Extensively oxidized low density lipoprotein (ox-LDL), a modulator of atherogenesis, down-regulates the lipopolysaccharide (LPS)-induced activation of the transcription factor NF-κB. We investigated whether 4-hydroxynonenal (HNE), a prominent aldehyde component of ox-LDL, represents one of the inhibitory substances. NF-κB activation by stimuli such as LPS, interleukin (IL)-1β, and phorbol ester, but not tumor necrosis factor (TNF), was reversibly inhibited by HNE in a dose-dependent manner in human monocytic cells, whereas AP-1 binding was unaffected. Using similar HNE concentrations, LPS-induced IκB and TNF or IL-8 promoter-dependent transcription was prevented. Furthermore, pretreatment with HNE suppressed TNF production but not lactate dehydrogenase levels. Under these conditions the binding of LPS to monocytic cells was not significantly affected. However, induced proteolysis of the inhibitory proteins IκB-α, IκB-β, and, at a later time point, IκB-ε was prevented. This is not due to inhibition of the proteasome, the major proteolytic activities of which remain unaffected, but rather to a specific prevention of the activation-dependent phosphorylation of IκB-α. This is the first report which demonstrates that HNE specifically inhibits the NF-κB/Rel system. Down-modulation of NF-κB-regulated gene expression may contribute at certain stages of atherosclerosis to low levels of chronic inflammation and may also be involved in other inflammatory/degenerative diseases.

The chronic inflammatory-fibroproliferative process of atherosclerosis is triggered or modulated by various substances accumulating in the damaged vessel wall, which have been shown to exert specific effects on transcriptional systems or signal transduction cascades (1). One of these substances is oxidized low density lipoprotein (ox-LDL) that has been found to display both positive and negative effects on gene expression (2–4). Ox-LDL represents a complex mixture of various components including lipid hydroperoxides, oxysterols, lysophosphatidylcholine, and aldehydes such as 4-hydroxynonenal (HNE) (5, 6). The specific substances within the ox-LDL molecule that are responsible for its effects on signaling/transcriptional regulation and the mechanisms involved are only partially known.

The pleiotropic transcription factor NF-κB has been suggested to play an important role in gene regulation during inflammatory and immune reactions in the atherosclerotic lesion (3, 7–10). NF-κB exists as a dimeric complex that is present in the cytosol in an inactive state bound to inhibitory proteins, collectively termed IκB (11–13). Several IκB proteins have been identified, including IκB-α (10, 14) and the more recently cloned IκB-β (15) and -ε (16). A variety of agents including inflammatory or lymphoproliferative cytokines and microbial pathogens (12, 17) induce the activation of NF-κB which is presumably facilitated by a network of kinases, some of which have been recently cloned, including IκB kinase (IKK)-α and -β (18–22), NF-κB-inducing kinase (23), and mitogen-activated protein kinase/ERK kinase kinase-1 (24). Activation of these kinases leads to the phosphorylation of IκB which is subsequently degraded in a ubiquitin-dependent step by the proteasome, a multicatalytic high molecular weight protease system (14, 25–27). The removal of the inhibitor IκB allows the translocation of the thus activated NF-κB dimer into the nucleus. NF-κB regulatory sequences have been found in promoters or enhancers of a variety of genes, e.g. coding for the proinflammatory cytokines tumor necrosis factor (TNF) and interleukin (IL)-1, chemokines such as IL-8 and monocyte chemotactic protein-1 (MCP-1), and diverse adhesion as well as immunoregulatory molecules (11–13).

