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Pharmacology

Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-κB

(multicatalytic proteinase complex/calpain/transcription factors/NO synthase induction/proteinase inhibitors)

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ABSTRACT **The objective of this study was to elucidate the role of the proteasome pathway or multicatalytic proteinase complex in the induction of immunologic nitric oxide (NO) synthase (iNOS) in rat alveolar macrophages activated by lipopolysaccharide. Macrophages were incubated in the presence of lipopolysaccharide plus test agent for up to 24 hr. Culture media were analyzed for accumulation of stable oxidation products of NO (NO_2^- + NO_3^-, designated as NO_X^-), cellular RNA was extracted for determination of iNOS mRNA levels by Northern blot analysis, and nuclear extracts were prepared for determination of NF-κB by electrophoretic mobility-shift assay. Inhibitors of calpain (α-*N*-acetyl-Leu-Leu-norleucinal; *N*-benzyloxycarbonyl-Leu-leucinal) and the proteasome (*N*-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-Bu)-Ala-leucinal) markedly inhibited or abolished the induction of iNOS in macrophages. The proteinase inhibitors interfered with lipopolysaccharide-induced NO_X^- production by macrophages, and this effect was accompanied by comparable interference with the appearance of both iNOS mRNA and NF-κB. Calpain inhibitors elicited effects at concentrations of 1–100 μM, whereas the proteasome inhibitor was 1000-fold more potent, producing significant inhibitory effects at 1 nM. The present findings indicate that the proteasome pathway is essential for lipopolysaccharide-induced expression of the iNOS gene in rat alveolar macrophages. Furthermore, the data support the view that the proteasome pathway is directly involved in promoting the activation of NF-κB and that the induction of iNOS by lipopolysaccharide involves the transcriptional action of NF-κB.**

The inducible immunologic isoform of NO synthase (iNOS) is responsible for the high-output production of NO by numerous cell types after exposure to lipopolysaccharide (LPS) and/or certain cytokines (1). Sufficient experimental evidence exists to warrant the working hypothesis that NF-κB is a major transcription factor involved in the inducible expression of the iNOS gene (2, 3). NF-κB is a ubiquitous transcription factor and pleiotropic regulator in the inducible expression of many genes that encode proteins targeted to the modulation of inflammatory and host defense processes in eukaryotic cells (4, 5). iNOS represents one such protein and the high-output production of NO results not only in initiating and/or exacerbating symptoms of acute and chronic inflammation but also in defense of the host against invading pathogens (6). In a recent study, we found that a variety of proteinase inhibitors prevent NO formation by activated rat alveolar macrophages by interfering with iNOS gene transcription (7). Nonselective proteinase inhibitors, however, cannot be used to elucidate precise mechanisms by which proteinases are involved in iNOS gene transcription. One particular proteinase inhibitor {N-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-Bu)-Ala-leucinal [Z-IE(*O*-*t*-Bu)A-leucinal]}, an inhibitor of the multicatalytic proteinase complex (MPC) or proteasome (8) and NF-κB activation (9), potently interfered with iNOS induction, thereby suggesting that this agent interferes with iNOS induction by preventing NF-κB activation.

The objective of the present study was to use certain relatively selective, peptidyl aldehyde, proteinase inhibitors to focus on the mechanism by which these compounds interfere with iNOS induction and to further test the hypothesis that NF-κB is the principal transcription factor involved in the LPS-induced expression of the iNOS gene. Calpains are calcium-dependent, extralysosomal cysteine proteinases that play physiological roles in modulating cell function such as signal-controlled, target-specific, limited proteolysis for the regulation of transcription factors (10). The proteasome is a calcium-independent, extralysosomal, multicatalytic proteinase complex (11) that is responsible for the turnover of abnormal and biologically active proteins and plays both general and specific roles in cellular proteolysis (12), such as activation of certain transcription factors (9, 13). Two main calpain inhibitors [α-*N*-acetyl-Leu-Leu-norleucine (calpain inhibitor 1); *N*-benzylcarbonyl-Leu-leucinal (Z-Leu-leucinal)] and one newly available proteasome inhibitor [Z-IE(*O*-*t*-Bu)A-leucinal] were studied for their mechanisms of action in preventing the induction of iNOS in LPS-activated rat alveolar macrophages.

