1 is involved in the phosphorylation and subsequent degradation of IκB-α, thus leading to the activation of IL-8 gene transcription.

Interleukin (IL)-8 is a cytokine that has chemotactic activity on neutrophils as well as T cells and basophils (1–3). IL-8 belongs to a chemokine family and is produced by a variety of cells stimulated with lipopolysaccharide (LPS) or proinflammatory cytokines, such as IL-1 or tumor necrosis factor-α (TNF-α) (4–8). Production of IL-8 is regulated at the transcriptional level through the activation of NF-κB complexes as well as C/EBP/NF-IL-6 or AP-1 complexes (9–11). NF-κB, originally identified as a transcription factor necessary for Igk gene expression (12), is a pleiotropic transcription factor that regulates the activation of various inflammatory genes (13–15). Recently, Ishikawa et al. (16, 17) reported the activation of a NF-κB complex in a cell-free system using the NF-κB binding site in the IL-8 gene and claimed that a staurosporine-sensitive kinase as well as tyrosine kinase is involved in the LPS-mediated NF-κB activation.

The phosphorylation-dephosphorylation of cellular proteins appears to be a major regulatory mechanism for cytokine production. In fact, inhibitors of protein phosphatases including okadaic acid (OA) or calyculin A (CA) are known to be good stimulators for IL-1 or TNF-α (18, 19). We therefore attempted to investigate the role of protein phosphorylation and/or dephosphorylation in IL-8 induction in a human promyelocytic cell line HL-60 subline, using OA and sodium orthovanadate (VA), specific inhibitors of phosphoserine/phosphothreonine phosphatase and tyrosine phosphatase, respectively. Our finding demonstrated that both OA and VA induced high levels of IL-8 production and its mRNA expression. Further, we analyzed the molecular mechanism of IL-8 gene activation by OA or VA in the HL-60 (C-15) cell line. We observed that mutation of the NF-κB binding site abolished the induction of CAT activity upon stimulation with OA or VA, indicating the essential role of NF-κB site also for the phosphatase inhibitor-induced IL-8 gene activation. In addition, an electrophoretic mobility shift assay (EMSA) revealed that OA or VA induced the formation of the NF-κB complexes. We also investigated the relationship of mitogen-activated protein kinase (MAPK) and IκB-α degradation during the IL-8 gene activation.

EXPERIMENTAL PROCEDURES

Reagents—OA, staurosporine, and genistein were obtained from Research Biochemicals International. VA was obtained from Sigma. Herbinycin A, H-7, H-8, KN62, and KN252a were obtained from Wako Pure Chemical Inc. (Osaka, Japan). [γ-32P]ATP, enhanced chemiluminescence reagents, and a p42/p44 MAPK enzyme assay system were obtained from Amersham (Tokyo, Japan). Monoclonal anti-phosphotyrosine antibody (mAb 4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal anti-MAPK (Erk-1/2) and anti-phospho-MAPK Abs were purchased from Biologs, Inc. Anti-Erk1 mAb was obtained from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated secondary Ab was purchased from DAKO (Denmark).

Cells and Cell Culture—An eosinophil-committed subline of the human promyelocytic leukemia cell line, HL-60 (C-15) (American Type Culture Collection [ATCC] CRL 1864, a gift of Dr. Steven Fischkoff) (20, 21) was used, since this subline produced higher levels of IL-8 than several other HL-60 cell lines. The HL-60 cell line was maintained in...
RFPI 1640 supplemented with 5% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine.

Measurement of Cytokine Production and Northern Blot Analysis—

Immunoreactive IL-8 was quantitated using an ELISA method as described elsewhere (22). This ELISA method detected at least 30 pg/ml of IL-8 and did not cross-react with other known members of the CXC and CC chemokine families. Total RNA was extracted from the cultured cells by 4 mM guanidine thiocyanate. Northern blot analysis was performed as described previously (23).

