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

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In unstimulated cells, transcription factor NF-κB is retained in the cytoplasm by interaction with the inhibitory protein, IκBα. Appropriate cellular stimuli activate IκB kinase 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 tetradeacanoylphorbol 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 IκB kinase (IKK) activity by direct reaction with IκB. Covalent adducts of HNE to IκB are detected on Western blots using antibodies against IKK or HNE-protein conjugates. Addition of dithiothreitol prevents HNE modification of IKK.

Aldehydes are products and propagators of oxidative stress (1). They are reactive electrophiles that form adducts to protein 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/S 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), the major toxic product of lipid peroxidation (1). HNE reacts with sulfhydryl 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-Myc (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, IκB. Dissociation of NF-κB from IκB 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 IκB 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 tetradeacanoylphorbol 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% fetal 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

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‡ 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.

1 The abbreviations used are: HNE, 4-hydroxy-2-nonenal; IKK, IκB kinase; TPA, tetradeacanoylphorbol acetate; IM, ionomycin; DTT, dithiothreitol; KLBD, kinase lysis buffer; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; p-IκBα, phosphorylated IκBα.
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 IAM (Calbiochem, San Diego, CA) was dissolved in MeSO. The final concentration of ethanol or MeSO in the medium was ≤0.1%.

Nuclear and cytoplasmic fractions were separated by centrifugation at 10,000 g for 10 min. The supernatant (cytoplasmic extract) was cleared by further centrifugation at 10,000 × 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), 5 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, 10 mM β-mercaptoethanol, and protease inhibitors). The proteins were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 0.1% Tween 20 and then incubated with anti-IkBα and anti-IKK polyclonal or anti-phospho-specific Ser-32 IKK antibodies (Calbiochem). The primary antibody complex was then stained with horseradish peroxidase-conjugated anti-rabbit or antimouse secondary antibodies (Amersham Pharmacia Biotech). Protein bands were visualized by enhanced chemiluminescence (ECL, Western blotting detection system, Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay—The double-stranded oligonucleotide probes used to assay NF-κB and AP-1/c-Jun binding were 5’-AGTTTCAGGACGGATCCAGGTGGCTTGATGAGCCGG ACAAGAGAATG-3’ and 5’-CCGCAGGGACCGACGT-3’, respectively (Promega, Madison, WI; bold letters indicate the binding site). DNA probes (3.5 pmol) were reacted with [γ-32P]ATP (10 μCi) in the presence of T4 polynucleotide kinase (10 units). Labeled probes were purified on a Sephadex G50 column (Amersham Pharmacia Biotech). Electrophoretic mobility shift assays were conducted with 20,000 cpm of oligonucleotide probe per sample (35 fmol of double-stranded oligonucleotide).

To assess NF-κB binding, nuclear extracts (5–10 μg) were incubated with the labeled DNA probe in the presence of poly(dI-dC) (2 μg), bovine serum albumin (2 μg), and Nonidet P-40 (0.5%) in 20 μL of reaction buffer (10 mM HEPES (pH 7.9), 20 mM KCl, 0.5 mM EDTA, 2.5 mM DTT, and 4% Ficoll) at room temperature for 10 min. Similarly, AP-1 binding was evaluated by incubating nuclear extracts (5–10 μg) in reaction buffer with 1.5 mM MgCl2 and 5 mM DTT in the presence of labeled DNA probe, poly(dI-dC) (2 μg), and bovine serum albumin (2 μg) at room temperature for 10 min. The specificity of binding was examined both by competition with unlabeled oligonucleotide and by supershift assay with respective antibodies. In supershift experiments, antibodies (1–2 μg) directed against NF-κB p50, NF-κB p65, or AP-1/c-Jun (Santa Cruz Biotechnology) were incubated with nuclear extracts for 45 min at 4 °C before addition of labeled probe. In competition experiments, antibodies were replaced with a 50-fold molar excess of unlabeled oligonucleotide.