MATERIALS AND METHODS

Reagents. Bacterial LPS (phenol-extracted *Escherichia coli*, serotype 0128:B12), optimized lactate dehydrogenase (LDH) UV-test, modified Eagle's medium without phenol red, 7.5% (wt/vol) sodium bicarbonate, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and Triton X-100 were obtained from Sigma. Ham's F-12 medium, L-glutamine, and Fungibact were purchased from Irvine Scientific. Fetal calf serum was obtained from PAA/Tissue Culture Biological and phosphate-buffered saline (PBS) was from GIBCO/BRL. Calpain inhibitor 1 and α-*N*-acetyl-Leu-Leu-methioninal(calpain inhibitor 2) were purchased from Boehringer Mannheim. Bio-Spin chromatography columns, high-strength analytical-grade agarose and 10× Tris/boric acid/EDTA (TBE) buffer were purchased from Bio-Rad. [α-^{32}P]dCTP (6000 Ci/mmol; 1 Ci =

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Abbreviations: NO, nitric oxide; iNOS, immunologic or inducible NO synthase; LPS, lipopolysaccharide; NO_X^-, nitrite plus nitrate; NMA, N^G-methyl-L-arginine; TPCK, N^α-*p*-tosyl-L-phenylalanine chloromethyl ketone; calpain inhibitor 1, α-*N*-acetyl-Leu-Leu-norleucinal; calpain inhibitor 2, α-*N*-acetyl-Leu-Leu-methioninal; MPC, multicatalytic proteinase complex; Z-IE(*O*-*t*-Bu)A-leucinal, *N*-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-Bu)-Ala-leucinal; Z-Leu-leucinal, *N*-benzyloxycarbonyl-Leu-leucinal; LDH, lactate dehydrogenase.

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37 GBq) and [γ-^{32}P]ATP (>5000 Ci/mmol) were purchased from Amersham. All other molecular biology grade chemicals were obtained from Sigma. N^G-Methyl-L-arginine (NMA) was synthesized as described (14). Z-IE(*O-t*-Bu)A-leucinal and Z-Leu-leucinal were prepared as described (8).

NO Synthase Induction in Rat Alveolar Macrophages. Rat alveolar macrophage cell line NR8383 [provided by R. J. Helmke (15)] was used as the source of macrophages. Cells were maintained continuously in Ham's F-12 medium supplemented with 15% heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% Fungibact (penicillin at 100 units/ml, streptomycin at 100 μg/ml, and amphotericin B at 2.5 μg/ml) in a humidified 37°C, 5% CO_2 95% air incubator. Floating macrophages were recovered by centrifugation of the medium (150 × *g*, 4°C, 10 min) and resuspended at a density of 10^6 cells per ml in modified Eagle's medium/2% heat-inactivated fetal calf serum/2 mM glutamine/1% Fungibact. In experiments designed for analysis of nitrite plus nitrate (NO_X^-), cells were plated in 12-well dishes (Costar), at 10^6 cells per well, activated by addition of LPS at 75 ng/ml and incubated for 24 hr. After incubation, the cell-free medium was aspirated and analyzed for NO_X^-. In experiments designed for determination of NO synthase activity, Northern blot analysis and electrophoretic mobility-shift assay, 10^7 cells were plated in 10-cm dishes (Fisher), activated with LPS, and then incubated for 24 hr, 6 hr, or 2 hr, respectively. Cells were then harvested by scraping and centrifugation of medium plus cells. Where indicated, test agents were added to cell cultures 30 min before activation with LPS.

NO Synthase Assay. NO synthase activity was determined by monitoring the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine by a modification of a described method (16). Briefly, macrophages were harvested as described above and homogenized at 4°C with the aid of a Teflon pestle, and the 20,000 × *g* (60 min, 4°C) supernatant was used to assay for iNOS activity essentially as described (16), except that the concentration of L-arginine substrate was raised to 0.1 mM to ensure that the V_{max} was attained in all samples to compare the samples for differences in enzyme quantity as reflected by differences in enzymatic activity.

Chemiluminescence Detection of NO. Concentrations of NO_X^- in samples of cell-free culture medium were determined by chemiluminescence detection exactly as described (17).

