J. Biochem. 129, 585–591 (2001)

A Pyrrolidinone Derivative Inhibits Cytokine-Induced iNOS Expression and NF-κB Activation by Preventing Phosphorylation and Degradation of IκB-α[1]

Koichi Katsuyama*,[2] and Yukio Hirata†

Nisshin Flour Milling Co., Ltd., 5-3-1, Tsurugaoka, Ohi-machi, Irumagun, Saitama 356-8511; and †Department of Clinical and Molecular Endocrinology, Graduate School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8519

Received November 7, 2000; accepted January 19, 2001

We previously showed that 1-[3-(3-pyridyl)-acryloyl]-2-pyrrolidinone hydrochloride (N2733) inhibits lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)–α secretion and improves the survival of endotoxemic mice. Since overproduction of nitric oxide (NO) by inducible NO synthase (iNOS) in vascular smooth muscle cells (VSMCs) is largely responsible for the development of endotoxemic shock, and iNOS gene expression is mainly regulated by LPS and inflammatory cytokines, we studied whether or not N2733 affects interleukin (IL)-1β–induced iNOS gene expression, NF-κB activation, and NF-κB inhibitor (IκB)–α degradation in cultured rat VSMCs. N2733 dose-dependently (10–100 μM) inhibited IL-1β–stimulated NO production, and decreased IL-1β–induced iNOS mRNA and protein expression, as found on Northern and Western blot analyses, respectively. Gel shift assay and an immunocytochemical study showed that N2733 inhibited IL-1β–induced NF-κB activation and its nuclear translocation. Western blot analyses involving anti-IκB-α and anti-phospho IκB-α antibodies showed that IL-1β induced transient degradation of IκB-α preceded by the rapid appearance of phosphorylated IκB-α, both of which were markedly blocked by N2733. N2733 blocked IL-1β–induced phosphorylated IκB-α even in the presence of a proteasome inhibitor (MG115). Immunoblot analysis involving anti-IκB kinase (IKK)-α and anti-phosphoserine antibodies revealed that N2733 inhibited IL-1β–induced IKK-α phosphorylation, whereas N2733 had no inhibitory effect on IL-1β–stimulated p42/p44 MAP kinase or p38 MAP kinase activity. Our results suggest that the inhibitory action of N2733 toward IL-1β–induced NF-κB activation and iNOS expression is due to its blockade of the upstream signal(s) leading to IKK-α activation, and subsequent phosphorylation and degradation of IκB-α in rat VSMCs.

Key words: IκB-α, IL-1β, inducible nitric oxide synthase, NF-κB.

NO has diverse physiological roles, including in vasodilation, neurotransmission, and mediation of immune responses (*1*). NO production from L-arginine and molecular oxygen is catalyzed by NO synthases (NOS). Three isoforms exist; namely two constitutive NOS isoforms which are expressed mainly in neurons (*2*) and the endothelium (*3*), and an inducible NOS (iNOS), which can be induced by bacterial lipopolysaccharides (LPS) and certain inflammatory cytokines in a variety of cells. NO production in vascular smooth muscle cells (VSMCs) is not detectable under normal conditions. However, following stimulation with inflammatory cytokines and LPS, excessive formation of NO after the induction of iNOS largely contributes to profound and intractable hypotension, a hallmark of endotoxin shock. Since VSMCs play a pivotal role in the regulation of vascular tonus, elucidation of iNOS gene regulation in VSMCs is critical for understanding the pathogenesis of endotoxin shock.

IL-1β is one of the key proinflammatory cytokines involved in the inflammation process (*4*, *5*). When it binds to its cell-surface receptor, IL-1β initiates a cascade of signaling events, including activation of p42/p44 mitogen–activated protein (MAP) kinase, p38 MAP kinase, Jun N-terminal kinase (JNK), and nuclear factor–kappa B (NF-κB), a key transcription factor responsible for the expression of many proinflammatory genes (*6*).

NF-κB is an inducible transcription factor that mediates signal transduction between the cytoplasm and nucleus in a variety of cells. In unstimulated cells, NF-κB is located in the cytoplasm as an inactive form complexed with an inhibitory protein, IκB-α. The conversion of NF-κB into the active nuclear form, composed of p50 and p65 (Rel-A) subunits, is induced by LPS or cytokines. These stimulants seem to activate NF-κB by inducing the phosphorylation and degradation of IκB-α, thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus. Recently, cytokine-responsive IκB kinase (IKK)–α, which acti-

[1] This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Phone: +81-492-67-3966, Fax: +81-492-67-3967

vates NF-κB through phosphorylation of Ser^{32} and Ser^{36} residues in IκB-α, has been identified (*7–11*). Following translocation into the nucleus, NF-κB binds to κB sites of DNA and activates gene expression.