Several laboratories, including ours, have shown that ox-LDL inhibits the LPS-induced activation of NF-κB (4, 28, 29) and the expression of the NF-κB target gene products TNF, IL-1α/β, and MCP-1 in monocyte/macrophages (30–32). One report describes the impairment of NF-κB activation by ox-LDL in vascular smooth muscle cells (33). The suppressive activity of ox-LDL on IL-1 and TNF expression was acquired only after extensive oxidation and was localized in the extract; HNE, 4-hydroxynonenal; IKK, IκB kinase; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; OA, okadaic acid; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; PSI, Z-Ile-Glu(OtBu)-Ala-Leu-CHO.
able lipid component (29–31, 33). HNE is one of the most prominent aldehyde substances generated during extensive lipid peroxidation of ox-LDL (5, 34, 35). Furthermore, HNE-modified lysine residues have been identified in the atherosclerotic lesion (36), and a variety of HNE effects in different cell types have been described (37–40). The aim of this study was to investigate whether HNE is able to modulate the activation of the NF-κB system by different stimuli or alter the expression of the NF-κB target gene product TNF. Furthermore, we examined to what extent this aldehyde affects B-cell as well as TNF and IL-8 promoter-dependent transcriptional activity. We also characterized the effects of HNE on the stimulus-induced proteolysis of IκB-α, -β, and -ε, on the three major peptidase activities of the proteasome, as well as on the phosphorylation status of IκB-α, in order to obtain more insight into the mechanisms involved.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Reagents—THP-1 monocytic cells (DSM, Braunschweig, Germany) were maintained in suspension in RPMI 1640 (Glutamax-1, low endotoxin) containing 7% fetal calf serum (FCS) (HyClone, Logan, UT), 2 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Eggenstein, Germany) as described (41). For the experiments, the cells were plated at a density of 3 × 10^6 per well in 6-well culture dishes. Peripheral blood mononuclear cells were isolated from blood samples of normal donors by the Ficoll-Paque method as described (42). Monocytes were isolated from mononuclear cells by adherence to achieve a purity of approximately 90% as determined by flow cytometry. The adherent monocytes were cultured overnight in the same medium as THP-1 cells with 10% FCS before the experiment was started. Mono Mac 6 cells (43) were maintained in 24-well plates at a density of 2 × 10^5 per ml in RPMI containing 2 mM L-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 1× nonessential amino acids (all from Life Technologies, Inc.), 1% OPI supplement containing oxalacetic acid, sodium pyruvate, and insulin (Sigma, Deisenhofen, Germany) and 10% FCS (Myosline). 4-Hydroxynonenal was synthesized at the Institute of Biochemistry, University of Graz, Austria (5, 34), and supplied to our laboratory for use in the present study. LPS (Escherichia coli 0111:B4), FTTC-IL-1β, TNF-α, phorbol 12-myristate 13-acetate (PMA), and okadaic acid (OA) were purchased from Sigma. The proteasome inhibitor PSI was purchased from Calbiochem (San Diego, California). Endotoxin-immune complex fractions (Triton X-100 were added at 1 μg/ml) and only reagents with an endotoxin content ≤ 10 pg/ml were used in the dye exclusion. A potential toxicity of the cell culture conditions applied was monitored by cell morphology and count, trypan blue dye exclusion, and the WST-1 cell toxicity test (Boehringer Mannheim, Germany). The activities (units/liter) of LDH in cytosolic extracts was measured in an automated colorimetric analysis system (Boehringer Mannheim/Hitachi 737) at 37°C.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Cytosolic extracts were isolated as described earlier (45). Electrophoresis was performed with 12.5% polyacrylamide gels (0.1% SDS) as described previously (41). The proteins were transferred to a nitrocellulose membrane using the wet blotting technique. After transfer, the nitrocellulose membranes were incubated with polyclonal antibodies against the carboxyl-terminal domains of the inhibitors IκB-α, –β (Santa Cruz Biotechnology, Heidelberg, Germany), and –ε (a kind gift from Prof. N. Rice, National Cancer Institute, Bethesda, Bethesda, Maryland) or with a monoclonal antibody against -actin (Sigma). To detect IκB phosphorylation we used a polyclonal phospho-specific anti-IκB-α antibody (Calbiochem) which detects IκB-α only when activated by phosphorylation at Ser-32. This incubation was followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany). Antibody binding to IκB proteins was visualized on x-ray film using the Western blot Chemiluminescent Reagent Plus (NEN Life Science Products). The protein size was confirmed by molecular weight standards (Amersham Pharmacia Biotech, Braunschweig, and Bio-Rad, Munich, Germany).

Pro tease Assay—Purified yeast 20 S proteasomes (kindly provided by Prof. R. Huber, Max-Planck-Institute of Biochemistry, Martinsried, Germany) (46) were incubated with either HNE or N-acetyl-Leu-Leu-nor leucinal in 20 mM Tris/His pH 7.4, for 1 h at 37°C. The assay was started by addition of fluorogenic substrates (Bachem, Heidelberg, Germany; dissolved in Tris buffer, 1% MeSO) for chymotrypsin-like (Suc- Leu-Leu-Val-Tyr-AMC, 8 μM), trypsine-like (Bz-Phe-Val-Arg-AMC, 8 μM), or peptideglutaminylpeptide hydrolyzing (Cbz-Leu-Leu-GLu-bNA, 40 μM) activities, and conducted at 37°C (where AMC is 1-amido-4- coumotoxybenzene; bNA is B-naphthylamide; Suc is succinyl; Bz is benzoyl; Cbz is benzyloxycarbonyl). The rates of substrate hydrolysis were determined using a luminescence spectrophotometer (Perkin-Elmer, Weiterstadt, Germany).

