**IL-1β–Induced Monocyte Chemoattractant Protein-1 Gene Expression in Endothelial Cells Is Blocked by Proteasome Inhibitors**

Graham C.N. Parry, Teresa Martin, Katherine A. Felts, Ronald R. Cobb

**Abstract**—Human monocyte chemoattractant protein-1 (MCP-1) is expressed by a variety of cell types in response to various stimuli. MCP-1 expressed by the endothelium plays an important role in cell migration and activation. MCP-1 is a major chemoattractant for monocytes, T lymphocytes, and basophils. In the present study, we present evidence that the proteasome complex is involved in mediating the interleukin (IL)-1β induction of MCP-1 in endothelial cells. We present evidence that a proteasome inhibitor, N-acetyl-leucinyl-leucinyl-norleucinal (norLeu), and the protease inhibitor tosyl-Phe-chloromethylketone (TPCK) block IL-1β induction of MCP-1 protein expression. norLeu and TPCK also blocked IL-1β–induced MCP-1 promoter-driven reporter gene expression as well as nuclear factor (NF)-κB–mediated reporter gene expression. The effects of norLeu were due to its inhibition of the proteasome rather than calpain, because other calpain inhibitors had no effect on MCP-1 expression. In contrast to TPCK, which blocked NF-κB translocation to the nucleus, norLeu had no effect on NF-κB nuclear translocation or IL-1β–induced phosphorylation of p65. This study demonstrates that the proteasome pathway is involved in IL-1β–induced MCP-1 gene expression in human endothelial cells. (*Arterioscler Thromb Vasc Biol. 1998;18:934-940.*)

**Key Words:** monocyte chemoattractant protein-1 ■ endothelial cells ■ proteasome inhibitors

The migration of monocytes across the vascular endothelium plays an important role during inflammation, immune surveillance, and atherogenesis. Although the mechanism of monocyte infiltration into sites of inflammation has not been fully characterized, human MCP-1 is believed to play an important role.1 MCP-1 is expressed by a variety of cell types, including monocytes, fibroblasts, vascular ECs, and smooth muscle cells in response to several different stimuli.1–12 High levels of MCP-1 mRNA have been found in the pathological foci of rheumatoid arthritis, glomerulonephritis, and atherosclerosis.7,10–12 Inhibition of MCP-1 synthesis at the level of transcription may provide a unique pharmacological approach in the treatment of several clinically important diseases.

The promoter region of the human MCP-1 gene has recently been cloned, sequenced,13,14 and shown to contain two putative consensus binding sites for the transcription factor NF-κB. Recently, Martin et al15 demonstrated that the NF-κB and activator protein-1 sites located 90 and 73 bp, respectively, upstream of the transcriptional start site coordinate regulation of IL-1β–stimulated MCP-1 gene expression in ECs. In addition, this NF-κB site is recognized by the p50/p65 heterodimer of the NF-κB/Rel family of transcription factors.

ECs have been shown to express NF-κB/Rel proteins that mediate the regulation of a variety of proinflammatory genes, including MCP-1, ICAM-1, VCAM-1, and E-selectin.15–19 Treatment of ECs with a variety of stimuli, including IL-1β and tumor necrosis factor-α, results in the rapid phosphorylation of IκBα, its subsequent proteolytic degradation, and the nuclear translocation of NF-κB.20–28 Previous studies have shown that the proteasome system is involved in the proteolytic degradation of IκBα and the precursor of p50.25,27 The proteasome is a calcium-independent, multicatalytic protease complex that is involved in the turnover of abnormal and biologically active proteins. This pathway has also been shown to play an important role in the degradation of several short-lived proteins.29–31

Given the importance of elevated levels of MCP-1 in the recruitment of monocytes as well as T lymphocytes33 to sites of inflammation, we investigated the role of the proteasome system in cytokine-induced MCP-1 gene expression. We used specific proteasome inhibitors to focus on the mechanism by which these compounds interfere with MCP-1 induction. We present evidence that the proteasome inhibitor norLeu and MG132 blocked IL-1β induction of MCP-1 protein expression as well as MCP-1 promoter activity. The nonspecific protease inhibitor TPCK also inhibited IL-1β–induced MCP-1 protein expression and promoter activity. In contrast to TPCK, norLeu did not block the IL-1β–induced nuclear translocation of NF-κB. In addition, norLeu did not block...
cytokine-induced IκBα degradation or phosphorylation of p65 in contrast to the results obtained with TPCK.