LDH Assay. LDH release into the cell culture medium was used to assess the cytotoxic effect of experimental conditions. LDH was measured by using a commercially available kit. Cytotoxicity was expressed as percentage of LDH activity from the cell-free medium compared to total LDH activity in corresponding cells plus medium (incubated with 1% Triton X-100 for 30 min at 23°C).

Northern Blot Analysis. RNA was isolated using commercially available kits (QIAshredder and RNeasy Total RNA; Qiagen). Northern blot analysis was performed by standard techniques (18) for formaldehyde-containing agarose gels. A mouse cDNA probe (19), CL-BS-mac-NOS, was labeled by random priming (Boehringer Mannheim) to a specific activity of >10^9 dpm/μg with [α-^{32}P]dCTP and added to the prehybridization solution (5× SSPE (1× SSPE is 0.18 M NaCl/10 mm phosphate pH 7.4/1 mM EDTA buffer containing 50% formamide, 5× Denhardt's solution, 0.1% SDS, heat-chilled salmon testes DNA at 50 mg/ml) at a concentration of 10^6 dpm/ml. Membranes were washed to a final stringency of 15 mM NaCl at 37°C and exposed overnight on Bio Max MR x-ray film using intensifying screens at −70°C. To normalize hybridization signals for variations in loading and/or transfer, membranes were initially visualized for 18S rRNA by methylene blue staining. Densitometry of the bands was performed on the autoradiography film using a Hewlett–Packard flat-bed scanner and National Institutes of Health Image densitometry software (v. 1.55).

Electrophoretic Mobility-Shift Assay. Nuclear extracts were prepared using high-salt buffer as described (20). Assays were conducted using the NF-κB binding protein detection system kit available from Boehringer Mannheim. The NF-κB double-stranded oligonucleotide corresponding to a tandem repeat of the NF-κB binding site consensus sequence (-GGGGACTT-TCC-) was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Unincorporated nucleotides were removed by passage through Bio-Spin chromatography columns. Standard binding reactions were performed by incubating nuclear extracts (20 μg) in 25 μl of 10 mM Tris·HCl, pH 7.5, containing 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 20% (vol/vol) glycerol, 2 μg of sonicated salmon sperm DNA and 50,000 dpm of ^{32}P-labeled NF-κB oligonucleotide (≈0.25 ng) for 20 min at room temperature (23–25°C). DNA–protein complexes were resolved on 6% nondenaturing polyacrylamide gels that were electrophoresed at 12 V/cm for 1 hr in low-ionic-strength buffer (0.5× TBE) at 4°C. Two control reactions were included in each assay: (*i*) a negative control (radiolabeled oligonucleotide only), and (*ii*) a positive control (2 μg of HeLa nuclear extract containing active NF-κB binding protein). In addition, various concentrations of unlabeled specific competitor oligonucleotide were added to some reactions to demonstrate specificity. In the experiment illustrated in Fig. 4, for example, 4 μg of competitive oligonucleotide added to the nuclear extract just before addition of the radiolabeled oligonucleotide markedly suppressed the binding of the radiolabeled oligonucleotide to NF-κB.

Protein Determination. Protein concentrations in NR8383 macrophage supernatant fractions (for assay of iNOS activity) were determined by the Bradford, Coomassie brilliant blue method as described by Bio-Rad. Bovine serum albumin was used as the standard.

Statistical Analysis. The data illustrated in Figs. 1 and 2 were analyzed statistically using one-way ANOVA and the Bonferroni *t* test for unpaired values.

RESULTS

The inducible expression of the iNOS gene in rat alveolar macrophages can be stimulated by LPS alone or by a combi-