Preparation of the Cell Lysates and Immunoprecipitation—Cells (10⁶) were lysed with lysis buffer (50 mM HEPES (pH 7.5), 0.5% Triton X-100, 100 mM NaN₃, 10 mM sodium phosphate, 2 mM EDTA, 2 mM sodium molybdate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml phenylmethylsulfonyl fluoride) by stirring for 1 h at 4°C. After centrifugation for 20 min at 15,000 rpm at 4°C, protein concentration was determined, and the samples were stored at −80°C. For the immunoprecipitation, 5 × 10⁶ cells were suspended in 1 ml of lysis buffer, and the lysate was centrifuged for 20 min at 15,000 rpm. Twenty μl of a slurry of pansorbin (Calbiochem) and 3 μg of anti-IκB-α Ab (kindly provided by Dr. Nancy Rice, NCI-Frederick Research and Development Center, Frederick, MD) were added to the supernatant and incubated overnight at 4°C. The precipitates spun down were washed three times with lysis buffer and were then boiled for 5 min in Laemmli sample buffer. The sample was electrophoresed in 10% polyacrylamide gel and transferred onto a membrane filter followed by the Western blot using anti-IκB-α Ab and peroxidase-labeled anti-rabbit IgG, as described elsewhere (16, 17).

Electrophoresis and Immunoblotting—An equal volume of 2 × Laemmli sample buffer was added to the cell lysate prepared as described above. Samples were boiled for 10 min, and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% bovine serum albumin in phosphate-buffered saline for 1 h and then incubated with anti-phosphotyrosine or anti-MAPK or anti-phospho-MAPK Abs for 1 h at room temperature. After incubation with secondary Ab coupled to horseradish peroxidase, detection was made by the relative mobilities of prestained molecular weight markers.

MAPK Assay—MAPK activity was measured using the p42/44 MAPK enzyme assay system according to the manufacturer’s instruction (Amersham). Cell lysates (15 μl) prepared as described above were incubated with a substrate peptide (human EGF receptor) in 10 μl of kinase buffer (10 mM Tris at pH 7.5, 50 mM NaF, 100 μM Na3VO4, 10 μM okadaic acid, 0.1 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) by stirring for 1 h at 4°C. After centrifugation for 15,000 rpm at 4°C, protein concentration was determined, and the samples were stored at −80°C. For the immunoprecipitation, 5 × 10⁶ cells were suspended in 1 ml of lysis buffer, and the lysate was centrifuged for 20 min at 15,000 rpm. Twenty μl of a slurry of pansorbin (Calbiochem) and 3 μg of anti-IκB-α Ab (kindly provided by Dr. Nancy Rice, NCI-Frederick Research and Development Center, Frederick, MD) were added to the supernatant and incubated overnight at 4°C. The precipitates spun down were washed three times with lysis buffer and were then boiled for 5 min in Laemmli sample buffer. The sample was electrophoresed in 10% polyacrylamide gel and transferred onto a membrane filter followed by the Western blot using anti-IκB-α Ab and peroxidase-labeled anti-rabbit IgG, as described elsewhere (16, 17).

Electrophoretic Mobility Shift Assay (EMSA)—An equal volume of 2 × Laemmli sample buffer was added to the cell lysate prepared as described above. Samples were boiled for 10 min, and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 3% bovine serum albumin in phosphate-buffered saline for 1 h and then incubated with anti-phosphotyrosine or anti-MAPK or anti-phospho-MAPK Abs for 1 h at room temperature. After incubation with secondary Ab coupled to horseradish peroxidase, detection was made using the enhanced chemiluminescence system. Molecular sizes were determined by the relative mobilities of prestained molecular weight markers.

MAPK Assay—MAPK activity was measured using the p42/44 MAPK enzyme assay system according to the manufacturer’s instruction (Amersham). Cell lysates (15 μl) prepared as described above were incubated with a substrate peptide (human EGF receptor) in 10 μl of kinase buffer. The reaction was initiated of 5 μl of [γ-32P]ATP (220TBq/mmol) in a final volume of 25 μl. Reactions were terminated after 30 min at 30°C by the addition of Laemmli sample buffer. The phosphorylation of the substrate protein was examined after SDS-polyacrylamide gel electrophoresis, followed by autoradiography. GST-IκB-α fusion protein was prepared as described elsewhere (16, 17).