Methods

Cells and Cell Culture
The human EC line ECV304 was purchased from ATCC (Rockville, Md.). HUVECs were purchased from Clonetics (San Diego, Calif.) and used between passages 1 and 6. ECV304 and HUVECs were maintained in EGM medium (Clonetics).

Chemicals and Reagents
All chemicals were purchased from Sigma Chemical Co, with the exception of E64, which was purchased from Boehringer Mannheim, and MG132, which was from Calbiochem. Compounds were solubilized in DMSO. IL-1β was purchased from R&D Systems. All oligonucleotides were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems).

ELISA
HUVECs (passage ≤6) were plated on gelatin-coated, 96-well tissue culture dishes (CoStar) and incubated at 37°C until confluent. The cells were then incubated with the test compound in EGM for 30 minutes. The medium was removed and replaced by medium containing the test compound plus 0.5 ng/mL IL-1β and incubated for another 6 hours. The supernatants were then collected and analyzed for MCP-1 protein concentration with the MCP-1 Quantikine kit, following the manufacturer’s instructions (R&D Systems).

RT-PCR
HUVECs were treated in a manner analogous to that described for the ELISA. Total RNA was isolated with RNAzol B by following the procedures of the manufacturer (Bio-TechX). One microgram of total RNA was used for the RT reaction with the GeneAmp RT-PCR kit (Perkin Elmer). Human β-actin primers were purchased from Clontech and amplified a 838-bp product. The PCR primers for MCP-1 amplified a 500-bp product and contained the following sequences: 5'-AGCATGAAAGTCTTGGCCCTCCTG-3' and 5'-AT-TACTTAAAGCTATAATGTTCAC-3'. The PCR parameters were as recommended by Clontech for their Amplimer sets. The amplified fragment was cloned into the KpnI site of pGLneo (Promega) to create pGLneoMCP-1. Each plasmid was then purified by a CsCl gradient, with standard procedures. p(κB)LUC contains four copies of a murine Igκ-kB site cloned upstream of a minimal SV40 promoter.

Luciferase Assays
gPNeoMCP-1 was transfected into ECV304 cells using lipofectin and following the instructions of the manufacturer (Life Technologies). Stably transfected cells were selected by resistance to genetin at 300 μg/mL (Life Technologies). Transient transfections were performed as described. pCMVβ (Clontech) was used as a transfection control. β-Galactosidase levels were determined with the Galacto-Light assay system (Tropix) and exhibited <20% variation between samples.

The stably transfected cell line (ECV MCP-1) was plated in gelatin-coated, 96-well Microlite tissue culture dishes (Dynatech Laboratories) and incubated overnight at 37°C. Cells were treated in a manner analogous to that described for the ELISA. Each sample was assayed in triplicate. After the 6-hour incubation, the cells were washed with PBS, lysed by the addition of 20 μL of lysis buffer per well (Analytical Luminescence Laboratories), and stored at −20°C overnight. Samples were then brought to room temperature and analyzed for luciferase production with an ML9000 luminometer (Analytical Luminescence Laboratories).

Cell Viability
Percent viability of HUVECs treated with the test compound was determined by using the alamarBlue Assay (BioSource International) according to the manufacturer’s instructions. Fluorometer readings were taken after a 6-hour incubation as well as a visual inspection of the cells after treatment with the test compound. Test compound concentrations with a cell viability of <80% were considered toxic.

