IκB Kinase, a Molecular Target for Inhibition by 4-Hydroxy-2-nonenal*

Chuan Ji, Kevin R. Kozak, and Lawrence J. Marnett‡
From the Vanderbilt-Ingram Cancer Center and Center in Molecular Toxicology, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received for publication, February 8, 2001, and in revised form, March 9, 2001
Published, JBC Papers in Press, March 16, 2001, DOI 10.1074/jbc.M101266200

In unstimulated cells, transcription factor NF-κB is retained in the cytoplasm by interaction with the inhibitory protein, IκBa. Appropriate cellular stimuli activate IκBa by phosphorylation, ubiquitination, and proteolytic degradation, which allows NF-κB to translocate to the nucleus and modulate gene expression. 4-Hydroxy-2-nonenal (HNE), a major lipid peroxidation product, inhibits activation of NF-κB in the human colorectal carcinoma cell line (RKO) and human lung carcinoma cell line (H1299). Pretreatment of cells with HNE dose-dependently suppresses tetradecanoylphorbol acetate (TPA)/ionomycin (IM)-induced NF-κB DNA binding activity and transactivation of luciferase-based reporter constructs. HNE pretreatment has no effect on TPA/IM-induced AP-1 DNA binding activity. HNE inhibits TPA/IM-induced degradation of IκBα in both H1299 and Jurkat T cells. The accumulation of IκBα parallels the inhibition of its phosphorylation. At doses that inhibit IκBα degradation, HNE inhibits IκBα kinase (IKK) activity by direct reaction with IKK. Covalent adducts of HNE to IKK are detected on Western blots using antibodies against IKK or HNE-protein conjugates. Addition of dithiothreitol prevents HNE modification of IKK. Thus, HNE is an endogenous inhibitor of NF-κB activation that acts by preventing IKK activation and subsequent IκBα degradation.

Aldehydes are products and propagators of oxidative stress (1). They are reactive electrophiles that form adducts to proteins and DNA that have been detected in tissues from healthy human beings and individuals with various diseases (2–6). Consequently, aldehydes modulate the activities of numerous proteins, induce mutations, and alter cell cycle progression (7–12). For example, malondialdehyde, a major carbonyl product of lipid peroxidation, is mutagenic and carcinogenic and induces cell cycle arrest at the G1/S and G2/M checkpoints (7). The G2/M arrest in human colon and lung cancer cells (RKO and H1299, respectively) is caused by induction of the cyclin-dependent kinase inhibitor, p21, whereas the G2/M arrest appears to be due to a reduction in the level of the cdc2 kinase. Thus, alteration of gene expression triggered by protein or DNA damage may contribute to the range of biological effects exerted by aldehydes.

A panoply of pathophysiological responses is also exerted by 4-hydroxynonenal (HNE),1 the major toxic product of lipid peroxidation (1). HNE reacts with sulphydryl and amino groups and leads to inactivation of DNA polymerases, dehydrogenases, and various transporters, inter alia (13). It also causes cell cycle arrest and apoptosis (8–10). HNE treatment of cells alters the expression of several transcription factors including c-Myb (12), c-Myb (14), and c-Jun (15), suggesting that it may have more global effects on protein expression and cell function. The induction of c-Jun by HNE is associated with activation of JNK kinase and p38 kinase, perhaps by H2O2 modulation of upstream signaling pathways (15, 16).

A major signaling pathway associated with inflammation and oxidative stress is mediated by the transcription factor NF-κB (17–19). NF-κB consists of heterodimers of two polypeptides, p50 and p65, which are members of a family of proteins related to the proto-oncogene c-rel (20, 21). Inactive NF-κB is located in the cytosol, bound to its inhibitory protein, IκBα. Dissociation of NF-κB from IκBα is a critical step in NF-κB activation that leads to translocation of NF-κB to the nucleus, enabling DNA binding and transactivation (22). This process is triggered by sequential phosphorylation and ubiquitination of IκBα, followed by digestion of the ubiquinated protein by the proteasome (23–25). The enzyme that catalyzes the ubiquitination of phosphorylated IκBα is constitutively active. Hence, in most cases, the key event for NF-κB activation is phosphorylation of two serine residues at the N terminus of IκBα by IκB kinase (IKK) (23, 24).