**RESULTS**

**Induction of IL-8 Production by OA or VA**—When HL-60 was stimulated with OA or VA, a significant amount of IL-8 was produced in the culture supernatants. HL-60 cultured without any exogenous stimuli released a marginal level of IL-8 (<0.5 ng/ml). It should be noted that both OA and VA were effective at a narrow range of concentration with maximal activation at 100 nM and 80 μM, respectively (Fig. 1). The effect of OA and VA was limited by their toxicity, which became apparent at concentrations greater than 100 nM (OA) and 200 μM (VA). Since induction of IL-8 was greatest at 100 nM (OA) or 80 μM (VA), these concentrations were used in further experiments to study the effect of these stimulants. As determined by kinetic analysis, IL-8 production induced by OA or VA was detectable at 3–6 h with maximal levels at 12–24 h (data not shown). OA increased IL-8 both in the supernatants and in cell lysate, with each 200- and 100-fold increase above unstimulated control. The effect of OA on IL-8 production was similar to that of OA, although the magnitude of IL-8 production was lower than that of OA. IL-8 production induced by OA or VA was still much higher than the levels induced by TNF-α (Fig. 2). These results indicated that both OA and VA by themselves are effective stimulants for IL-8 production. When the simultaneous addition of OA and VA was tested, no significant synergism, only an additive effect, was observed (data not shown).

Although we studied the direct effect of phosphatase inhibi-
tors on the undifferentiated HL-60 cells, TNF-α (but not IL-1α/β) induced only a moderate level of IL-8 by this cell line. Therefore, we examined whether phosphatase inhibitors modulate TNF-α-induced IL-8 production. As shown in Fig. 2, additive or weak synergistic effects were observed between TNF-α (20 ng/ml) and lower doses of OA (20 nM) and VA (10 µM), suggesting that phosphatase inhibitor did not interfere with TNF-α-induced IL-8 production, but rather acted cooperatively.

We also examined the effect of OA and VA on the normal human blood monocytes. As shown in Table I, both OA and VA increased IL-8 production only marginally, with a 1.3-1.7-fold increase above control, in which unstimulated monocytes spontaneously produced high levels of IL-8. Rather, higher doses of OA (50–100 nM), which were optimal for IL-8 production by HL-60 cells, inhibited IL-8 production, suggesting the differential effects of phosphatase inhibitors on the primary monocytes and leukemic cell line.

IL-8 mRNA Expression by OA or VA Stimulation—To confirm whether the increase of IL-8 was accompanied by the transcription of the IL-8 gene, we examined the expression of IL-8 mRNA. Northern blot analysis showed that IL-8 mRNA was detected as early as 1 h, and OA elicited a dramatic induction of IL-8 mRNA at 6–12 h (Fig. 3). The increase of IL-8 mRNA peaked at 12 h of OA treatment and declined at 24 h. Similarly, VA elicited a moderate but significant induction of IL-8 mRNA without a significant decline of IL-8 mRNA at 24 h.

To determine if OA and VA contributes to IL-8 mRNA stabilization, we measured the half-life of IL-8 mRNA, by incubating the cells with OA or VA for 3 h, followed by the treatment with actinomycin D. The half-life (t<sub>1/2</sub>) of IL-8 mRNA from unstimulated cells was approximately 5 h, while those of OA- or VA-treated cells were prolonged more than 10 h (data not shown). These results unambiguously indicated that both OA and VA increased stability of IL-8 mRNA markedly, thus contributing to the sustained expression of IL-8 mRNA.

Effects of Protein Kinase Inhibitors on IL-8 Induction by OA or VA—To investigate which type of protein kinase(s) was involved in the stimulation with OA or VA, HL-60 cells were treated with staurosporine, a nonspecific protein kinase inhibitor. Staurosporine at 100 nM completely blocked the increase of IL-8 by OA or VA (Fig. 4), suggesting that a basal level of protein phosphorylation is necessary for the action of OA or VA. Protein kinase C inhibitors, H-7 and K252a, markedly blocked IL-8 production induced by OA or VA with an IC<sub>50</sub> of H-7 and K252a at 10 and 20 µM, respectively (Fig. 4). HA1004, an analog of H-7, a weak protein kinase C inhibitor, was inactive even at 100 µM (data not shown). In addition, the tyrosine kinase inhibitors, genistein and herbimycin A, significantly inhibited the increase of IL-8 production by OA or VA, with the IC<sub>50</sub> of genistein at 6–10 µM and that of herbimycin A at 1 µM, indicating that tyrosine phosphorylation is essential for the IL-8 induction by OA or VA. On the other hand, treatment with KN-62, a calmodulin-dependent kinase inhibitor, had no effect. Similarly, H-8, PKA, and cGMP-dependent protein kinase inhibitors showed only marginal inhibitory effects on OA and VA stimulation.