Binding activity was analyzed by electrophoretic mobility shift assay using a 4–5% polyacrylamide gel and Tris, glycine, EDTA buffer (5 mM Tris (pH 8.4), 9 mM glycine, and 0.2 mM EDTA). Visualization and quantification of radioactive bands were performed using a PhosphorImager (Molecular Dynamics).

Luciferase Assay—Cells were plated in 6-well plates at a density of 4 × 10^5 cells per well 18–24 h prior to transfection. Cells were transfected with 0.5 μg of reporter construct in a pGL2 luciferase-expressing vector (Promega) using LipofectAMINE reagent (Life Technologies, Inc.). The plasmid was constructed with 6× NF-κB binding sites upstream of the SV40 promoter. Thus, the luciferase reporter gene was under NF-κB control. Cells were exposed 18–24 h after transfection to TPA/IM (0.08 μM/2 μM) or TPA/IM plus HNE (10–80 μM) for 6 h and lysed in luciferase reporter lysis buffer (Promega). Total cell lysates (5–10 μg of protein) were determined for luciferase activity by the luciferase assay reagent (Promega) in a Monolight 2010 luminometer (Analytical Luminescence Laboratories).
competition with unlabeled probe and supershift with anti-
AP-1 antibody (Fig. 1B, lanes 9 and 10, respectively). The
supershift in this particular experiment was relatively weak.
Clearly, concentrations of HNE that completely prevent DNA
binding by NF-κB have no effect on DNA binding by AP-1.

**HNE Blocks NF-κB Transactivation in H1299 Cells**—To correlate HNE effects on NF-κB transactivation with DNA binding inhibition, an NF-κB-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 μM HNE providing complete suppression and higher doses decreasing luciferase activity to below unstimulated levels (Fig. 2). Thus, HNE inhibited both NF-κB DNA binding and NF-κB transactivation activity. Parallel experiments with RKO cells produced similar results, as shown in the lower panel of Fig. 2.

**HNE Blocks IκBα Degradation in H1299 Cells and Jurkat T Cells**—NF-κB activation requires degradation of the inhibitory protein, IκBα (26, 27). Consequently, HNE inhibition of NF-κB DNA binding and transactivation activities could result from the inhibition of IκBα degradation. To test this possibility, the effects of TPA/IM stimulation and HNE treatment on IκBα 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 IκBα protein (Fig. 3). For H1299 cells, the reduction in the level of IκBα 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 IκBα 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 IκBα concentrations resulted from an induction of IκBα phosphorylation followed by a degradation of phosphorylated IκBα (p-IκBα, Fig. 3B, lanes 1–4). In contrast, no detectable p-IκBα 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 IκBα concentration (Fig. 3, A and B, lanes 5–8). In addition, HNE pretreatment completely abolished the formation of p-IκBα in Jurkat T cells. Thus, it appears likely that HNE treatment prevents IκBα degradation by inhibition of IκBα phosphorylation.

**HNE Inhibits IKK Activity in Jurkat T Cells**—IKK activity is required for IκBα phosphorylation (28–30). Thus, one possible mechanism to explain the inhibitory effect of HNE on TPA/IM stimulation of NF-κB 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/IM:
IM. Total cell extracts were prepared, and IKK activity was determined using a fusion protein of I\(\kappa\)B and glutathione S-transferase (I\(\kappa\)B-GST) as substrate. Kinase activity was evaluated by incorporation of \(32^P\) into the fusion protein substrate. Incubation of the IKK substrate with TPA/IM-stimulated cell extracts resulted in a time-dependent increase in I\(\kappa\)B phosphorylation (Fig. 4, lanes 1–4). Pretreatment of cells with HNE significantly inhibited the formation of \(32^P\)-labeled I\(\kappa\)B-GST. This suggests that HNE inhibition of NF-\(\kappa\)B activation is due to inhibition of IKK activity.