LPS Binding and Flow Cytometry—Mono Mac 6 cells, grown in LPS-negative medium as described, were incubated with or without HNE at 10^6 cells per ml for 1 h at 37°C. After washing, the cells were resuspended in FBS (25 μl) and with and without LPS at 10 μg/ml and 5 min later FACS-LPS was added at 1 μg/ml. After overnight incubation at 4°C, cells were washed, and 10^5 cells per sample were analyzed using an EPICS Version 753 flow cytometer (Coulter Electronics, Krefeld, Germany). The percentage of positive cells was determined by subtraction of the histogram for the LPS-competent sample from the histogram of the noncompetent sample. Specific mean fluorescence intensity is the delta mean of the competent and uncompetent histogram in channels.

Kinase Assays—Kinase assays were performed essentially as described (47). Cytosolic extracts were subjected to immunoprecipitation in TNNF buffer (200 mM NaCl, 20 mM Tris-Cl, pH 7.5, 1% Triton X-100, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, antipain, aprotinin, pepstatin A, chymostatin 0.75 μg/ml each), the kinase reaction was carried out in kinase buffer for 30 min at room temperature in 20 μl of binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, 1 mg/ml bovine serum albumin, 0.2% Nonidet P-40, 50 μg of poly(dI-dC)/μl). Samples were run in 0.25 x TBE buffer (10 x TBE is as follows: 890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0) on nondenaturing 4% polyacrylamide gels. The binding of Sp-1 and AP-1 was also analyzed by EMSA using specific consensus oligonucleotides (Promega, Heidelberg, Germany) that were labeled with [γ-32P]ATP (>5,000 Ci/mmol, NEN Life Science Products) and T4 polynucleotide kinase (Boehringer Mannheim). Gels were dried and analyzed by autoradiography.

Transfection of THP-1 Cells—The following reagents and plasmids were used for transfections: 5 μg of the luciferase reporter plasmid containing 3 copies of a prototypical (-GGGACCTTTCC-)3)kB site (45); TNF-kp, luci, comprising 1108 base pairs of the TNF promoter region (45); pGL2-IL-8, containing 420 base pairs of the IL-8 promoter region (4) or pGL2basic (Promega) lacking any insert. These individual plasmids were transiently co-transfected with a constitutively active Renilla luciferase control plasmid (pRLSK, Promega) into THP-1 cells using a DEAE-dextran-based protocol (4, 44). After transfection, cells were plated out at a density of 2 × 10^7/ml RPMI with 7% FCS in a 6-well plate and incubated for 2 days. After this time, the cells were first preincubated with HNE for 1 h followed by a 5-h LPS stimulation. Subsequent to stimulation the cells were lysed and the luciferase activity was determined using the Luciferase Assay system (Promega). The results are expressed as firefly luciferase relative light units (RLU) divided by the RLU values obtained for the Renilla luciferase.

Determination of TNF and LDH—The concentration of TNF protein in the medium taken from experimental cultures was measured by sandwich immunoassay according to the manufacturer’s instructions (Coulter Immunotech, Hamburg, Germany). The activity (units/liter) of LDH in cytosolic extracts was measured in an automated colorimetric analysis system (Boehringer Mannheim/Hitachi 737) at 37°C.

4-Hydroxynonenal Inhibits NF-κB

Transfection of THP-1 cells was performed by cotransfection with pGL2baic, a plasmid carrying a 420-base pair DNA fragment containing oxalacetic acid, sodium pyruvate, and insulin (Sigma, Deisenhofer, Germany) and 10% FCS (Myoclone). 4-Hydroxynonenal was synthesized at the Institute of Biochemistry, University of Graz, Austria (5, 34), and supplied to our laboratory for use in the present study. LPS (Escherichia coli 0111:B4), FTTC-IL-1β, TNF-α, phorbol 12-myristate 13-acetate (PMA), and okadaic acid (OA) were purchased from Sigma. The proteasome inhibitor PSI was purchased from Calbiochem (San Diego, California). Endotoxin-immune complex fractions (Triton X-100 were added at 1 μg/ml) and only reagents with an endotoxin content ≤ 10 pg/ml were used in the dye exclusion. A potential toxicity of the cell culture conditions applied was monitored by cell morphology and count, trypan blue dye exclusion, and the WST-1 cell toxicity test (Boehringer Mannheim, Germany). The activities (units/liter) of LDH in cytosolic extracts was measured in an automated colorimetric analysis system (Boehringer Mannheim/Hitachi 737) at 37°C.
NF-κB binding was observed. EMSA was carried out, and the Sp-1 binding, as shown.