These results suggested that protein kinase C as well as tyrosine kinase are involved in the stimulation of IL-8 production by OA or VA. However, no direct evidence was obtained on the involvement of the cAMP- and cGMP-dependent protein kinases and calmodulin-dependent kinases.

Effect of OA and VA on Tyrosine Phosphorylation—To delineate the target proteins involved in protein phosphorylation, lysates of OA- or VA-stimulated cells were prepared, electrophoresed, and immunoblotted with an anti-phosphotyrosine Ab (clone 4G10). Stimulation with OA or VA for 1 h induced increased tyrosine phosphorylation of several proteins, particularly a protein with an apparent molecular mass of 44 kDa (p44) (Fig. 5). It should be of note that no significant tyrosine phosphorylation of p44 was detected in lysates of unstimulated cells. Assuming that p44 might be a member of the MAPK family, which plays a crucial role in signal transduction from receptor tyrosine kinases (24), cell lysates were subjected to immunoblotting with anti-MAPK or phospho-MAPK Abs. When lysates of OA or VA-stimulated cells were electrophoresed and immunoblotted with rabbit anti-MAPK (rat Erk1/2) Ab, 44-kDa (Erk1) and 42-kDa (Erk2) bands were consistently

**Table I**

Induction of IL-8 in human blood monocytes treated with OA or VA with or without LPS stimulation

| OA | IL-8 production (ng/ml) | VA | IL-8 production (ng/ml) |
|----|------------------------|----|------------------------|
| nM | No LPS | 1 µg/ml LPS | µM | No LPS | 1 µg/ml LPS |
| 0  | 50 ± 2.5 | 55 ± 1.0 | 0  | 51 ± 2.1 | 55 ± 2.1 |
| 5  | 85 ± 4.2<sup>a</sup> | 72 ± 2.5<sup>b</sup> | 10 | 55 ± 5.7 | 66 ± 3.0<sup>a</sup> |
| 10 | 64 ± 3.6<sup>c</sup> | 91 ± 2.1<sup>d</sup> | 50 | 58 ± 2.5<sup>e</sup> | 70 ± 3.6<sup>f</sup> |
| 20 | 60 ± 4.2<sup>c</sup> | 56 ± 4.5 | 100 | 68 ± 4.6<sup>c</sup> | 76 ± 4.6<sup>d</sup> |
| 50 | 30 ± 5.6<sup>c</sup> | 29 ± 2.1<sup>d</sup> | 200 | 55 ± 5.1 | 82 ± 4.7<sup>e</sup> |
| 100| 24 ± 4.5 | 22 ± 2.6 | 500 | 65 ± 5.1 | 82 ± 4.7<sup>e</sup> |

<sup>a</sup> p < 0.005 compared with the value without OA or VA.
<sup>b</sup> p < 0.01 compared with the value without OA or VA.
<sup>c</sup> p < 0.05 compared with the value without OA or VA.
The values are the means of three independent determinations, and each S.E. was less than 5% of the mean.

OA or VA Stimulation Induces MAPK Activity—In accordance with the above finding, we investigated whether MAPK activity was induced by OA and VA treatment, i.e. lysates from OA- or VA-stimulated cells were prepared and assayed for MAPK activity. Time kinetics of the MAPK activity by 100 nM OA or 80 μM VA stimulation were shown in Fig. 7. After a 30-min stimulation with OA or VA, MAPK activity rapidly increased, reaching a maximal level at 1 h, which was maintained thereafter. Thus, a good correlation was observed between OA- or VA-induced tyrosine phosphorylation and MAPK activity. It should be noted that herbimycin A (5 μg/ml), an inhibitor of tyrosine kinases, inhibited MAPK activity almost completely (data not shown).