EMSA
Nuclear extracts were prepared from HUVECs as previously described. Protein concentrations in nuclear extracts were 1 to 5 mg/mL, as determined by the bicinchoninic acid protein assay (Pierce Chemical Co). The oligonucleotide containing the prototypical kB binding site from the murine Igκ gene (italics), 5'-CA-GAGGGACTTTCCGAGA-3', was radiolabeled with [α-32P]dCTP (>3000 Ci/mmol, Amersham) as described. This prototypical NF-κB binding site has been shown to bind to p50/p65 heterodimers in human ECs. Analysis of binding was performed as described.

To quantify the effect of norLeu on the nuclear translocation of NF-κB, an oligonucleotide containing an Sp1 binding site (italics), 5'-ATTCGATCGGCGGCGCGGCGAC-3', was used as an internal control. The intensity of each protein/DNA complex was determined by using a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics Inc). The intensity of the NF-κB complex was calculated relative to the intensity of the Sp1 complex. In all determinations, the labeled oligonucleotide was in excess, and measurements were made in the linear range of the PhosphorImager screen.

Western Blot Analysis
HUVEC protein extracts were prepared according to the procedures described in the Boehringer Mannheim immunoprecipitation kit. Proteins were fractionated by electrophoresis on a 10% SDS–polyacrylamide gel (Novex) and transferred to NitroBind (Micron Separations, Inc). IκBα protein was detected with a 1:1000 dilution of an anti-IκBα antibody (Santa Cruz Biotechnology) and an anti-rabbit–horseradish peroxidase conjugate (Promega) according to the ECL protocol (Amersham).

p65 Phosphorylation and Immunoprecipitation
Confluent monolayers of HUVECs in a T25 flask were washed once with PBS. The cells were then incubated in phosphate-free RPMI 1640 medium containing 100 mCi/mL of [32P]PO4 (400 to 800 mCi/mL, ICN) for 2 hours. HUVEC monolayers were pretreated with the inhibitors for 30 minutes. Cells were then stimulated with IL-1β (0.5 ng/mL) for 15 minutes. After incubation the cells were
MCP-1 Gene Expression Is Blocked by Proteasome Inhibitors

Results

Previous studies have shown that proteasome inhibitors prevent cytokine-induced nuclear translocation of NF-κB in HeLa and Jurkat cells. To address the role of the proteasome in the IL-1β-induced upregulation of MCP-1 in ECs, HUVEC monolayers were treated with the proteasome inhibitor norLeu (also known as a calpain I inhibitor). As shown in Figure 1A, this compound inhibited the IL-1β–induced production of MCP-1 protein expression at a concentration of 100 μmol/L. A less potent proteasome inhibitor and a calpain II inhibitor, N-acetyl-leucinyl-leucinyl-methylionyl (allMet), was much less effective in inhibiting the induction of MCP-1. The calpain inhibitors (2S,3S)-trans-epoxysuccinyl-l-leucylamido-3-methylbutane ethyl ester (Trans) and (2S,3S)-trans-epoxysuccinyl-l-leucinylamido-3-methoxybutane (data not shown), did not show any significant inhibition of MCP-1 protein expression (Figure 1A). However, the serine protease inhibitor TPCK was effective in blocking IL-1β–induced MCP-1 protein expression (Figure 1B). The proteasome-specific inhibitor MG132 was also effective in blocking IL-1β–induced MCP-1 expression (Figure 1B).

RT-PCR analysis was performed on total RNA from HUVECs to elucidate whether the proteasome inhibitors had any effect on MCP-1 mRNA accumulation. HUVECs were treated in a similar manner as described for the ELISA. As shown in Figure 2, a 100 μmol/L pretreatment with norLeu or a 50 μmol/L pretreatment with TPCK almost completely inhibited the IL-1β–induced accumulation of MCP-1 mRNA. These inhibitors had no effect on IL-1β–induced plasminogen activator inhibitor-1 expression, which is induced in HUVECs by IL-1β in an NF-κB–independent manner.