Com then activated with various stimuli as follows: LPS (1 μg/ml), BO (10 pg/ml) for 1 h, PMA (50 ng/ml) for 4 and 12 h, and TNF (1.6 ng/ml). The level of phosphorylated IκB was determined by EMSA. In these primary monocytes the LPS-induced NF-κB activation was also inhibited by HNE in a dose-dependent manner (Fig. 1B). In addition, experiments were performed with monocytic Mono Mac 6 cells, which were also incubated with or without HNE followed by a 1-h LPS stimulus (100 ng/ml). EMSA showed an HNE dose-dependent inhibition of LPS-induced NF-κB activation (50 μM HNE, inhibition below base line; 25 μM HNE, 62% inhibition).

We then tested whether HNE (25 μM) modulates the activation of NF-κB by other stimuli such as IL-1β, PMA, and TNF. Our experiments demonstrated a marked inhibition of NF-κB activation induced by a 1-h incubation with 10 pg/ml IL-1β (Fig. 1C). Furthermore, pretreatment with HNE also significantly affected both short and long term incubation with PMA (50 ng/ml). In contrast, the activation of NF-κB by treatment with TNF (0.5 to 4.0 ng/ml) for 0.5 or 1 h was not affected by 25 μM HNE (Fig. 1C, and data not shown), which suggests selective and differential inhibitory actions of HNE.

A potential toxic effect of HNE on monocytic cells was monitored by cell morphology and count, trypan blue dye exclusion, and the WST-1 cell toxicity test. HNE was not found to be toxic at the concentrations and conditions applied in our study (50 and 25 μM HNE, no decrease in metabolic activity after 24 h incubation compared with the untreated control using the WST-1 test).

**HNE Inhibits Temporarily and Is Active When Added Subsequent to the Stimulus**—The next experiments tested whether the inhibition of NF-κB by pretreatment with HNE is reversible. THP-1 cells were pretreated with HNE (25 μM), washed intensively, and transferred to a new culture dish. At different time points LPS was added for a 1-h incubation period. When LPS was added immediately or 2 h after removal of HNE, a significant inhibition of NF-κB was detected (Fig. 2A). However, when cells were activated with LPS 4 h after HNE removal, a reduction of the inhibitory effect was already observed. The LPS-induced stimulation of NF-κB at 24 h after the HNE treatment was no longer impaired, which indicates that the effect of HNE is temporary and reversible. It should be mentioned that the observation that addition of LPS does not activate NF-κB even 2 h after HNE is removed also demonstrates that the effect of this aldehyde is not due to an interaction with the stimulatory molecule in the supernatant.

Furthermore, a different incubation condition was used, i.e. HNE was added together with or varying times (10 min steps) after the addition of LPS. These experiments showed that HNE (25 μM) was still effective when given to the cells as late as approximately 20 min after LPS (Fig. 2B).

**κB as Well as TNF and IL-8 Promoter-dependent Transcription Is Prevented by HNE**—In order to examine whether HNE selectively prevents κB-dependent transcription in our system, THP-1 cells were transiently transfected with a luciferase reporter construct bearing three copies of a prototypic κB site (45). Incubation with LPS dramatically induced the transcriptional activity of this construct (Fig. 3A). This effect was strongly inhibited by preincubation with HNE. In contrast,
HNE showed no effect on the expression plasmid pRLtk (25 μg, no inhibition of transcriptional activity). A similar approach was used to test if HNE affects the transcription of both the TNF and IL-8 gene which contain regulatory κB sites in their respective promoters (12, 48). Preincubation with HNE inhibited the transcriptional activity of both the TNF and IL-8 promoter construct in a dose-dependent manner with a marked inhibition with 25 μg (Fig. 3, B and C).

**HNE Suppresses the Production of TNF Protein**—Next we investigated whether the inhibition of transcriptional activity by HNE has a corresponding impact on protein production. For this purpose we monitored the accumulation of TNF in the supernatant. Incubation of cells with LPS induced a dramatic increase in the levels of TNF protein which was inhibited in a dose-dependent manner by HNE with a marked effect at 25 and 50 μg and a partial inhibition at 12.5 μg (Fig. 4A). This was accompanied by an inhibition of LPS-induced TNF mRNA expression, as demonstrated by Northern blotting (data not shown). A similar inhibition of TNF production by HNE was observed ex vivo in isolated human adherent monocytes (Fig. 4B). The level of the constitutively expressed intracellular protein LDH was unaffected by the HNE treatment used in this study (25 and 50 μg, no inhibition).