We previously reported that a pyrrolidinone derivative, N2733, is a potent inhibitor of the immune response, inflammation, and endotoxic shock (*12*). N2733 inhibits LPS-induced tumour necrosis factor (TNF)-α gene expression in human myeloid THP-1 cells *in vitro*, and the administration of N2733 inhibits LPS-induced TNF-α production and improves the survival of endotoxemic mice *in vivo* (*12*). However, it remains undetermined whether or not N2733 affects cytokine-induced NF-κB activation and whether or not it modulates iNOS expression. These observations led us to examine whether or not N2733 affects IL-1β–induced iNOS expression and NO production *via* the NF-κB–dependent pathway in VSMCs.

MATERIALS AND METHODS

Materials—Human recombinant IL-1β was purchased from Cistron (Pine Brook, NJ). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL (Grand Island, NY), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and the Alamar Blue assay kit from Wako Pure Chemical (Osaka), phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT), poly (dI-dC), leupeptin, pepstatin, and aprotinin from Sigma Chemical (St. Louis, MO), MG115 and PD98059 from Calbiochem (San Diego, CA), SB203580 from Alexis Biochemicals (San Diego, CA), [^{3}H]leucine and [α-^{32}P]dCTP from Amersham International (Tokyo), and dNTP and a Klenow fragment of DNA polymerase I from Takara Shuzo (Shiga), and N2733 was synthesized in our laboratory.

Cell Culture—VSMCs from the thoracic aortae of 12-week-old Wistar rats were prepared by the explant method, and then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C under a humidified atmosphere of 95% air–5% CO_2 as described previously (*13*). Subcultured VSMCs (15–25th passages) were used in the experiments.

Determination of Nitrite/Nitrate (NO_x)—Confluent VSMCs (10^6 cells/well) were preincubated with serum-free DMEM in the absence or presence of N2733 for 60 min and then stimulated with IL-1β (10 ng/ml) for 15 h; the NO_x concentrations in the conditioned media were measured with an autoanalyser (TCI-NOX 100; Tokyo Kasei Kogyo, Tokyo) as described (*14*). In brief, samples premixed with the carrier solution (0.07% EDTA and 0.3% NH_4Cl) were passed through a copperized cadmium reduction column to reduce NO_3^- to NO_2^-, which reacts with Griess reagent (1% sulfonamide/0.1% *N*-1-naphtylethylenediamine dihydrochloride/5% HCl). The absorbance at 540 nm was measured with a flow-through visible spectrophotometer (Model S/3250; Soma-Kogaku, Tokyo). NO_3^- was used as a standard.

Measurement of Cell Viability and Protein Synthesis—Cell viability was assessed by means of the Alamar Blue assay kit (*12*). Confluent VSMCs (1×10^6 cells/ml/well) were incubated with or without N2733 for 24 h, and then 100 μl of the Alamar Blue solution was added. After 3 h treatment, supernatants were measured at 570/600 nm. Protein synthesis was assessed as the incorporation of [^{3}H]leucine into cells (*15*). In brief, confluent VSMCs (1×10^6 cells/well) were pretreated with or without N2733 for 60 min and then stimulated with IL-1β (10 ng/ml) for 2 h. After stimulation, 1.5 μCi [^{3}H]leucine was added and the cells were further incubated for 6 h. After incubation, trichloroacetic acid–insoluble radioactivity was measured with a liquid scintillation counter.

Electrophoretic Mobility Shift Assay (EMSA)—Cell stimulation with IL-1β and EMSA was carried out as we previously described (*16, 17*). Briefly, confluent VSMCs (5×10^6 cells/dish) pretreated with or without N2733 for 60 min were stimulated with IL-1β for 2 h, washed with ice-cold phosphate-buffered saline (PBS), and harvested in 0.4 ml ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 5 μg/ml leupeptin). After 15 min incubation at 0°C, 25 μl 10% Nonidet P-40 was added, followed by centrifugation at 10,000 ×*g* for 1 min. The nuclei pellets were collected, resuspended in 30 μl hypertonic extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1 mM PMSF), and then centrifuged at 10,000 ×*g* for 10 min, and the resulting supernatant was subjected to EMSA. The single-stranded oligonucleotides (forward: 5′-TGGGGACTCTCC-3′, complement: 5′-AAGGGAGAGTCC-3′) corresponding to the NF-κB binding sequence of a downstream region (−107 to −97) of the rat iNOS gene promoter (*18*) were annealed at 65°C for 15 min, and then filled with [α-^{32}P]dCTP (111 TBq/mmol), dNTP, and a Klenow fragment of DNA polymerase I. Nuclear proteins (10 μg) were incubated with 20,000 cpm ^{32}P-labeled NF-κB double-stranded oligonucleotide and 1 μg poly (dI-dC) in EMSA buffer (10 mM Tris-HCl, pH 7.5, 2% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 50 mM NaCl) for 30 min and then subjected to polyacrylamide gel electrophoresis. The gel was then dried and autoradiographed. To examine the specificity of the NF-κB binding protein, the gel shift assay was performed in parallel with the same samples in the presence of a 100-fold excess of unlabeled oligonucleotide as a competitor. For the gel supershift assay, the nuclear protein was preincubated for 30 min with goat polyclonal antibodies against the human NF-κB p50 or p65 subunit (Santa Cruz Biotechnology, Santa Cruz, CA).