We report here that treatment of RKO and H1299 cells with HNE leads to a dramatic loss of DNA binding and transcriptional activation by NF-κB in cells treated with tetradecanoylphorbol acetate (TPA) and ionomycin (IM). The loss of NF-κB activity is due to stabilization to the IκBα–NF-κB complex, which results from a decrease in the rate of turnover of IκBα. The prevention of IκBα turnover is attributable to the inhibition of IKK caused by direct reaction with HNE. These findings indicate that HNE is a potent inhibitor of the NF-κB-dependent cell signaling.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Chemical Treatment—Human colorectal carcinoma cells (RKO) were maintained in McCoy’s 5A medium (Hyclone, Logan, Utah). Human large cell lung carcinoma cells (H1299) were maintained in F-12 medium (Hyclone), and human lymphoma Jurkat T cells were maintained in RPMI (Hyclone). RKO and H1299 cells were grown in the presence of 10% bovine serum, and Jurkat T cells were grown in the presence of 10% heat-inactivated fetal bovine serum. All media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in 5% CO2 at 37°C. RKO and H1299 cells were plated 18–24 h prior to chemical

1 The abbreviations used are: HNE, 4-hydroxy-2-nonenal; IKK, IκB kinase; TPA, tetradecanoylphorbol acetate; IM, ionomycin; DTT, dithiothreitol; KLB, kinase lysis buffer; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; p-IκBα, phosphorylated IκBα.

* This work was supported by Research Grant CA47479 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence and requests for reprints should be addressed. Tel.: 615-343-7329; Fax: 615-343-7534; E-mail: marnett@toxicology.mc.vanderbilt.edu.
exposure and were 50–70% confluent at a density of 7 × 10^5/ml at the time of treatment. HNE (a generous gift from V. Amarnath, Vanderbilt University) and TPA (Sigma) were dissolved in 70% ethanol, and IM (Calbiochem, San Diego, CA) was dissolved in Me2SO. The final concentration of ethanol or Me2SO in the medium was ≤0.1%.

Nuclear Extract—Preparation of Nuclei and Total Cell Extract—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 1 mM DTT, and the protease inhibitors antipain (5 μg/ml), leupeptin (5 μg/ml), pepstatin A (5 μg/ml), chymostatin (5 μg/ml), phenylmethylsulfonyl fluoride (50 μg/ml), (Sigma) for 30 min at 4°C. The nuclei were collected by addition of Nonidet P-40 (Roche Molecular Biochemicals)) for 20 min at 4°C. The cell lysates (final concentration, 0.4%), cell lysates were incubated on ice for 10 min. Nuclear and cytoplasmic fractions were separated by centrifugation at 10,000 × g. The supernatant (cytoplasmic extract) was cleared by further centrifugation at 100,000 × g in a microcentrifuge for 10 min (cytosolic extract). The pellets were washed once with buffer A and resuspended in buffer B (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 25% glycerol, and protease inhibitors (as above). The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 × g for 10 min. The supernatant containing nuclear proteins was collected. For isolation of total cell extracts, cells were lysed in kinase lysis buffer (KLB: 40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM Na3VO4, 0.3 mM DTT, and protease inhibitors (as above)). The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 × g for 10 min. The supernatant containing nuclear proteins was collected. For isolation of total cell extracts, cells were lysed in kinase lysis buffer (KLB: 40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM Na3VO4, 0.3 mM DTT, and protease inhibitors (as above)). The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 × g for 10 min. The supernatant containing nuclear proteins was collected. For isolation of total cell extracts, cells were lysed in kinase lysis buffer (KLB: 40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM Na3VO4, 0.3 mM DTT, and protease inhibitors (as above)). The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 × g for 10 min. The supernatant containing nuclear proteins was collected. For isolation of total cell extracts, cells were lysed in kinase lysis buffer (KLB: 40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM Na3VO4, 0.3 mM DTT, and protease inhibitors (as above)). The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 × g for 10 min. The supernatant containing nuclear proteins was collected. For isolation of total cell extracts, cells were lysed in kinase lysis buffer (KLB: 40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM Na3VO4, 0.3 mM DTT, and protease inhibitors (as above)). The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 × g for 10 min. The supernatant containing nuclear proteins was collected.
effects of TPA/IM stimulation and HNE treatment on IkappaB
phoretic mobility shift assay using nuclear extracts prepared from these cells was analyzed by an electro-
coli oligonucleotides containing NF-kappaB and AP-1 recognition sequences. H1299 cells, either untreated or pretreated with HNE (40 
mu M) for 30 min, were incubated with TPA plus ionomycin (TPA/IM, 0.08 
uM/2 
uM) at 37°C for the time indicated. The binding activity of the nuclear extracts prepared from these cells was analyzed by an electrophoretic mobility shift assay using gamma-32P-labeled NF-kappaB (A) or AP-1 (B) oligonucleotide probes. Equal amounts of nuclear extract were added to each lane. The specificity of binding was determined either by adding a 50-fold molar excess of unlabeled oligonucleotides as competitor or by supershift assays with respective antibodies. The results are represent-
tive of three independent experiments.