**Prevention of IκB-α, -β, and -ε Proteolysis by HNE Is Stimulus-dependent**—In the following stage of the study we investigated whether HNE could affect the activation-induced proteolysis of the IκB inhibitor proteins which trap the NF-κB dimer in the cytosol (10, 14). Incubation of THP-1 cells with LPS over 1 h led to a significant proteolysis of IκB-α (Fig. 5A). HNE (25 μg) when added −1 h relative to LPS significantly prevented the LPS-stimulated proteolysis of IκB-α. A similar effect of HNE on LPS-induced IκB-α proteolysis was observed in Mono Mac 6 cells (data not shown). Pretreatment with the proteasome inhibitor PSI also inhibited IκB-α degradation (Fig. 5A) as observed earlier (45). However, addition of PSI resulted in an accumulation of the phosphorylated form of IκB-α, an effect not seen with HNE. Consistent with the data observed with EMSA, the TNF-induced IκB-α proteolysis was not impaired by 25 μg HNE, neither after a 30-min (data not shown) nor after a 1-h incubation interval with this cytokine (Fig. 5B). Stimulus-induced proteolysis of IκB-β (15) was also inhibited by HNE as shown by a dose-response experiment (Fig. 5C). Furthermore, we investigated the fate of the recently cloned IκB-ε (16) fol-

**FIG. 2. Effects of different HNE treatment on NF-κB activation.** A, the inhibitory effect of HNE on NF-κB activation is reversible. THP-1 cells were treated with HNE (25 μg) for 1 h, extensively washed, and stimulated with LPS (1 μg/ml) at the times indicated. EMSA was carried out, and the brackets indicate NF-κB or Sp-1, respectively. B, HNE remains effective when added subsequent to LPS stimulation. THP-1 cells were stimulated with LPS (1 μg/ml) and HNE (25 μg) was added at the times indicated relative to LPS stimulation. EMSA revealed the NF-κB binding activity of the nuclear extracts (bracket). Com, competition experiments were performed as described for Fig. 1A.

**FIG. 3. HNE inhibits κB- as well as TNF and IL-8 promoter-dependent transcription.** THP-1 cells were co-transfected with 4 μg of the appropriate reporter plasmid and 0.2 μg of the constitutively expressed pRLtk Renilla plasmid, left for 2 days, and then incubated with HNE for 1 h followed by LPS stimulation for 5 h. The results are presented as firefly luciferase relative light units (RLU) divided by the respective relative light unit values obtained for the Renilla luciferase. A, the effect of HNE on the LPS (1 μg/ml)-induced activation of the prototypic κB-driven reporter plasmid 3×κB. luc and the control plasmid pGL2 was monitored. B, TNF promoter construct (TNFkop.luci)-transfected cells were incubated with increasing concentrations of HNE and stimulated with 1 μg/ml LPS. The results are presented as percent sample values of the LPS control (100%). C, an IL-8 promoter-driven luciferase reporter plasmid (pGL2-IL-8) was transfected into the cells, which were subsequently treated with HNE as above followed by LPS (1 μg/ml or 0.1 μg/ml). Co, control.
Fig. 4. TNF protein production is suppressed by HNE. THP-1 cells (A) or human adherent monocytes (B) were treated for 1 h with the indicated concentration of HNE and subsequently stimulated for 5 h with LPS (1 µg/ml, THP-1 cells; 100 ng/ml, adherent monocyes). The culture supernatants were analyzed for TNF content in an ELISA. The data (mean ± S.D., n = 3) are shown as percent of TNF protein production in the samples in comparison to the control (LPS) value. The actual level of TNF measured in LPS-stimulated control supernatants varied from 90 to 300 pg/ml for THP-1 cells and 2 to 12.45 ng/ml for adherent monocytes.

following treatment with LPS in the absence/presence of HNE. In contrast to IκB-α or -β, the LPS-induced degradation of IκB-ε in THP-1 cells occurred at a later time point, i.e. 90 and 120 min (Fig. 5D). This decrease of IκB-ε was also impaired by 25 µM HNE.