We next investigated the effects of norLeu on the transcriptional regulation of MCP-1. For these studies a contiguous 559-bp fragment of the human MCP-1 promoter including the transcriptional start site was cloned into a luciferase reporter gene plasmid. This plasmid, pGLneoMCP-1, was then used to stably transfect ECV304 human ECs. The level of IL-1β–induced luciferase production from the pGL-neoMCP-1 plasmid in the stably transfected cell line was similar to that seen in HUVECs transiently transfected with the same plasmid (data not shown). Therefore, the stably transfected cell line was used in these experiments. The stably transfected cell line was then treated with the test compounds, and their effects on luciferase production were measured. As shown in Figure 3, norLeu significantly inhibited MCP-1 promoter-driven luciferase production. MG132 also significantly inhibited MCP-1 promoter-driven luciferase production. The effects of norLeu were due to its inhibition of the proteasome rather than of calpain, because other calpain inhibitors, eg, (2S,3S)-trans-epoxysuccinyl-l-leucinylamido-3-methylbutane ethyl ester, did not block the IL-1β–induced MCP-1 promoter activity (Figure 3).

Previous results have shown that NF-κB and activator protein-1 coordinately regulate the cytokine-induced MCP-1 gene expression in ECs. Therefore, we investigated the effects of norLeu on NF-κB–mediated promoter activity. As shown in Figure 4, norLeu and TPCK inhibited cytokine-induced, NF-κB–dependent promoter activity in a dose-dependent manner.

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prototypical site has been shown to bind to p50/p65 heterodimers in human ECs. TPCK completely abolished IL-1β–induced nuclear translocation of NF-κB (Figure 5). In contrast, norLeu had no effect on nuclear translocation or the DNA binding ability of NF-κB at a concentration that completely inhibited MCP-1 gene expression. To more precisely characterize the effect of norLeu on the nuclear translocation of NF-κB, an Sp1 oligonucleotide was used as an internal control, and protein-DNA complexes were quantified by using a PhosphorImager. In three independent experiments, the intensity of the NF-κB complex in the norLeu-treated extracts was 120 ± 14% (mean ± SD) of that in the extracts treated with IL-1β alone. Thus, norLeu did not inhibit nuclear translocation of NF-κB in IL-1β–stimulated ECs. Consistent with this observation, norLeu did not block IL-1β–induced degradation of IκBa, whereas TPCK did block this degradation (Figure 6), although a small amount of the phosphorylated form of IκBa was observed in the cells treated with norLeu in these experiments.

Recent evidence has shown that p65 is strongly phosphorylated during the activation of NF-κB. Further studies revealed that p65 contains two transactivation domains in the carboxy terminus, TA1 and TA2. Stimulation of cells results in the phosphorylation of TA2 and is correlated with increased transcriptional activity, whereas TA1 is constitutively phosphorylated. To determine whether norLeu treatment inhibited the activity of nuclear p50/p65 complexes by blocking phosphorylation of p65, we investigated the phosphorylation of p65 in IL-1β–stimulated HUVECs. p65 was immunoprecipitated from unstimulated and stimulated cells and detected by Western blotting with an anti-p65 antibody (Santa Cruz Biotechnology) to confirm p65 recovery from the extracts. IL-1β induced significant phosphorylation of p65 in HUVECs. This inducible phosphorylation was not affected by norLeu treatment but was inhibited by TPCK treatment (Figure 7). Immunoprecipitation of p65 was blocked by the addition of the peptide (Santa Cruz Biotechnology) that was used to raise the antibody.

Discussion

In the present study we investigated the role of the proteasome complex in the IL-1β–mediated induction of MCP-1 gene expression in human ECs. The proteasome inhibitors norLeu and MG132 and the protease inhibitor TPCK blocked IL-1β–induced MCP-1 gene expression. We present evidence that norLeu did not block cytokine-induced nuclear translocation of NF-κB–Rel complexes and that the proteolytic degradation of IκBa was not affected. However, the protease inhibitor TPCK did block nuclear translocation of NF-κB, and IκBa proteolytic degradation was also inhibited. Furthermore, norLeu did not block IL-1β–induced phosphorylation of p65. However, IL-1β–stimulated, NF-κB–mediated transcription was inhibited by norLeu. Therefore, these studies
suggest that norLeu blocks NF-κB–mediated transcription either by affecting the functional activity of the NF-κB/Rel proteins or by blocking the function of an accessory protein necessary for NF-κB activation.