Fig. 1. Effect of HNE on the binding of nuclear proteins to oligonucleotides containing NF-kappaB and AP-1 recognition sequences. H1299 cells, either untreated or pretreated with HNE (40 
mu M) for 30 min, were incubated with TPA plus ionomycin (TPA/IM, 0.08 
uM/2 
uM) at 37°C for the time indicated. The binding activity of the nuclear extracts prepared from these cells was analyzed by an electrophoretic mobility shift assay using gamma-32P-labeled NF-kappaB (A) or AP-1 (B) oligonucleotide probes. Equal amounts of nuclear extract were added to each lane. The specificity of binding was determined either by adding a 50-fold molar excess of unlabeled oligonucleotides as competitor or by supershift assays with respective antibodies. The results are represent-
tive of three independent experiments.

HNE Blocks NF-kappaB Transactivation in H1299 Cells—To correlate HNE effects on NF-kappaB transactivation with DNA binding inhibition, an NF-kappaB-dependent, luciferase-expressing vector was employed. Twenty-two h after transient transfection with the luciferase reporter, H1299 cells were stimulated with TPA/IM or treated with HNE and TPA/IM for 6 h. Control cells were not treated with HNE or with TPA/IM. Cell extracts were prepared and analyzed for luciferase activity. TPA/IM treatment induced a 3-fold increase in luciferase activity relative to untreated cells (Fig. 2). HNE treatment suppressed the TPA/IM-induced increase in luciferase activity in a dose-dependent manner, with 20 
mu M HNE providing complete suppression and higher doses decreasing luciferase activity to below unstimulated levels (Fig. 2). Thus, HNE inhibited both NF-kappaB DNA binding and NF-kappaB transcriptional activation. Parallel experiments with RKO cells produced similar results, as shown in the lower panel of Fig. 2.

HNE Blocks IkappaB Degradation in H1299 Cells and Jurkat T Cells—NF-kappaB activation requires degradation of the inhibitory protein, IkappaB (26, 27). Consequently, HNE inhibition of NF-kappaB DNA binding and transactivation activities could result from the inhibition of IkappaB degradation. To test this possibility, the effects of TPA/IM stimulation and HNE treatment on IkappaB degradation were evaluated. Treatment of H1299 or Jurkat T cells with TPA/IM for 0–30 min at 37°C resulted in a rapid decrease in cellular IkappaB protein (Fig. 3). For H1299 cells, the reduction in the level of IkappaB protein appeared maximal by 5 min, and some increase was evident by 30 min (Fig. 3A, lanes 1–4). For Jurkat T cells, the reduction in the level of IkappaB protein was detectable at 5 min, with complete disappearance evident in 20 min (Fig. 3B, lanes 1–4). In Jurkat cells, the TPA/IM-mediated decrease in cellular IkappaB concentrations resulted from an induction of IkappaB phosphorylation followed by a degradation of phosphorylated IkappaB (p-IkappaB, Fig. 3B, lanes 1–4). In contrast, no detectable p-IkappaB was found in TPA/IM-treated H1299 cells (Fig. 3A, lanes 1–4). Pretreatment of cells with HNE prevented the TPA/IM-mediated reduction of IkappaB concentration (Fig. 3, A and B, lanes 5–8). In addition, HNE pretreatment completely abolished the formation of p-IkappaB in Jurkat T cells. Thus, it appears likely that HNE treatment prevents IkappaB degradation by inhibition of IkappaB phosphorylation.

HNE Inhibits IKK Activity in Jurkat T Cells—IKK activity is required for IkappaB phosphorylation (28–30). Thus, one possible mechanism to explain the inhibitory effect of HNE on TPA/IM stimulation of NF-kappaB activity is that HNE inhibits IKK activity. To test this possibility, Jurkat T cells, with or without a 30-min pretreatment with HNE, were stimulated with TPA/
Addition of 30 μM HNE to immune complexes of IKK produced bands that migrated more slowly than IKK protein by HNE. Incubations of immune complexes of IKK with HNE resulted in clear inhibition of IKK activity. When parallel incubations were conducted in the presence of an excess of the HNE scavenging agent DTT (1 mM), only a modest decline in IKK activity was detected at the higher HNE concentration.