Northern Blot Analysis—Confluent VSMCs (5×10^6 cells/dish) pretreated with or without N2733 for 60 min were stimulated with IL-1β for 6 h and then total RNAs were extracted by the acid guanidinium thiocyanate-phenol-chloroform method (*19*). The total RNAs (20 μg) separated by formaldehyde/1.1% agarose gel electrophoresis were transferred to a Magna Graph nylon membrane (Micron Separations, Minnetonka, MN). The cDNA probe for rat iNOS recently cloned from rat endothelial cells (*20*) was labeled with [α-^{32}P]dCTP (111 TBq/mmol) by the random-primed labeling method. RNA immobilized on the membrane was hybridized with the labeled probes. The membrane was washed finally in 0.1× SSPE (15 mM NaCl, 1 mM NaH_2PO_4, and 0.1 mM EDTA)–0.5% SDS and autoradiographed.

Western Blot and Immunoblot Analyses—Western blot analyses were performed essentially as described (*16, 17*). Confluent VSMCs (5×10^6 cells/dish) were pretreated with or without N2733, MG115, PD98059, or SB203580 for 60 min, stimulated with IL-1β for the indicated times for IκB-α, phospho IκB-α, IKK-α, phospho p42/p44 MAP kinase,

phospho p38 MAP kinase, and iNOS. Cells were lysed in 62.5 mM Tris-HCl, pH 6.8 (10% glycerol, 2% SDS, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM PMSF). The cell lysates were boiled, and the extracted proteins were separated on a 12.5% (for IκB-α, phospho IκB-α, IKK-α, phospho p42/p44 MAP kinase, and phospho p38 MAP kinase) or 7.5% (for iNOS) SDS–polyacrylamide gel and then transferred to Hybond ECL nitrocellulose membranes (Amersham), which were incubated with rabbit polyclonal antibodies for human IκB-α (1:1,000; Santa Cruz Biotechnology), rabbit polyclonal antibodies for human phospho IκB-α (Ser^{32}) (1:1,000; New England Biolabs., Beverly, MA), rabbit polyclonal antibodies for murine IKK-α (1:500; Santa Cruz Biotechnology), rabbit polyclonal antibodies for human phospho p42/p44 MAP kinase (1:1,000; New England Biolabs.), rabbit polyclonal antibodies for human phospho p38 MAP kinase (1:1,000; New England Biolabs.), or mouse monoclonal antibodies for murine iNOS (1:1,000; Transduction Laboratories, Lexington, KY) at 4°C overnight. After extensive washing, the secondary antibody (donkey anti–rabbit IgG or sheep anti–mouse IgG horseradish peroxidase, Amersham) was applied for 1 h, and then exposure was performed with an Enhanced Chemiluminescence (ECL) kit (Amersham).

For immunoblot analysis of phospho IKK-α, confluent VSMCs (5×10^6 cells/dish) were pretreated with or without N2733, stimulated with IL-1β for 5 min, and then lysed in 0.4 ml lysis buffer (pH 7.4, containing 20 mM Tris-HCl, 150 mM NaCl, 2.5 mM EDTA, 1.0% Triton-X, 0.1% SDS, 0.1% deoxycholic acid, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM PMSF) (*15*, *21*). Lysates were centrifuged at 10,000 ×*g* for 10 min, and the supernatants were incubated with mouse monoclonal anti-phosphoserine antibodies (3 μg, Alexis Biochemicals) at 4°C for 2 h. The reaction mixture was subsequently incubated with 10% immobilized protein G (Pierce, Rockford, IL) at 4°C overnight and then centrifuged at 10,000 ×*g* for 1 min. The immunocomplex pellet was washed 4 times with the lysis buffer, and then resuspended in 62.5 mM Tris-HCl, pH 6.8 (10% glycerol, 4% SDS, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM PMSF) and boiled for 10 min. After centrifugation at 10,000 ×*g* for 5 min, the extracted immunocomplex was separated on a 12.5% SDS–polyacrylamide gel and then transferred to Hybond ECL nitrocellulose membranes, which were incubated with rabbit polyclonal antibodies for murine IKK-α (1:500; Santa Cruz Biotechnology) at 4°C overnight. After extensive washing, the secondary antibody (donkey anti–rabbit IgG horseradish peroxidase) was applied for 1 h, and exposure was performed with an ECL kit.