Proteolytic Activities of the Proteasome Are Not Affected by HNE—Inhibition of IκB proteolysis could be due to prevention of proteasome peptidase activities (25). Therefore, we examined the effects of HNE on the three major catalytic activities of this protease complex. 20 S proteasome particles were preincubated with 100 µM HNE for 1 h. This was followed by addition of fluorogenic substrates for chymotrypsin-like, trypsin-like, or peptidylglutamyl peptide hydrolyzing activity. Table I demonstrates that none of these activities were significantly impaired by pretreatment with HNE, whereas the chymotrypsin-like activity was significantly inhibited by the proteasome inhibitor N-Ac-Leu-Leu-norleucinal. It should be mentioned that the chymotrypsin-like activity has been previously identified as mainly responsible for IκB degradation (25). In a different approach we pretreated the fluorogenic substrates with HNE before they were added to the assay, which also did not affect the proteasomal functions tested (data not shown).

LPS Binding in the Presence of HNE—In these experiments we wanted to determine whether pretreatment with HNE affects the binding of LPS to monocyte cells. Unfortunately, with the sensitivity of available methods, non-differentiated THP-1 cells do not show detectable LPS binding (49), and this was confirmed in our studies using E. coli LPS-FTTC (data not shown). In order to study the effects of HNE on LPS binding, we therefore turned to the more mature cell line Mono Mac 6 (43). In a representative series of experiments, LPS binding was detected on 25.7 ± 3.0% of these cells with an average intensity of 19.1 ± 3.0 channels (n = 3). Preincubation with even the highest concentration of HNE used in this study (50 µM) resulted only in a moderate reduction of LPS binding to 21.8 ± 5.1% (n = 3; 15% inhibition) and 15.8 ± 4.4 channels (17% inhibition).

HNE Specifically Blocks Phosphorylation of IκB—Activation of NF-κB occurs via phosphorylation of IκB-α at the serines at position 32 and 36 (50). This has been shown to enable conjugation with ubiquitin followed by proteasome-mediated degradation of IκB, resulting in the release of active NF-κB (10, 25). To determine IκB phosphorylation, we used a phospho-specific anti-IκB-α-antibody that detects IκB-α only when activated by phosphorylation at Ser-32. In the first set of experiments we examined the phosphorylation status of IκB-α over time following LPS stimulation. In unstimulated cells no signal was detected (Fig. 6A). Activation of cells with LPS led to an increase of phosphorylated IκB-α reaching a plateau between 30 and 40 min. This effect was markedly reduced in HNE (25 µM)-pretreated cells (Fig. 6B). An opposite result was observed in the presence of the proteasome inhibitor PSI, where a dramatic

Fig. 5. Degradation of IκB-α, -β, and -ε is selectively prevented by HNE. A, THP-1 cells were incubated with or without HNE (25 µM) for 1 h and then stimulated for different times with LPS (1 µg/ml). Cytosolic extracts were analyzed by Western blot analysis. IκB-α is indicated by the arrow. The asterisk depicts a 1-h incubation with the proteasome inhibitor PSI (10 µM), followed by LPS (1 h). The accumulation of the phosphorylated form of IκB-α in the presence of PSI is shown by the double arrow. B, cells were incubated as in A but with a 1-h stimulation with either LPS (1 µg/ml) or TNF (1.6 ng/ml). IκB-α is shown by the arrow. C, cells were pretreated with increasing concentrations of HNE followed by LPS stimulation. The level of IκB-β (arrow) was monitored by Western blot analysis. D, THP-1 cells were incubated in the presence/absence of HNE (25 µM) and then activated with LPS for different time intervals. IκB-ε is depicted by the arrow.
20 S proteasomal particles were incubated with 100 μM HNE or 10 μM N-Ac-Leu-Leu-norleucinal (chymotrypsin-like activity) and 100 μM N-Ac-Leu-Leu-norleucinal (trypsin-like and PGPH activity) for 1 h, followed by addition of the fluorogenic substrates. The rate of substrate hydrolysis in the absence of HNE or ALLN, respectively, was defined as the 100% control value, and data are presented as mean ± S.D. (n = 2). The abbreviations are explained under “Experimental Procedures.”

| Proteasomal activity | Enzyme | Substrate | Substrate hydrolysis |
|----------------------|--------|-----------|----------------------|
| Chymotrypsin-like    | nM     | Succ-Leu-Leu-Val-Tyr-AMC, 8 μM | 102 ± 11 |
| Trypsin-like         | 5      | Bz-Phe-Val-Arg-AMC, 8 μM | 101 ± 6 |
| PGPII                | 5      | Chz-Leu-Leu-Glu-βNA, 40 μM | 96 ± 8 |