Western blots were performed to probe for the modification of IKK protein by HNE. Incubations of immune complexes of IKK with HNE produced bands that migrated more slowly than IKK as well as bands that migrated at the anticipated size for a dimer of IKK subunits (−220 kDa) (Fig. 5A, lower two panels, lanes 4–5). Thus, incubation mixtures of IKK with HNE contained HNE-modified IKK molecules, some of which migrated as cross-linked protein dimers. Comparison of the kinase assay bands in Fig. 5A with the Western blots in the lower panels indicates that the formation of the HNE-IKK conjugates correlated with the loss of IKK activity. When parallel incubations of immune complexes of IKK and HNE were conducted in the presence of an excess of DTT, only trace amounts of slower migrating forms of IKK were detected; no higher molecular size HNE-IKK complexes were evident on gel electrophoresis (Fig. 5B, lower two panels, lanes 4–5). These results demonstrate that HNE reacts covalently with IKK, which prevents IkBα degradation and NF-κB activation.

DISCUSSION

In the present studies, we show that TPA/IM stimulates IkBα phosphorylation and subsequent degradation, resulting in NF-κB activation. This finding is consistent with previous observations that NF-κB activation is responsive to a wide range of activators that lead to phosphorylation and degradation of IkBα (19, 26, 27, 31, 32). Our experiments demonstrate that pretreatment of human cancer cells or Jurkat T cells with HNE leads to the inhibition of the NF-κB signaling pathway. HNE prevents IkBα phosphorylation and subsequent degradation, reducing NF-κB DNA binding activity and NF-κB transactivation. These results are in good agreement with the findings that HNE modulates NF-κB activation by inhibiting IkBα phosphorylation and subsequent proteolysis in human monocytic cells (33).

Interestingly, the complete process of IkBα phosphorylation and subsequent degradation following treatment of cells with TPA/IM was only observed in Jurkat T cells. Phosphorylation of IkBα was not observed in H1299 cells even though its TPA/IM-stimulated degradation was obvious (Fig. 3). Three possibilities may explain the inability to detect p-IkBα in H1299 cells. TPA/IM-induced phosphorylation of IkBα may occur at a residue other than Ser-32 or Ser-36, so that the phosphorylated protein may not be recognized by the antibody employed in these studies. This possibility has been documented with anoxia, which stimulates phosphorylation of IkBα at Tyr-42 and NF-κB activation without proteasome-mediated degradation of IkBα (34). A second possibility is that activation of NF-κB in H1299 cells results from phosphorylation-independent IkBα degradation. For example, UV irradiation leads to IkBα degradation without phosphorylation in HeLa cells, 293 cells, and human fibroblasts (35, 36). Finally, the kinetics of IkBα phosphorylation and IkBα degradation in H1299 cells may prevent a detectable steady-state concentration of p-IkBα from accumulating.
Phosphorylation of IκB requires IKK activity (22). IKK is a complex, which contains two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), along with a regulatory protein, IKKγ (37–40). In our experimental conditions, both IKKα and IKKβ were immunoprecipitated by anti-IKKα antibody (data not shown). Thus, the IKK activity represented the combination of IKKα and IKKβ. A variety of stimuli modulate the signal transduction pathways that lead to activation of upstream kinases including NF-κB-inducing kinase and mitogen-activated protein kinase kinase kinase 1. These kinases are responsible for phosphorylation and activation of IKK (29, 30, 41). HNE did not inhibit any of these upstream kinases, and in fact, a brief survey indicated that it stimulated the activity of ERK1, ERK2, JNK1, and JNK2 (data not shown). This is consistent with a previous finding of stimulation of p38 kinase activity by HNE (42).

The effects of HNE are directly on IKK activity and appear to result from covalent modification of IKK protein. Aspirin, salicylate, and sulindac inhibit IKKβ activity by competing for binding to ATP (43, 44), whereas anti-inflammatory cyclopane tenone prostaglandins inhibit NF-κB activation by covalently modifying Cys-179 on the activation loop of IKKβ, leading to substantially reduced IKKβ activity (45). It is well known that HNE can rapidly react with proteins containing sulphydryl groups by Michael addition; so it is possible that HNE inhibits IKK activity by direct reaction with a cysteine residue (16, 46). To test this possibility, we conducted an in vitro assay to assess the effect of HNE on IKK activity and protein modification. Our results demonstrated that HNE induced the loss of IKK activity concomitant with the formation of higher molecular size forms of IKK (Fig. 5A). A prominent band was detected at a molecular size corresponding to cross-linked homodimers or heterodimers of IKK subunits. The higher molecular size band on SDS-PAGE gels reacted with antibodies specific for IKKα and with antibodies specific for a Michael addition product of HNE with protein residues. This is consistent with the formation of an HNE-mediated cross-link of IKK protein subunits. The activation domains of IKKα or IKKβ are believed to be located in close proximity to each other in the IKK complex, which might place the cysteine residues of the two activation domains close enough to enable cross-link formation (22, 45).