Immunocytochemical Staining—Immunocytochemical staining was performed as we previously described (*16*, *22*). Briefly, subconfluent cells grown on a LAB-TEK Chamber Slide (Nalge Nunc Int., Tokyo) were treated with IL-1β in the absence or presence of N2733 for 2 h, fixed with 3.7% formaldehyde-PBS for 10 min at room temperature, and then washed with PBS for 2 min. Goat polyclonal antibodies specific for the NF-κB p50 and p65 subunits were used. Immunostaining was visualized with an indirect immunoperoxidase avidin-biotin-peroxidase kit (Vector Lab., Burlingame, CA).

RESULTS

N2733 Inhibits IL-1β-Induced NO_x Production and iNOS Expression—We studied whether or not N2733 affects cytokine-stimulated NO production in rat VSMCs. As shown in Fig. 1A, IL-1β (10 ng/ml) increased NO_x production during 15 h incubation by 13-fold compared with control cells, this effect being suppressed by N2733 in a dose-dependent manner (10–100 μM). N2733 (50–100 μM) affected neither the cell morphology, viability, nor protein synthesis (data not shown).

We next examined whether or not N2733 affects iNOS mRMA and protein expression induced by IL-1β in rat VSMCs (Fig. 1B). Northern blot analysis with rat iNOS cDNA as a probe revealed that IL-1β (10 ng/ml) induced iNOS mRNA expression (4.5 kb), this effect being suppressed by N2733 (100 μM) (Fig. 1B, upper panel). Western blot analysis with specific anti–murine iNOS antibodies gave a distinct band of 130 kDa-NOS protein after stimulation with IL-1β, this band also being decreased by N2733 (100 μM) (Fig. 1B, bottom panel). These data indicate that N2733 blocks IL-1β–induced iNOS mRNA and protein expression, and subsequent NO production.

N2733 Blocks IL-1β-Induced NF-κB Activation—To determine whether or not N2733 affects NF-κB activation induced by IL-1β in rat VSMCs, EMSA was performed with synthetic oligonucleotides corresponding to the downstream NF-κB site of the rat iNOS promoter as a probe. The addition of IL-1β (10 ng/ml) induced NF-κB activation at 30 min, which peaked at 1–2 h, as observed on EMSA (*16*, *17*). IL-1β caused a distinct shifted band, while there was no distinct band for the control cells (Fig. 2A). The specificity of the NF-κB binding gel shift assay was examined by means of coincubation with excess unlabeled probe as a competitor. These bands were completely eliminated with the presence of a 100-fold molar excess of unlabeled oligomers. For the characterization of NF-κB subunits, spe-

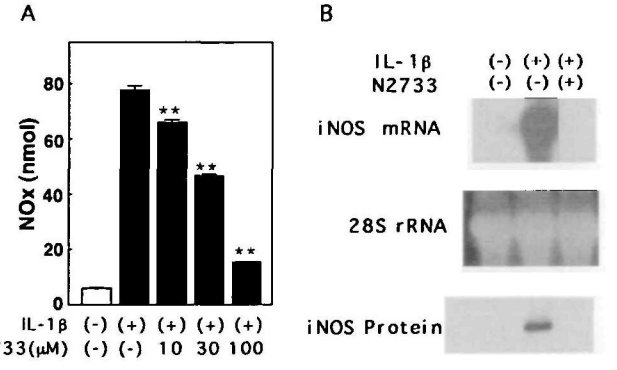

Fig. 1. **Effect of N2733 on IL-1β–induced NO production, iNOS mRNA and protein expression in rat VSMCs.** (A) Confluent cells pretreated with N2733 at the indicated concentrations were stimulated with or without IL-1β (10 ng/ml) at 37°C for 15 h; the concentrations of NO_x released into media were measured. Each column represents the mean ± SE (n = 3). (B) Cells treated with N2733 (100 μM) were stimulated with IL-1β (10 ng/ml) for 6 h for Northern blotting and 15 h for Western blotting, respectively. Northern blot analysis for iNOS mRNA (upper panel), and 28S ribosomal RNA (second panel); Western blot analysis for iNOS protein (lower panel).

cific antibodies for the human p50 and p65 subunits were examined. The IL-1β–induced NF-κB protein-DNA complexes were supershifted by the anti-p50 antibody, while the anti-p65 antibody caused a supershifted band in addition to a decrease in the shifted band (Fig. 2A). The IL-1β–induced NF-κB activation levels in N2733 (100 μM)-treated cells were markedly reduced by about 80% compared with those in nontreated cells (Fig. 2B).