Requirement of NF-κB Binding Sites for IL-8 Gene Activation by OA or VA—Since it has been demonstrated that both TNF-α- and IL-1-induced IL-8 gene activation requires the NF-κB site in the 5′-flanking region of the IL-8 gene (9–11), we determined whether the NF-κB site as well as AP-1 and C/EBP sites are prerequisite as well for the OA- or VA-induced IL-8 gene activation. Since CAT activity transfected with −133-CAT expression vectors was normally induced by OA or VA (Fig. 8B), the mutants of the AP-1, C/EBP, or NF-κB sites were constructed as shown in Fig. 8A. Mutation of AP-1 or C/EBP/NF-IL-6 had little effect on the induction of CAT activity upon stimulation with OA or VA, indicating that these sites were not essential for gene regulation by these agents. On the other hand, mutation of the NF-κB site completely abolished the induction of CAT activity upon stimulation with OA or VA (Fig. 8B), indicating the essential role of the NF-κB site for IL-8 gene activation by these phosphatase inhibitors, as was the case in IL-1-induced IL-8 production in the glioblastoma cell line T98G (11).

Induction of NF-κB Complex Formation by OA or VA—To delineate the role of a nuclear factor, NF-κB, in the OA- and VA-induced IL-8 mRNA transcription, we performed an EMSA using nuclear extracts from the OA- or VA-stimulated HL-60 cells. Nuclear extracts from OA-treated HL-60 cells contained NF-κB complex as indicated, utilizing an authentic NF-κB oligo probe (Fig. 9, lane 2), whereas no specific NF-κB-DNA complex formation was observed in extracts from unstimulated cells (lane 1). The specificity of the complex was confirmed by competition experiments using an unlabeled NF-κB probe or an unlabeled mutated NF-κB probe. The band disappeared in the presence of an excess amount of an unlabeled NF-κB probe (lane 4), whereas the band did not disappear in the presence of an excess amount of unlabeled mutated NF-κB probe (lane 3). Moreover, a labeled mutated NF-κB probe failed to form a NF-κB complex (lane 5). VA-stimulated nuclear extracts gave results similar to those of OA. These results indicated that a specific NF-κB complex occurred following OA or VA stimulation.

Effect of OA and VA on the Phosphorylation and Degradation of IκB-α—Finally, we investigated the fate of IκB-α after the treatment of cells with OA or VA. Anti-IκB-α Ab detected a 38-kDa band (IκB-α) on Western blots in extracts from unstimulated HL-60 cells (Fig. 10), while no corresponding band was seen with the control antibody (data not shown). Within 10–20 min after OA stimulation, the IκB-α band almost disappeared, indicating degradation of this component. In contrast, the decrease of IκB-α following treatment of VA was slower and not complete as compared with OA-treatment.

In addition, we examined whether IκB-α could serve as a substrate of Erk1. Erk1 was prepared by immunoprecipitating OA-stimulated HL-60 cells and was used to phosphorylate re-
combinant GST-I\(\kappa\)B-\(\alpha\) protein. In an immune complex kinase assay, Erk1 effectively phosphorylated GST-I\(\kappa\)B-\(\alpha\) (Fig. 11A, lane 2) but not control GST (Fig. 10A, lane 1). A time course study showed phosphorylation peaking at 20 min after OA stimulation (Fig. 11B). These results indicated that GST-I\(\kappa\)B-\(\alpha\) could be a substrate of Erk1. Since Erk1 prepared from OA-stimulated, but not from unstimulated, HL-60 cells markedly enhanced phosphorylation of GST-I\(\kappa\)B-\(\alpha\), it is quite possible that OA induces activation of Erk1 to phosphorylate I\(\kappa\)B-\(\alpha\), followed by the degradation of I\(\kappa\)B-\(\alpha\).