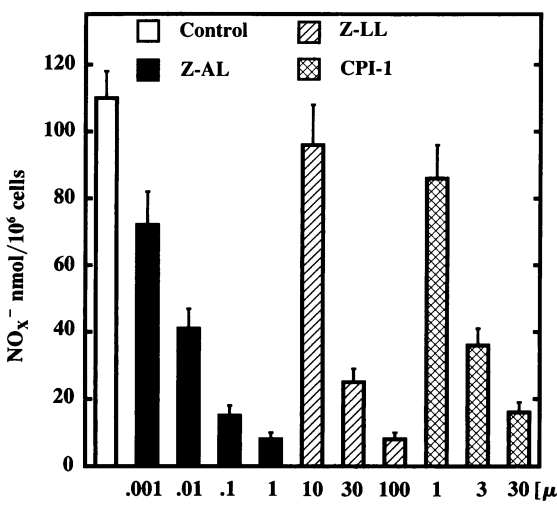

FIG. 1. Concentration-dependent inhibitory effects of proteinase inhibitors on the production of NO by LPS-activated macrophages. NR8383 cells were incubated for 24 hr in the absence or presence of Z-IE(*O-t*-Bu)A-leucinal (Z-AL), Z-Leu-leucinal (Z-LL) or calpain inhibitor 1 (CPI-1) at the indicated concentrations. Test agents were added to cell cultures 30 min prior to addition of LPS at 75 ng/ml. Cell-free media were analyzed for NO_2^- + NO_3^- (NO_X^-) as described. NO_X^- accumulation in cell culture medium in the absence of added LPS was consistently 7–8 μM (data not shown). Data are expressed as the mean ± SEM of six determinations from three separate experiments. All values (except those for 10 μM Z-LL and 1 μM CPI-1) are significantly different ($P < 0.01$) from the value for control.

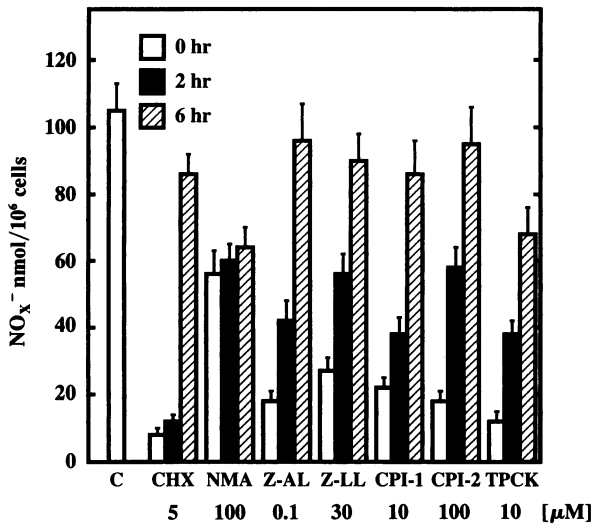

FIG. 2. Time course of inhibitory effects of proteinase inhibitors, cycloheximide and NMA, on the production of NO by LPS-activated macrophages. NR8383 cells were incubated for 24 hr in the absence or presence of cycloheximide (CHX), NMA, Z-IE(*O-t*-Bu)A-leucinal (Z-AL), Z-Leu-leucinal (Z-LL), calpain inhibitor 1 (CPI-1), calpain inhibitor 2 (CPI-2) or TPCK at the indicated concentrations. Test agents were added to cell cultures at 0 hr (30 min before addition of LPS at 75 ng/ml, 2 hr or 6 hr after LPS addition. Cell-free media were analyzed for NO_X^- as described. NO_X^- accumulation in cell culture media in the absence of added LPS was consistently 7–8 μM (data not shown). Data are expressed as the mean ± SEM of six determinations from three separate experiments. All values for test agents corresponding to 0 hr and 2 hr are significantly different ($P < 0.01$) from the control (C) value. The 6-hr values for NMA and TPCK are significantly different ($P < 0.05$) from the control value, whereas all other 6-hr values are not significantly different ($P > 0.05$) from the control value. Values at 2 hr for Z-AL, Z-LL, CPI-1, CPI-2, and TPCK are significantly different ($P < 0.05$) from values at both 0 hr and 6 hr.