Previous studies have demonstrated that NF-κB/Rel proteins act in concert with activator protein-1 in the cytokine-mediated induction of MCP-1. The results presented in this study demonstrate that MCP-1 gene expression can be blocked without interfering with nuclear translocation of NF-κB. Similar effects of proteasome inhibitors have been presented for VCAM-1 and ICAM-1 gene expression. In addition, phosphatidylcholine-specific phospholipase C inhibitors have been shown to block cytokine-induced VCAM-1 and ICAM-1 gene expression in a similar manner. In this study, we also present evidence that cytokine-induced phosphorylation of p65 occurs even when the functional activity of NF-κB is blocked. Our results indicate that the proteasome inhibitors block transcription of the MCP-1 gene; however, we cannot exclude the possibility that MCP-1 mRNA may be destabilized by this inhibitor. Furthermore, although previous studies have shown that NF-κB activation is required for maximal MCP-1 gene transcription, inhibition may occur by effects on other, undescribed factors regulating the MCP-1 gene.

The effects of norLeu and TPCK on MCP-1 gene expression were specific for IL-1β induction of proinflammatory genes, which contain NF-κB binding sites. Previous results have shown that norLeu and TPCK will block ICAM-1 and VCAM-1 gene expression but not plasminogen activator inhibitor-1 expression. Plasminogen activator inhibitor-1 gene expression is induced in human ECs by IL-1β via an NF-κB–independent mechanism. Other inhibitors of calpain...
did not block IL-1β–induced MCP-1 gene expression. Because these other inhibitors of calpain did not block the cytokine induction of MCP-1, one can conclude that inhibition of calpain cannot account for the inhibitory effects of norLeu. Therefore, it appears that the effects of norLeu on the proteasome complex account for the inhibition of MCP-1 gene expression in response to cytokine stimulation. In contrast to these observations, Read et al.40 suggested that inhibition of induced EC gene expression by the proteasome inhibitor MG132 was due to blocking of NF-κB nuclear translocation. However, only partial inhibition of nuclear translocation was observed at doses of MG132 that were significantly higher than those required to inhibit gene expression. We did not observe any inhibition of NF-κB nuclear translocation in norLeu-treated cells at doses that blocked MCP-1 gene expression. This result suggests that inhibition of NF-κB activity occurred by a novel mechanism.

Recent studies have shown that p65 is phosphorylated upon activation of NF-κB.42,43 The TA2 transactivation domain is constitutively phosphorylated, whereas the TA2 trans-activation domain is rapidly phosphorylated after phorbol ester stimulation.43 This induced phosphorylation of the TA2 domain results in an enhancement of transactivation.45 The results presented here demonstrate that norLeu does not inhibit IL-1β–induced phosphorylation of p65. However, it is possible that the changes in the phosphorylation pattern of p65 are modified by inhibition of the proteasome, which could not have been detected in our experiments. In addition, norLeu did not completely block NF-κB–mediated transcription in transiently transfected ECs. These results are similar to those observed with inhibitors of 5-lipoxygenase, which inhibited NF-κB–mediated transcription without interfering with nuclear translocation or p65 phosphorylation.44 Taken together, these results imply that it is possible to inhibit the transactivation by NF-κB without interfering with phosphorylation or nuclear translocation.

This study identifies the importance of the proteasome complex in the regulation of cytokine induction of MCP-1 in ECs. Our results clearly demonstrate that phosphorylation of p65 and nuclear translocation of NF-κB are not sufficient for the induction of the MCP-1 gene. Because MCP-1 appears to play a significant role in inflammation, the proteasome complex may represent a target for therapeutic intervention in pathophysiological states associated with high levels of MCP-1.

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References


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