**Fig. 6. IκB-α phosphorylation is inhibited by HNE.** A, the LPS-induced phosphorylation of IκB-α was investigated using an antibody that detects IκB-α only when activated by phosphorylation at Ser-32 (arrow). THP-1 cells were stimulated for varying times with LPS (1 μg/ml), and cytosolic extracts were analyzed by Western blot. The asterisk marks a 1-h incubation with the proteasome inhibitor PSI (10 μM), followed by LPS. B, HNE (25 μM) was added to the cells 1 h prior to LPS stimulation for 30 or 40 min. The cytosolic extracts were analyzed as in A. In the same extracts the level of α-actin was investigated (arrow). C, cells were pretreated with HNE as described in B before they were stimulated with TNF (1.6 ng/ml) for 5 min. The cytosolic extracts were examined as described above.

**TABLE I**

Effect of HNE and ALLN on proteolytic activities of the proteasome

| Proteasomal activity | Enzyme Substrate | Substrate hydrolysis |
|----------------------|------------------|----------------------|
| Chymotrypsin-like    | nM Succ-Leu-Leu-Val-Tyr-AMC, 8 μM | 102 ± 11 |
| Trypsin-like         | 5 Bz-Phe-Val-Arg-AMC, 8 μM | 101 ± 6 |
| PGPII                | 5 Chz-Leu-Leu-Glu-βNA, 40 μM | 96 ± 8 |

**DISCUSSION**

The presented report demonstrates that HNE, a prominent aldehyde component of atherogenic ox-LDL (2, 3, 34, 35), specifically and reversibly inhibits the activation of NF-κB and κB-dependent transcription in a dose range expected to be found in extensively oxidized forms of LDL (5). Furthermore, TNF and IL-8 promoter-dependent transcription as well as the production of TNF mRNA and protein was dose-dependently reduced in the presence of HNE which implies that the effect of HNE on the NF-κB system is indeed associated with functional consequences (12, 48). The inhibitory actions of HNE parallel those of ox-LDL on NF-κB and target gene expression, including TNF, reported previously in cells of the monocytic lineage (4, 28–32).

HNE inhibited the activation of NF-κB by a variety of stimuli, i.e. LPS, IL-1β, and PMA. To further elucidate how HNE modulates cellular function, we used LPS as a stimulus in most of the experiments. This mediator is a potent activator of monocytic cells (48) and has been used earlier as a tool to study the effects of ox-LDL on monocyte/macrophage gene expression (4, 28–32). More importantly, certain forms of LPS may have some pathophysiological relevance in atherogenesis since LPS from Gram-negative *Chlamydia pneumoniae* has recently been shown to be present in the atherosclerotic lesion (53, 54). Furthermore, it has been suggested that infection of cells with *C. pneumoniae* modulates gene expression in this environment (53, 55). Since to our knowledge it is not possible to get a large amount of *C. pneumoniae* LPS, we used the commercially available *E. coli* LPS. We are aware that there are differences between both forms of LPS (56). However, it should be mentioned that LPS from a different strain of *Chlamydia, i.e. trachomatis*, has been shown to significantly activate NF-κB (56).
If one assumes that some forms of \( C. pneumoniae \) LPS or other substances such as IL-1 (1) activate NF-\( \kappa \)B and related target cytokine expression in the atherosclerotic lesion, what is then the pathophysiological significance of the suppressive effect of HNE on monocyte/macrophage inflammatory gene expression? The following speculative scenario could be envisaged. An antigen (e.g., \( C. pneumoniae \))-induced production of cytokine mediators may be required to allow the coordinated orchestration of immune cell behavior between monocyte/macrophages and T cells in the atherosclerotic lesion (57) with the aim of removing \( C. pneumoniae \) or any other foreign material and finally resolving inflammation. This cytokine fine-tuned monocyte/macrophage/T cell interaction may be impaired by ox-LDL-related compounds such as HNE, allowing the development of a state of chronic, low level inflammation, which is a characteristic feature of the atherosclerotic lesion (1–5).