DISCUSSION

We have demonstrated in this paper that both OA and VA, two potent protein phosphatase inhibitors, were effective stimulants for IL-8 production in HL-60 cells both at transcriptional and post-transcriptional levels. In addition, a nonspecific kinase inhibitor, staurosporine, blocked OA- or VA-induced IL-8 production, indicating the requirement of a basal level of protein phosphorylation for this activity. Because there were no essential differences between VA and OA stimulation and no synergistic actions were observed between these stimuli, both stimuli appear to activate the same or a very similar pathway resulting in the induction of IL-8 production.

Numerous exogenous and endogenous stimuli such as endotoxin, stresses, viruses, and bacteria as well as IL-1 and TNF-\(\alpha\) stimulate IL-8 gene activation in a variety of cells (1, 5, 7–10) in which transcriptional activation of the IL-8 gene is fairly well understood (9–11). The CAT assay experiments revealed that, in the HL-60 cell line as well, the NF-\(\kappa\)B site was prerequisite for the appropriate activation of the IL-8 gene by OA or VA stimulation, similar to TNF-\(\alpha\) and IL-1 stimulation, as has been described elsewhere (9–11). In addition, EMSA revealed that OA or VA induced the formation of the NF-\(\kappa\)B complexes. However, the role of protein phosphorylation in IL-8 gene activation has not yet been clearly defined. Of interest is the finding that inhibitors of protein phosphatases including OA or calyculin A induce IL-1 or TNF-\(\alpha\) in various cell systems (18, 19). Furthermore, it has been shown that OA treatment results in immediate phosphorylation of a variety of proteins in many cell types including fibroblasts and that OA mimics gene expression induced by IL-1 and TNF-\(\alpha\) (25). In this study, we demonstrated that OA or VA caused tyrosine phosphorylation of several proteins in HL-60 cells. We have assumed that tyrosine phosphorylation of MAPK is one of the key signal transduction pathways leading to IL-8 gene activation in HL-60 cells. To prove the assumption, we tried to identify a tyrosine-phosphorylated 44-\(k\)Da protein in OA- or VA-stimulated HL-60 cells. Immunoblots using anti-phosphotyrosine, MAPK, and phosphorylation of MAPK Abs indicated that the tyrosine-phosphorylated 44-\(k\)Da protein was Erk1, a member of the MAPK family (24). The involvement of c-Jun N-terminal kinase/stress-activated protein kinase or p38 MAPK homologues, which also are members of MAPK family (26–28), cannot be completely ruled out in this study, although the anti-phospho-MAPK Ab used detects tyrosine-phosphorylated MAPK and does not cross-react with the corresponding phosphorylated tyrosine of either c-Jun N-terminal kinase/stress-activated protein kinase or p38 MAPK. More detailed study is required to elucidate the involvement of the c-Jun N-terminal kinase/stress-activated protein kinase or p38 MAPK family.

Members of the MAPK family are active only when they are concomitantly phosphorylated on both tyrosine and threonine residues (29). While only tyrosine phosphorylation was measured in this report, the finding that MAPK activity was increased by OA or VA strongly suggests that the threonine residue was also phosphorylated. Nishida et al. (30) have demonstrated that MAPK activation from the ligand-receptor interaction involves Ras, Raf, and MAPK kinase with subsequent activation of MAPK. We have found that OA- or VA-induced tyrosine phosphorylation of p44 correlates with increased MAPK activity. Moreover, herbimycin A, a tyrosine kinase inhibitor, caused a decrease in MAPK activity. Thus, MAPK activity is thought to be down-regulated by phosphotyrosine dephosphorylation. It is known that both the Thr-183 and Tyr-185 residues in Erk1/Erk2 are phosphorylated and activated by various stimulations (31–32). Phospho-Thr-183 of...
Erk1/Erk2 may be dephosphorylated by phosphatase 2A, leaving phospho-Tyr-185, which is dephosphorylated by an unknown phosphotyrosine phosphatase (33). One of the targets of OA and VA action might be the inhibition of the dephosphorylation of this Erk1/2. Alternatively, OA- or VA-induced alteration in IL-8 gene expression may be due to the phosphorylation of transcription factors and the subsequent alteration in their DNA binding properties. Some transcription factors (Elk-1, Sap-1) are good substrates for Erk1/Erk2 in vitro (34, 35). Identification of the substrates of MAPK in this cell system will provide important information concerning the mechanism of IL-8 production.