**HNE Blocks IKK Activity in Vitro**—To clarify whether HNE inhibition of IKK activity occurs by direct interaction with IKK, an in vitro assay for HNE-mediated inhibition of IKK activity was developed. Immune complexes of IKK were precipitated, then incubated with HNE in the presence or absence of DTT for 10 min at 30 °C, and assayed for IKK activity, as shown in Fig. 5. Treatment of immune complexes of IKK with HNE in the absence of DTT caused dose-dependent inhibition of IKK activity (Fig. 5A). Addition of 30 \(\mu\)M HNE resulted in clear inhibition of IKK activity, and 60 \(\mu\)M HNE completely inhibited activity. In fact, the higher dose of HNE lowered IKK activity to below basal levels. When parallel incubations were conducted in which immune complexes were treated with HNE 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 I\(\kappa\)B degradation and NF\(\kappa\)B activation.

**DISCUSSION**

In the present studies, we show that TPA/IM stimulates I\(\kappa\)B phosphorylation and subsequent degradation, resulting in NF-\(\kappa\)B activation. This finding is consistent with previous observations that NF-\(\kappa\)B activation is responsive to a wide range of activators that lead to phosphorylation and degradation of I\(\kappa\)B (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-\(\kappa\)B signaling pathway. HNE prevents I\(\kappa\)B phosphorylation and subsequent degradation, reducing NF-\(\kappa\)B DNA binding activity and NF-\(\kappa\)B transactivation. These results are in good agreement with the findings that HNE modulates NF-\(\kappa\)B activation by inhibiting I\(\kappa\)B phosphorylation and subsequent proteolysis in human monocytic cells (33).

Interestingly, the complete process of I\(\kappa\)B phosphorylation and subsequent degradation following treatment of cells with TPA/IM was only observed in Jurkat T cells. Phosphorylation of I\(\kappa\)B 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-I\(\kappa\)B in H1299 cells. TPA/IM-induced phosphorylation of I\(\kappa\)B 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 I\(\kappa\)B at Tyr-42 and NF-\(\kappa\)B activation without proteasome-mediated degradation of I\(\kappa\)B (34). A second possibility is that activation of NF-\(\kappa\)B in H1299 cells results from phosphorylation-independent I\(\kappa\)B degradation. For example, UV irradiation leads to I\(\kappa\)B degradation without phosphorylation in HeLa cells, 293 cells, and human fibroblasts (35, 36). Finally, the kinetics of I\(\kappa\)B phosphorylation and p-I\(\kappa\)B degradation in H1299 cells may prevent a detectable steady-state concentration of p-I\(\kappa\)B from accumulating.
HNE Inhibition of NF-κB

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 cycloartenone 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 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 inhibits HNE-induced cross-link formation and loss of enzyme activity (42). In our experimental conditions, bothIKKα 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).

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 Δ2, which is a decomposition product of prostaglandin Δ2, 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 Δ2 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 (H, 30 μM) for 30 min were incubated with TP/EM (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 30 or 60 μM HNE or vehicle in the absence of DTT at 30 °C for 10 min. The kinase activities associated with the immunoprecipitated IKK complex were determined using IκBα-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 (IκBα-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 antiserum to IKKα or to HNE-modified protein. The third panel of A and B represents the detection of HNE-modified IKK molecules with an antiserum 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.