Our data show that HNE prevented the degradation of the cytosolic NF-\( \kappa \)B inhibitor proteins IxB-\( \alpha \) (10) and -\( \beta \) (15) following 1 h of LPS stimulation. It is of note that ox-LDL is also capable of inhibiting LPS-induced IxB-\( \alpha \) degradation, as shown earlier by our laboratory (4). Surprisingly, a significant degradation of the recently cloned IxB-\( e \) (16) was only observed after a longer LPS incubation period of 90 and 120 min in THP-1 cells in the present study. This LPS-induced slow decrease was also impaired by pretreatment with HNE. It should be mentioned that the regulation of IxB-\( e \) is only partially understood in monocyte cells and requires further investigation. IxB proteolysis is mediated by the proteasome, and the chymotrypsin-like proteasome activity has been demonstrated to be most important for IxB degradation (25). Therefore, we tested the effect of HNE on the three major peptidase activities of this multicatalytic enzyme complex (26, 27). Our experiments demonstrated that preincubation with HNE did not modulate these activities. Stimulus-induced degradation of IxB by the proteasome requires the phosphorylation of these inhibitor proteins at specific residues (50). For example IxB-\( \alpha \) is phosphorylated following activation at the amino-terminal Ser-32 and Ser-36 (50). This was the reasoning behind our examination of whether HNE interferes with the phosphorylation of IxB. Indeed, using a phospho-specific antibody we were able to demonstrate that HNE specifically prevented the LPS-induced phosphorylation of IxB-\( \alpha \). At this point it should be mentioned that our binding studies showed that even at the highest concentration of HNE only a marginal reduction in LPS binding was achieved. This indicates that the profound effect of HNE on NF-\( \kappa \)B is not due to an interference with the LPS-binding properties of the cells. Hence, HNE appears to inhibit IxB phosphorylation at a signaling stage located downstream from the LPS receptor level.

Various stimuli activate the NF-\( \kappa \)B-system by engaging different receptor/signaling pathways (58, 59). This involves the independent/simultaneous activation of a network of kinases constituting several hierarchical modules that ultimately leads to the phosphorylation of IxB (10, 50). In our study, HNE was able to inhibit the activation of NF-\( \kappa \)B by LPS, IL-1\( \beta \), and PMA but not by TNF, which indicates that the signaling pathways induced by the tested stimuli exhibit different sensitivity to HNE. Interestingly, TNF, as well as okadaic acid given \textit{in vitro}, still activated phosphorylation/proteolysis of IxB-\( \alpha \) in the presence of HNE. Furthermore, kinase assays showed that HNE was correspondingly unable to inhibit LPS-induced IKB-\( \beta \) activity when added \textit{in vitro} directly to the kinase assay step, but when cells were treated with HNE prior to the assay, the kinase activity was reduced. This could mean that the signalosome (IKK-\( \alpha \), IKK-\( \beta \), NF-\( \kappa \)B-inducing kinase, additional proteins), suggested to represent the bona fide IxB kinase (18–23), may be not directly impaired by this aldehyde but rather a more upstream receptor proximal step. Several molecules are involved in NF-\( \kappa \)B activation, such as TNF receptor-associated factor 6 (60) or mitogen-activated ribosomal S6 protein kinase (p90\( \text{rsk} \)) (61), which presumably are not engaged by TNF but may participate in signaling by other stimuli. For example, dominant-negative p90\( \text{rsk} \) has been shown to interfere with phorbol ester-induced, but not with TNF-induced, degradation of IxB-\( \alpha \) \textit{in vitro} (61). In this context, it is also interesting to cite recent work which suggests that LPS specifically induces an IL-1 receptor-like NF-\( \kappa \)B signaling cascade (62). On the other hand, it should be mentioned that TNF activates several (kinase) pathways such as TNF receptor-associated factor 2-, mitogen-activated protein kinase (p90\( \text{rsk} \)), and ERK kinase kinase-1, or spinogymelinase-associated cascades (24, 58, 59, 63) which may not be activated by the other stimuli and not be affected by HNE. These various pathways offer potential points of divergence in the signaling events following LPS/IL-1\( \beta \)/PMA or TNF stimulation and thereby the possibility of a differential effect of HNE.

In conclusion, our data suggest that the aldehyde HNE is one of the active components in ox-LDL responsible for the inhibitory capacities of this lipoprotein on the NF-\( \kappa \)B system. Treatment with HNE appears to block selectively signaling events that are required for IxB phosphorylation, thereby preventing NF-\( \kappa \)B activation. The inhibition of NF-\( \kappa \)B-regulated gene expression may contribute at certain stages of atherosclerosis to the low level of chronic inflammation. Besides atherosclerosis, HNE is potentially involved in other degenerative diseases such as liver cirrhosis (64) or neurodegenerative processes including Alzheimer’s (65, 66) and Parkinson’s disease (67). Therefore, the inhibition of NF-\( \kappa \)B/Rel and regulated gene expression may be a process relevant to a broader field of chronic inflammatory/degenerative disease.

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