nation of LPS plus interferon γ to obtain a potentiated response (16). In view of the finding that LPS, but not interferon γ, causes activation of NF-κB (21) and because this study focuses on the relationship between NF-κB activation and iNOS induction, LPS alone was used to activate macrophages. Incubation of macrophages for 24 hr in the presence of LPS at 75 ng/ml caused about a 15-fold increase in NO production, as assessed by NO_X^- accumulation in the cell culture medium (Fig. 1). Proteinase inhibitors were tested by addition to cell cultures at 30 min before addition of LPS, and incubations were continued for 24 hr after LPS addition. The two calpain inhibitors (calpain inhibitor 1; Z-Leu-leucinal) and the proteasome inhibitor [Z-IE(*O-t*-Bu)A-leucinal] produced concentration-dependent inhibitory effects on NO production by LPS-activated macrophages (Fig. 1). The order of potencies as inhibitors of NO production was Z-IE(O-t-Bu)A-leucinal >> calpain inhibitor 1 > Z-Leu-leucinal. These inhibitory effects were not attributed to cytotoxicity as LDH release from intact cells above control values (LPS alone; 8–10% of total) was not observed, and cell adhesion was not affected. In addition, the proteinase inhibitors did not exert any appreciable direct or indirect cofactor effect on iNOS catalytic activity, as assessed by enzymatic assays in which test agents were incubated in reaction mixtures containing macrophage cytosol extract plus all necessary substrates and cofactors for iNOS, as described previously (22). Under these conditions, 100 μM NMA caused nearly 100% inhibition of iNOS catalytic activity.

These observations suggested that the proteinase inhibitors interfered with the induction of iNOS. Pilot experiments were conducted to ascertain whether the proteinase inhibitors could prevent the expression of iNOS, as assessed by the recovery of iNOS catalytic activity from macrophages after 24-hr incubation. Cells were harvested and homogenized, and the cytosolic fraction was assayed for iNOS activity by determining the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine (see *Materials and Methods*). The three proteinase inhibitors used in the experiments described above produced concentration-dependent inhibitory effects on the recovery of iNOS catalytic activity from LPS-activated macrophages (data not shown). The iNOS activity recovered from control cells was 962 ± 71 pmol of L-citrulline/min per mg of protein. Calpain inhibitor 1 (10 μM), Z-Leu-leucinal (30 μM), and Z-IE(*O-t*-Bu)A-leucinal (0.1 μM) inhibited iNOS recovery by 82 ± 11%, 86 ± 9%, and 92 ± 7%, respectively. In view of these pilot data, a time-course analysis was conducted to ascertain the time after cell activation by LPS at which the proteinase inhibitors exert their influence. The effects of the proteinase inhibitors were compared with the effects of cycloheximide, an inhibitor of protein synthesis, and NMA, an inhibitor of NOS catalytic activity. Fig. 2 illustrates that cycloheximide inhibited NO production when added to macrophages within 2 hr of cell activation by LPS, whereas NMA elicited similar effects whether added at 0, 2, or 6 hr after LPS. These data were expected as translation of mRNA into protein is largely complete by 6 hr after LPS addition, whereas NMA inhibits iNOS catalytic activity at all time points. The calpain inhibitors (Z-Leu-leucinal, calpain inhibitor 1, calpain inhibitor 2) and the proteasome inhibitor [Z-IE(*O-t*-Bu)A-leucinal] produced marked inhibition when added just before LPS (0 hr) but produced significantly less inhibition when added 2 hr after LPS. This result is consistent with an effect of these agents on very early events such as transcription. None of these proteinase inhibitors produced significant inhibitory effects when added 6 hr after LPS. TPCK, a highly nonselective protease inhibitor, displayed a similar pattern but also caused significant inhibition when added 6 hr after LPS. This latter effect is likely attributed to nonspecific inhibition of various intracellular processes and highlights the hazards of using nonselective protease inhibitors to assess mechanisms of action.

These findings were consistent with the view that the proteinase inhibitors interfere with the inducible expression of the iNOS gene. Accordingly, the influence of these agents on iNOS mRNA expression was assessed by Northern blot analysis. The calpain and proteasome inhibitors produced concentration-dependent inhibitory effects on iNOS gene expression (Fig. 3). Although not shown, Z-Leu-leucinal produced no apparent effect at 10 μM but caused about a 50% reduction in band intensity at 100 μM. Based on densitometric analysis of the iNOS bands after correcting for variations in technique as assessed by 18S rRNA recovery, Z-IE(*O-t*-Bu)A-leucinal was