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REFERENCES

1. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Free Radic. Biol. Med. 11, 81–128
2. Esterbauer, H. (1993) Am. J. Clin. Nutr. 57, (suppl.) 779–785
3. Marnett, L. J. (1999) Mutat. Res. 424, 83–95
4. Chaudhary, A. K., Nokobe, M., Reddy, G. R., Yeola, S. N., Morrow, J. D., Blair, I. A., and Marnett, L. J. (1994) Science 265, 1580–1582
5. Nath, R. G., and Chung, F.-L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7491–7495
6. Chung, F. L., Nath, R. G., Ocando, J., Nishikawa, A., and Zhang, L. (2000) Am. J. Clin. Nutr. 72, 81–128
7. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Free Radic. Biol. Med. 11, 81–128
8. Esterbauer, H. (1993) Am. J. Clin. Nutr. 57, (suppl.) 779–785
9. Marnett, L. J. (1999) Mutat. Res. 424, 83–95
10. Liu, W., Kato, M., Akhand, A. A., Hayakawa, A., Suzuki, H., Miyata, T., Kurikawa, K., Hotta, Y., Ishikawa, N., and Nakashima, I. (2000) J. Cell Sci. 113, 635–641
11. Faniz, V. M., Barrera, G., Martinotti, S., Farace, M. G., Giglioni, B., Frati, L., Manzari, V., and Dianzani, M. U. (1999) Cancer Res. 60, 1507–1511
12. Ji, C., Rouzer, C. A., Marnett, L. J., and Pietenpol, J. A. (1998) Carcinogenesis 19, 1275–1283
13. Kirichenko, A., Li, L., Morandi, M. T., and Holi, A. (1996) Toxicol. Appl. Pharmacol. 141, 416–424
14. Kruuman, I., Bruce-Keller, A. J., Breeden, D., Waeg, G., and Mattson, M. P. (1997) J. Neurosci. 17, 5089–5100
15. Liu, W., Kato, M., Akhand, A. A., Hayakawa, A., Suzuki, H., Miyata, T., Kurikawa, K., Hotta, Y., Ishikawa, N., and Nakashima, I. (2000) J. Cell Sci. 113, 635–641
16. Faniz, V. M., Barrera, G., Martinotti, S., Farace, M. G., Giglioni, B., Frati, L., Manzari, V., and Dianzani, M. U. (1999) Cancer Res. 60, 1507–1511
17. Ji, C., Rouzer, C. A., Marnett, L. J., and Pietenpol, J. A. (1998) Carcinogenesis 19, 1275–1283
18. Mercurio, F., and Manning, A. M. (1999) J. Biol. Chem. 274, 27339–27342
19. Janssen-Heininger, Y. M., Poynter, M. E., and Baeuerle, P. A. (2000) Science 287, 1295–1304
20. Okumura, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10312–10317
21. Baeuerle, P. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 585–588
22. Yamamoto, Y., Yin, M. J., and Gaynor, R. B. (1998) Cell 95, 875–884
23. Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999) Science 284, 309–313
24. Cordle, S. R., Donald, R., Read, M. A., and Hawiger, J. (1993) J. Biol. Chem. 268, 11803–11810
25. Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baeuerle, P. A. (1993) Nature 365, 182–185
26. Page, S., Fischer, C., Baumgartner, B., Haas, M., Kreusel, U., Loidl, G., Hayn, M., Ziegler-Heitbrock, H. W., Neumeier, D., and Brand, K. (1999) J. Biol. Chem. 274, 11611–11618
27. Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B., Mueller-Dieckmann, C., Farshidfar, D., Rosci, B., Auberger, P., Baeuerle, P. A., and Beynon, J. F. (1996) Cell 86, 787–798
28. Li, N., and Karin, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13012–13017
29. Bender, K., Gottlicher, M., Whiteside, S., Rahmsdorf, H. J., and Herrlich, P. (1998) EMBO J. 17, 5170–5181
30. Delhase, M., Hayakawa, M., Baeuerle, P. A., Zandi, E., and Karin, M. (1999) Nature 398, 548–554
31. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbas, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–866
32. Yamamoto, Y., Gussi, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) Cell 93, 1231–1240
33. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) Nature 395, 297–300
34. Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5357–5362
35. Uchida, K., Shiraiishi, M., Naito, Y., Torii, Y., Nakamura, Y., and Osawa, T. (1997) J. Biol. Chem. 272, 4909–4912
36. Yin, M. J., Yamamoto, Y., and Gaynor, R. B. (1998) Nature 396, 77–80
37. Yamamoto, Y., Yin, M. J., Lin, K. M., and Gaynor, R. B. (1999) J. Biol. Chem. 274, 27307–27314
38. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. G. (2000) Nature 403, 103–108
39. Friguet, B., Stadtman, E. R., and Szweda, L. I. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 21639–21643
40. Castrillo, A., Diaz-Guerra, M. J., Hortaleno, S., Martin-Sanz, P., and Bosca, I. (2000) Mol. Cell. Biol. 20, 1692–1698
41. Li, L., Hamilton, R. F., Jr., and Holi, A. (1999) Am. J. Physiol. 277, L550–L557