Immunohistochemical staining with anti-p50 and p65 antibodies demonstrated that nonstimulated cells exhibited diffuse but faint distribution of immunoreactivity of both p50 and p65 within the cytoplasm (Fig. 3, A and D), whereas exposure of rat VSMCs to IL-1β (10 ng/ml) resulted in extensive accumulation of immunoreactive p50 and p65 within the nucleus (Fig. 3, B and E), these effects being prevented by pretreatment with N2733 (100 μM) (Fig. 3, C and F). These data confirmed that N2733 indeed blocks IL-1β–induced nuclear translocation of NF-κB.

N2733 Prevents IL-1β–Induced IκB-α Degradation and Phosphorylation—To determine whether or not IL-1β causes IκB-α degradation in rat VSMCs, Western blot analysis with anti–IκB-α antibodies was performed (Fig. 4). The addition of IL-1β (10 ng/ml) resulted in a rapid (within 15 to 30 min) decrease in the IκB-α protein level, which then returned to the baseline level within 60 min (Fig. 4A). Pretreatment with N2733 (100 μM) completely prevented the

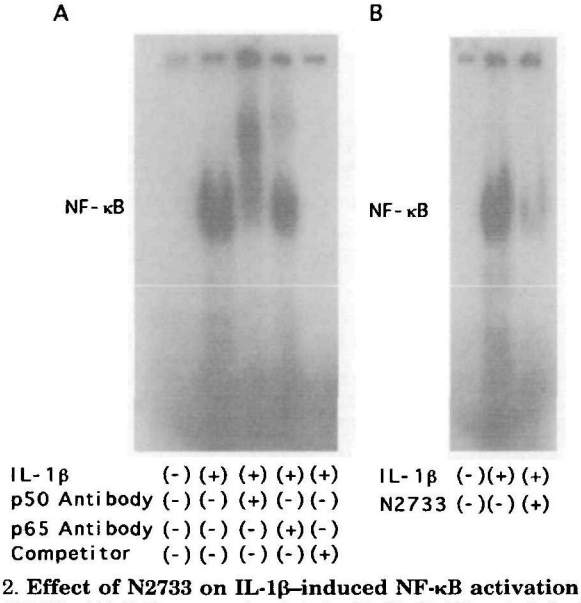

Fig. 2. **Effect of N2733 on IL-1β–induced NF-κB activation in rat VSMCs.** (A) Cells were stimulated with IL-1β (10 ng/ml) for 2 h for EMSA. Nuclear proteins (10 μg) were pretreated without (−) or with (+) antibodies against the NF-κB p50 or p65 subunit, or a 100-fold excess of unlabeled probe prior to EMSA. (B) Cells pretreated with or without N2733 (100 μM) were stimulated with IL-1β (10 ng/ml) for 2 h.

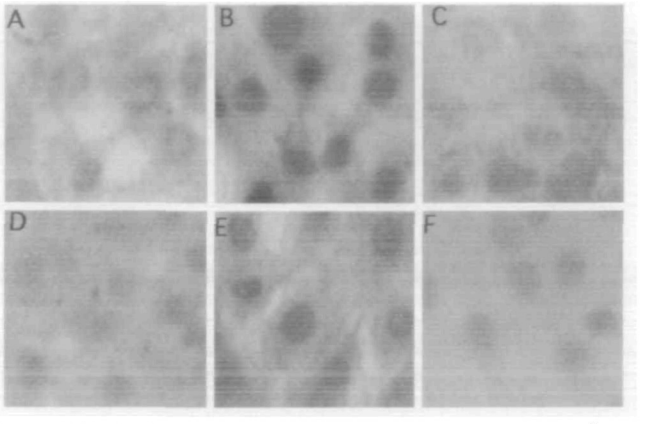

Fig. 3. **Effect of N2733 on IL-1β–induced nuclear translocation of NF-κB, examined by an immunohistochemical method.** Cells pretreated with or without N2733 (100 μM) were stimulated with IL-1β (10 ng/ml) for 2 h, fixed with 3.7% formaldehyde-PBS, and then stained with anti-p50 antibodies (A, B, C) or anti-p65 antibodies (D, E, F). A, D, control; B, E, IL-1β; C, F, IL-1β plus N2733.

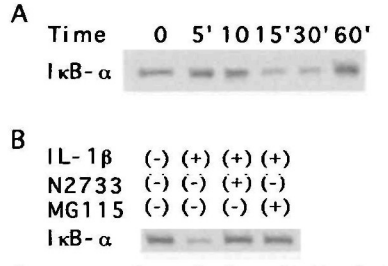

Fig. 4. **Effect of N2733 on degradation of IκB-α by IL-1β in rat VSMCs.** Confluent cells were incubated with IL-1β (10 ng/ml) (A) for the indicated times, or (B) for 15 min with (+) or without (−) N2733 (100 μM). Cell lysates were subjected to Western blot analysis with anti–IκB-α antibodies.