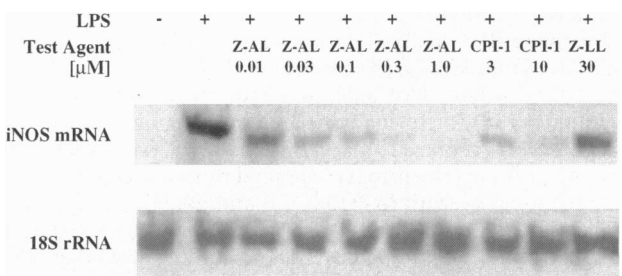

FIG. 3. Inhibitory influence of proteinase inhibitors on the transcriptional expression of iNOS mRNA in LPS-activated macrophages. NR8383 cells were incubated for 6 hr in the absence or presence of Z-IE(*O-t*-Bu)A-leucinal (Z-AL), Z-Leu-leucinal (Z-LL), or calpain inhibitor 1 (CPI-1) at the indicated concentrations. Test agents were added to cell cultures 30 min before addition of LPS at 75 ng/ml. Cells were harvested, total RNA was isolated, and Northern blot hybridizations for iNOS mRNA were performed as described. 18S rRNA was visualized by methylene blue staining. Data illustrated are from a single experiment and are representative of a total of three separate experiments.

≈100- to 300-fold more potent than calpain inhibitor 1 and 3000-fold more potent than Z-Leu-leucinal.

The data indicate clearly that the proteinase inhibitors interfere with LPS-induced iNOS gene expression in rat alveolar macrophages, and that Z-IE(O-t-Bu)A-leucinal is by far the most potent. As LPS induces iNOS gene largely by mechanisms involving NF-κB activation, the next experiment was designed to ascertain the extent to which the proteinase inhibitors interfere with the activation of NF-κB, as assessed by electrophoretic mobility-shift assay. As illustrated in Fig. 4, LPS caused marked activation of NF-κB that was inhibited by the proteinase inhibitors tested. As with the previous experiments, Z-IE(*O-t*-Bu)A-leucinal was found to be the most potent agent tested.

DISCUSSION

Evidence has accumulated to support the view that NO possesses proinflammatory properties and that the high-output production of NO catalyzed by iNOS is likely involved in mediating both acute and chronic inflammation (6, 23). Moreover, inhibitors of iNOS catalytic activity produce anti-inflammatory effects (24). Based on the early evidence that NO might be proinflammatory and that NF-κB plays an active role in the inducible expression of many genes that encode proinflammatory proteins, we took an indirect approach to ascertain whether the iNOS gene could also be under transcriptional control by NF-κB. An established inhibitor of NF-κB activation, pyrrolidine dithiocarbamate (25), was tested for its capacity to interfere with NO production by cytokine-activated rat alveolar macrophages. This antioxidant thiol inhibited NO production, thereby suggesting that NF-κB is involved in the inducible expression of the iNOS gene (26). Subsequent studies were confirmatory (27) and the promoter of the murine gene encoding iNOS was shown to contain an NF-κB consensus site upstream from the TATA box that is necessary to confer inducibility by LPS (2, 3). More recently, studies from this (7) and another laboratory (28) showed that various proteinase inhibitors interfere with NO production by cytokine-activated macrophages by mechanisms involving inhibition of transcriptional expression of the iNOS gene. Because proteinases are intimately involved in the activation of NF-κB (29–32), the objective of the present study was to determine whether selective proteinase inhibitors prevent iNOS induction by interfering with NF-κB activation.

The activation of NF-κB entails a cascade of intracellular events resulting in the liberation of free NF-κB in the cytoplasm, after which time the NF-κB translocates into the nucleus and binds to the appropriate DNA to promote transcription (33). One of the requisite intracellular events for NF-κB activation is proteolytic cleavage of a large cytosolic protein complex consisting of NF-κB bound to another protein termed IkB-α, thereby resulting in liberation of free NF-κB (31, 32). Inhibition of this proteolytic pathway would result in inhibition of NF-κB activation. Although various nonselective serine and cysteine proteinase inhibitors can interfere with NF-κB activation (9, 31), these agents likely interfere with multiple pathways that are sensitive to inhibition by nonselective proteinase inhibitors. For example, TPCK, other chloromethyl ketones, and related proteinase inhibitors interfere with the phosphorylation of IkB-α (34), a prerequisite step to the cleavage of IkB-α from NF-κB (32, 35). Therefore, nonselective proteinase inhibitors interfere with iNOS induction likely by multiple mechanisms that lead to prevention of NF-κB activation.