The importance of the reaction of HNE with cysteine residues is suggested by the observation that treatment with DTT inhibited HNE-induced cross-link formation and loss of enzyme activity. DTT is a dithiol that is used as a reducing agent to protect free protein thiols from oxidation; it is commonly added to enzyme assays or purification buffers for this purpose. DTT inhibited HNE-induced cross-link formation and loss of enzyme activity. DTT is a dithiol that is used as a reducing agent to protect free protein thiols from oxidation; it is commonly added to enzyme assays or purification buffers for this purpose. DTT also reacts with α,β-unsaturated carbonyl compounds such as HNE. Its inclusion in the present experiments abolished modification and inhibition of IKK in cell-free extracts. We believe this accounts for previous reports that HNE does not inhibit IKK activity (33).

We demonstrate here that the key target in HNE modification of NF-κB activity is IKK. Inhibition of IKK activity by this major product of lipid peroxidation occurs through covalent modification of the constituent proteins. Because NF-κB stimulates transcription in response to oxidative stress, HNE modification may limit the magnitude of this transcriptional response. A similar role was recently proposed for 15-deoxyprostaglandin D2, which is a decomposition product of prostaglandin D2, a product of arachidonic acid metabolism in inflammatory cells (45, 47). Furthermore, a related reaction with IKK may account for the previously noted inhibition of NF-κB by acrolein (48). HNE is structurally related to 15-deoxyprostaglandin D2 and acrolein, because it contains an α,β-unsaturated carbonyl compound capable of reacting as a bifunctional electrophile. In this way, it may serve as an endogenous factor that

**Fig. 5.** HNE inhibits IKK activity by directly reacting with IKK in vitro. Jurkat T cells either untreated or pretreated with HNE (30 μM) for 30 min were incubated with TPA/IM (0.04 μM/3 μM) at 37 °C for 10 min. Total cell extracts (250 μg/lane) were immunoprecipitated with IKKα antibody and analyzed for the effect of HNE on IKK activity in vitro. A, the IKK immune complex was incubated with 0 or 60 μM HNE or vehicle in the absence of DTT at 30 °C for 10 min. B, the IKK immune complex was incubated with 30 or 60 μM HNE or vehicle in the presence of DTT (1 mM) at 30 °C for 10 min. The kinase activities associated with the immunoprecipitated IKK complex were determined using 1×GST fusion protein as substrate and are displayed in the top panels of A and B. Immune complexes of IKK corresponding to equal volumes of cell extracts were loaded in each lane. Equal amounts of the substrate (1×GST-GST) were present in each assay, as confirmed by ink staining and immunoblotting of the membranes (second panel of A and B). Individual samples were divided in two, and separate PAGE gels were run for Western blotting. After Western transfer, the blots were visualized with antisera to IKKα or to HNE-modified protein. The third panel of A and B represents the detection of IKK molecules with an antisem against IKKα. The lower panel of A and B represents the detection of HNE-modified IKK molecules with an antisem that recognizes HNE-modified protein conjugates. The amounts of IKK immune complexes added to each reaction corresponded to equal amounts of cell lysate. These complexes contained comparable amounts of IKK protein, as judged by the Western blots in A and B, lanes 1–3. Incubation with HNE may alter immune reactivity; so the amounts of IKK detected in A and B, lanes 4–5, may not accurately reflect IKK content. The results are representative of three independent experiments.
regulates the inflammatory response associated with oxidative stress.

Acknowledgments—We thank Lawrence D. Kerr for generously providing an NF-κB-dependent luciferase construct and V. Amarnath for providing advice. We are especially thankful to Carol Rouzer for a critical reading and editorial suggestions.

REFERENCES

IkB Kinase, a Molecular Target for Inhibition by 4-Hydroxy-2-nonenal
Chuan Ji, Kevin R. Kozak and Lawrence J. Marnett

doi: 10.1074/jbc.M101266200 originally published online March 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101266200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 23 of which can be accessed free at
http://www.jbc.org/content/276/21/18223.full.html#ref-list-1