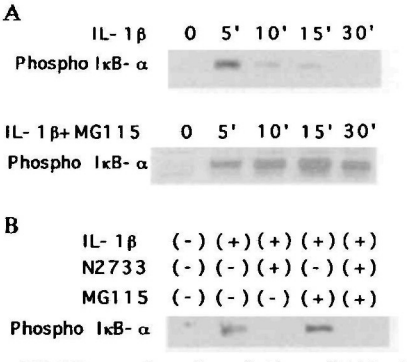

Fig. 5. **Effect of N2733 on phosphorylation of IκB-α by IL-1β in rat VSMCs.** Confluent cells were incubated with IL-1β (10 ng/ml) (A) for the indicated times, or (B) for 5 min with (+) or without (−) N2733 (100 μM). Cell lysates were subjected to Western blot analysis with anti–phospho IκB-α antibodies.

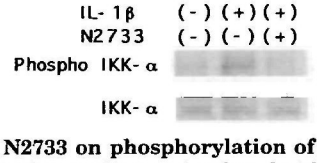

Fig. 6. **Effect of N2733 on phosphorylation of IKK-α by IL-1β in rat VSMCs.** Confluent cells were incubated with IL-1β (10 ng/ml) for 5 min with (+) or without (−) N2733 (100 μM). Cell lysates were subjected to immunoprecipitation with anti-phosphoserine antibodies and then to Western blotting with anti–IKK-α antibodies (upper panel). For total IKK-α detection, cell lysates were subjected to Western blot analysis with anti–IKK-α antibodies (bottom panel).

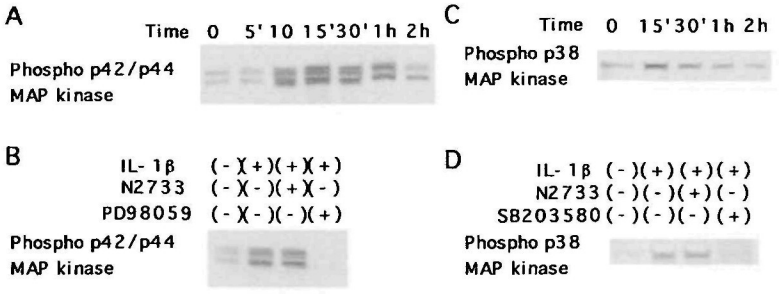

Fig. 7. **Effect of N2733 on phosphorylation of MAP kinases by IL-1β in rat VSMCs.** Confluent cells were incubated with IL-1β (10 ng/ml) (A, C) for the indicated times, or (B) for 15 min for phospho p42/p44 MAP kinase or (D) 30 min for phospho p38 MAP kinase with (+) or without (−) N2733 (100 μM). Cell lysates were subjected to Western blot analyses with anti–phospho p42/p44 MAP kinase and anti–phospho p38 MAP kinase antibodies.

IL-1β–induced decrease in the IκB-α level in a similar manner to in the case of MG115 (10 μM), a proteasome inhibitor. These data suggest that N2733 inhibits the cytokine-stimulated degradation of IκB-α.

To determine whether or not IL-1β causes IκB-α phosphorylation in rat VSMCs, Western blot analysis with anti-phospho Ser^{32} of IκB-α antibodies was performed (Fig. 5, A and B). The addition of IL-1β (10 ng/ml) resulted in rapid phosphorylation of Ser^{32} of IκB-α, which peaked at 5–10 min and then decreased by 30 min (Fig. 5A, upper panel). Pretreatment with MG115 (10 μM) stabilized the IL-1β–induced phosphorylation of IκB-α (Fig. 5A, bottom panel). In contrast, N2733 (100 μM) blocked the IL-1β–induced increase in the phosphorylated IκB-α level with or without MG115 (Fig. 5B). These data suggest that N2733 inhibits cytokine-stimulated rapid phosphorylation of IκB-α prior to its degradation.

N2733 Prevents IL-1β–Induced IKK-α Phosphorylation—To determine whether or not N2733 affects IL-1β–activated IKK-α in rat VSMCs, immunoblot analyses with anti–IKK-α and anti-phosphoserine antibodies were performed (Fig. 6). IL-1β (10 ng/ml) caused rapid (within 5 min) phosphorylation of Ser residue(s) of IKK-α, this effect being inhibited in the presence of N2733 (100 μM); the total IKK-α level remained unchanged with or without N2733. These data suggest that N2733 inhibits cytokine-activated IKK-α.