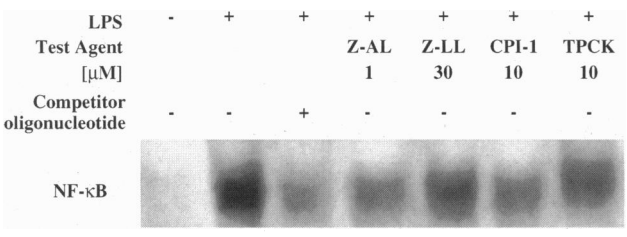

FIG. 4. Inhibitory influence of proteinase inhibitors on NF-κB activation in LPS-activated macrophages. NR8383 cells were incubated for 2 hr in the absence or presence of Z-IE(O-t-Bu)A-leucinal (Z-AL), Z-Leu-leucinal (Z-LL), calpain inhibitor 1 (CPI-1), or TPCK at the indicated concentrations. Test agents were added to cell cultures 30 min before addition of LPS at 75 ng/ml. Cells were harvested, nuclei were isolated and extracted, and electrophoretic mobility-shift assays were conducted as described. Binding assays were performed by using 20 μg of nuclear extract protein and 4 μg of competitive oligonucleotide where indicated. Data illustrated are from a single experiment and are representative of a total of three separate experiments.

Although both calpains and the proteasome serve important regulatory functions in transcriptional regulation, the proteasome or MPC rather than calpains appears to constitute the proteolytic pathway responsible for NF-κB activation (9). In the present study, the proteasome inhibitor, Z-IE(*O-t*-Bu)A-leucinal, was a very potent inhibitor of iNOS induction and NF-κB activation in LPS-treated macrophages. These observations support the views that NF-κB activation requires the proteolytic activity of the proteasome and that NF-κB activation is required for LPS-induced, iNOS gene expression in rat alveolar macrophages. Z-IE(*O-t*-Bu)A-leucinal and Z-Leu-leucinal are cell-permeable peptidyl aldehydes that are capable of inhibiting both serine and cysteine proteinases, but these agents display relatively high selectivity in comparison to chloromethyl ketones such as TPCK and related proteinase inhibitors (8). Z-Leu-leucinal is a potent inhibitor of calpain activity but only a relatively weak inhibitor of the proteasome (8). In contrast, Z-IE(*O-t*-Bu)A-leucinal is a potent and relatively selective inhibitor of the proteasome and is over 100-fold more potent than Z-Leu-Leucinal as an inhibitor of the chymotrypsin-like activity of the MPC or proteasome (8). In the present study, Z-IE(*O-t*-Bu)A-leucinal was approximately 1000-fold more potent than Z-Leu-leucinal as an inhibitor of NO production and iNOS expression in LPS-activated macrophages. Similarly, Z-IE(*O-t*-Bu)A-leucinal was much more potent than calpain inhibitors 1 and 2 in preventing iNOS induction. Calpain inhibitor 1 was ≈10-fold more potent than calpain inhibitor 2 in preventing iNOS induction, which is consistent with the report that the former is more potent than the latter as an inhibitor of the proteasome (36). These observations indicate clearly that the proteasome rather than calpain plays a key role in LPS-inducible expression of the iNOS gene. In view of the knowledge that the proteasome pathway is the principal proteinase responsible for NF-κB activation, the present data also provide further convincing evidence that LPS promotes iNOS gene induction by initiating the activation of NF-κB.

This study contributes to the expanding list of physiological functions of the proteasome proteolytic pathway (13). The magnitude of the inhibition of inducible iNOS expression by the peptidyl aldehydes correlated with their rank order of potency as inhibitors of the multicatalytic proteinase activity of the proteasome (8, 9, 36). This correlation suggests that induction of iNOS in macrophages occurs by the same inhibitor-sensitive step and that NF-κB activation is required for induction. Similar correlations were found and conclusions drawn in the recent studies demonstrating that peptidyl aldehyde proteinase inhibitors interfere with cytokine-induced, endothelial–leukocyte adhesion molecule expression (37) and class 1 major histocompatibility complex antigen processing (36). The present study highlights the importance of the macrophage proteasome pathway in the signal-transduction mechanisms involved in LPS-induced iNOS gene expression. Moreover, the proteasome pathway of NF-κB activation may

be a target for therapeutic intervention in pathophysiological states associated with high-output production of NO catalyzed by over-expressed iNOS.

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