In this study, a close association of MAPK activity with IκB-α degradation was observed, leading to NF-κB activation. NF-κB is retained in an inactive form by being associated with its inhibitor, IκB, in the cytoplasm in most types of cells. Stimulation of cells with LPS or with proinflammatory cytokines such as IL-1α/β and TNF-α causes phosphorylation of IκB, and the subsequent release of NF-κB from IκB permits translocation of NF-κB to the nucleus and binds to the NF-κB binding site (36–38). However, protein kinases and proteases involved in this IκB degradation remain to be investigated. Chen et al. (39) recently found that IκB-α was phosphorylated by a IκB kinase complex containing ubiquitin. On the other hand, several groups reported that protein kinase C (40) and double-stranded RNA-dependent protein kinase phosphorylate IκB-α in vitro (41). Furthermore, Kuno et al. (17) identified a novel 42-kDa IκB-α-kinase, which is distinct from MAPK. We found that Erk1 phosphorylated a GST-IκB-α protein in vitro. From these studies, it is probable that there exist several pathways following different stimuli leading to the phosphorylation of IκB-α.

Induction of IL-8 mRNA expression by OA or VA and the complete inhibition of mRNA synthesis by actinomycin D show that the regulation of IL-8 by OA or VA in HL-60 is primarily transcriptional. Furthermore, OA and VA increased the half-

![FIG. 8](image-url)

FIG. 8. A, the positions of mutations introduced to −133-CAT to create C/EBP mutant, AP-1 mutant, and NF-κB mutant are shown. B, the CAT activity in cell extract was determined using an equal amount of cell extracts. After thin layer chromatography, radioactivity was measured using the bioimage analyzer BAS 2000 (Fuji-film, Tokyo, Japan), and percentage of conversion (% Acetylation) was calculated as cpm in the acetylated form divided by total cpm.

![FIG. 9](image-url)

FIG. 9. EMSA using the NF-κB binding site in the IL-8 gene as probe. Nuclear extracts were prepared from the 10⁶ cells stimulated with OA or VA for 30 min as described under "Experimental Procedures" and tested for the NF-κB binding activity. Nuclear extracts were incubated with labeled oligonucleotide (0.5 ng) containing the NF-κB sequence in the absence (lane 2) and presence of 10 ng of unlabeled oligonucleotide containing the NF-κB (lane 4) or 10 ng of unlabeled oligonucleotide containing the mutated NF-κB sequences (lane 3). Nuclear extracts from OA- or VA-stimulated cells were incubated with labeled oligonucleotide (0.5 ng) containing the mutated NF-κB sequence (lane 5). Nuclear extracts from unstimulated cells (lane 1) were also tested.

![FIG. 10](image-url)

FIG. 10. Rapid degradation of IκB-α after OA or VA stimulation. Cells were stimulated with OA or VA for the indicated times. Anti-IκB-α Ab was used to immunoprecipitate the IκB-α protein as described elsewhere (16, 17). Immunoprecipitated IκB-α was immunoblotted with anti-IκB-α Ab.
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Figure 11. Phosphorylation IκB-α by the immunoprecipitated Erk1 in vitro. Recombinant GST (panel A, lane 1) or GST-IκB-α expressed in Escherichia coli (1 μg) (panel A, lane 2) was used as a substrate in an immune complex kinase assay with Erk1 prepared from OA-treated cells for 30 min. The kinetics of IκB-α phosphorylation by the immunoprecipitated Erk1 are indicated in panel B.

life of IL-8 mRNA. It has been reported that a 32-kDa protein has been identified that binds to the AU-rich 3'-untranslated region of short-lived cytokine genes such as TNF-α (42). Whether this protein is activated or induced by OA or VA and thereby selectively modulates mRNA stability remains to be determined. Inasmuch as OA and VA inhibit phosphoserine/phosphothreonine phosphatase and phosphotyrosine phosphatase, it is conceivable that OA and VA thus may prolong the half-life of phosphorylated proteins (MAPK and others) with regard to their signal transduction, leading to sustained IL-8 gene activation. Further experiments will be directed toward identifying the kinases that are involved in the induction of IL-8 gene activation.

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