N2733 Did Not Affect Cytokine-Stimulated p42/p44 MAP Kinase or p38 MAP Kinase—To determine whether or not p42/p44 MAP kinase and p38 MAP kinase are involved in the cytokine-stimulated IκB-α phosphorylation, Western blot analyses with anti–phospho p42/p44 MAP kinase and anti–phospho p38 MAP kinase antibodies were performed, respectively (Fig. 7). The addition of IL-1β (10 ng/ml) resulted in marked phosphorylation of Thr^{202}/Tyr^{204} of p42/p44 MAP kinase, which peaked at 10–60 min and then decreased by 2 h (Fig. 7A). Pretreatment with a MEK inhibitor, PD98059 (50 μM), completely blocked the IL-1β–induced phosphorylation of p42/p44 MAP kinase, whereas N2733 (100 μM) had no effect (Fig. 7B). IL-1β also induced transient phosphorylation of Thr^{180}/Tyr^{182} of p38 MAP kinase, which peaked at 15–30 min and then decreased by 2 h (Fig. 7C). Pretreatment with a p38 MAP kinase inhibitor, SB203580 (10 μM), completely inhibited the IL-1β–induced phosphorylation of p38 MAP kinase, whereas N2733 (100 μM) had no effect (Fig. 7D). These data suggest that N2733 does not affect cytokine-stimulated p42/p44 or p38 MAP kinases.

DISCUSSION

IL-1β is one of the key cytokines involved in the inflammation and immune responses (*4*, *5*, *23*). After binding to the IL-1 receptor (IL-1R), IL-1β initiates signaling cascades leading to the activation of several protein kinases, such as ceramide-activated protein kinase, p42/p44 MAP kinase, p38 MAP kinase, and Jun N-terminal kinase, and ultimately NF-κB activation, a pivotal transcription factor that regulates many proinflammatory genes including iNOS (*6*, *24*, *25*). However, little information is available as to how immediate signaling by IL-1β cause NF-κB activation and iNOS expression in VSMCs. The present experiments involving cultured rat VSMCs demonstrated that stimulation with IL-1β caused transient and rapid phosphorylation and degradation of IκB-α, followed by NF-κB activation and its nuclear translocation. Cytokine-responsive IKK-α, recently identified, phosphorylates two serine residues of IκB-α, *i.e.* Ser^{32} and Ser^{36} (*7–11*). Phosphorylation of these residues is a prerequisite for polyubiquitination and subsequent degradation of IκB-α by the 26S proteasome (*26*). TNF-α rapidly activates IKK-α, peaking at 5–10 min in HeLa cells (*27*). In the present study, IL-1β induced rapid (5–10 min) phosphorylation of the Ser^{32} residue of IκB-α, followed by transient (15–30 min) degradation and subsequent resynthesis of IκB-α in rat VSMCs. Furthermore, we demonstrated that IL-1β induced rapid (within 5 min) phosphorylation of Ser residue(s) of IKK-α. These data are consistent with our previous studies (*16*, *17*), suggesting that IL-1β rapidly stimulates IKK-α to phosphorylate the Ser^{32} residue of IκB-α, which is degraded by proteasome, thereby leading to NF-κB activation in rat VSMCs.

In our previous study, N2733, a pyrrolidinone derivative, was shown to have an anti-inflammatory action and to increase the survival rate of endotoxic shock mice (*12*), although the mechanism by which it acts remains unknown. Since several NSAIDs were recently shown to inhibit NF-κB activation and iNOS expression in a variety of cells (*28–32*), we investigated the effects of N2733 on cytokine-induced iNOS expression in rat VSMCs. The present study has clearly shown that N2733 dose-dependently blocked the IL-1β–induced NO production by inhibiting the expression of iNOS mRNA and protein, as determined by Northern and Western blot analyses, respectively. These data suggest that N2733 inhibits iNOS expression at the transcriptional level. The apparently discrepant inhibitory effects of N2733 on NO production and iNOS expression may be accounted for by the different experimental conditions (incubation times, sample collection, and extraction methods) employed.

The present study has further shown that N2733 markedly blocked cytokine-induced NF-κB activation and its nuclear translocation, as determined by EMSA and an immunocytochemical study, respectively. The apparent dis-

crepancy between the incomplete inhibition of NF-κB activation by N2733 and iNOS expression may be due to the different experimental conditions, as discussed above. Furthermore, N2733 prevented the rapid phosphorylation of IκB-α induced by IL-1β and the subsequent decrease in IκB-α, as determined by Western blot analyses with anti–IκB-α and anti–phospho IκB-α antibodies, respectively. Antioxidants have been reported to stabilize the NF-κB/IκB-α complex by scavengering reactive oxygen species including the superoxide anion (*33*). This reactive oxygen species generated by a variety of stimuli is postulated to mediate NF-κB activation *via* a redox-sensitive mechanism. N2733 is a pyrrolidinone derivative which may act as an antioxidant. However, antioxidants such as pyrrolidinedithiocarbamate (PDTC) and *N*-acetylcysteine failed to prevent IL-1β–induced NF-κB activation (*16*). Furthermore, N2733 exhibits no radical scavengering activity, as assessed by the 1-diphenyl-2-picrylhydrazyl radical method (data not shown). The inhibitory effect of N2733 on the cytokine-induced NF-κB activation and iNOS expression is most likely due to inhibition of IκB-α phosphorylation rather than the proteasome-mediated IκB-α degradation.

The present study has shown that IL-1β induced rapid phosphorylation of the Ser residue(s) of IKK-α. Our data are consistent with those of a previous study (*34*) showing that IKK-α is phosphorylated at Ser^{176} by NF-κB–inducing kinase (NIK). In the present study, N2733 inhibited IL-1β–induced IKK-α phosphorylation. Therefore, the inhibitory effect of N2733 on the cytokine-induced NF-κB activation is most likely due to suppression of the upstream signal(s) leading to IKK-α phosphorylation rather than direct inhibition of IKK-α activity.

IL-1β has been shown to stimulate p42/p44 and p38 MAP kinases, thereby leading to the phosphorylation of several key transcription factors, such as AP-1, Elk-1, and ATF-2 (*35*, *36*). In the present study, however, N2733 failed to affect the IL-1β–induced activation of both p42/p44 and p38 MAP kinases. Furthermore, N2733 did not affect IL-1β–induced c-Fos expression (unpublished observation). Taken together, it is suggested that N2733 may preferentially act on an NF-κB pathway distinct from the MAP kinase pathways.

Macrophages and monocytes stimulated with LPS produce high amounts of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (*37*, *38*), whose genes are mainly regulated by NF-κB (*5*, *39*). We previously reported that N2733 dose-dependently inhibited LPS-stimulated TNF-α mRNA and protein expression in human myeloid THP-1 cells (*12*). N2733 (30–100 mg/kg) also improved the survival of LPS-induced endotoxemic mice. Thus, our present data support the assumption that the beneficial effect of N2733 on the septic shock model is due to inhibition of the NF-κB activation pathway linked to proinflammatory genes including the iNOS gene.

In conclusion, we have demonstrated that the inhibitory effect of N2733 on IL-1β–stimulated NO production and iNOS expression in rat VSMCs is mediated through inhibition of the phosphorylation of IKK-α to decrease the phosphorylation and degradation of IκB-α, thereby leading to inhibition of NF-κB activation. N2733 may have therapeutic potential as to use for the treatment of endotoxic shock by inhibiting NF-κB activation and subsequent blockade on iNOS expression.

REFERENCES

1. Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051–3064
2. Bredt, D.S., Hwang, P.M., and Snyder, S.H. (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* **347**, 768–770
3. Lamas, S.P., Marsden, P., Li, G., Tempst, P., and Michel, T. (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. USA* **89**, 6348–6352
4. Dinarello, C.A. (1994) The interleukin-1 family: 10 years of discovery. *FASEB J.* **8**, 1314–1325
5. Stylianou, E. and Saklatvala, J. (1998) Interleukin-1. *Int. J. Biochem. Cell Biol.* **30**, 1075–1079
6. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **398**, 252–256
7. DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388**, 548–554
8. Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) IKK-1 and IKK-2: cytokine-activated IκB kinase essential for NF-κB activation. *Science* **278**, 860–866
9. Woronicz, J.D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D.V. (1997) IκB kinase β: NF-κB activation and complex formation with IκB kinase-α and NIK. *Science* **278**, 866–869
10. Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997) The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell* **91**, 243–252
11. Regnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z.D., and Rothe, M. (1997) Identification and characterization of an IκB kinase. *Cell* **90**, 373–383
12. Katsuyama, K., Kojima, R., Yokoyama, S., Yanai, M., Sueda, N., Sugita, M., Momose, K., and Yamada, H. (1998) N2733, 1-[3-(3-pyridyl)-acryloyl]-2-pyrrolidinone hydrochloride inhibits LPS-induced TNF-α production and improves survival in endotoxemic mice. *Biosci. Biotechnol. Biochem.* **62**, 2177–2181
13. Hirata, Y., Yoshimi, H., Takaichi, S., Yanagisawa, M., and Masaki, T. (1988) Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett.* **239**, 13–17
14. Kanno, K., Hirata, Y., Emori, T., Ohta, K., Eguchi, S., Imai, T., and Marumo, F. (1992) L-Arginine infusion induces hypotension and increases diuresis/natriuresis with concomitant increased urinary excretion of nitrite/nitrate and cyclic GMP in human. *Clin. Exp. Pharmacol. Physiol.* **19**, 619–625
15. Iwasaki, H., Eguchi, S., Shichiri, M., Marumo, F., and Hirata, Y. (1998) Adrenomedullin as a novel growth-promoting factor for cultured vascular smooth muscle cells: Role of tyrosine kinase-mediated mitogen-activated protein kinase activation. *Endocrinology* **139**, 3432–3441
16. Katsuyama, K., Shichiri, M., Marumo, F., and Hirata, Y. (1998) NO inhibits cytokine-induced iNOS expression and NF-κB activation by interfering with phosphorylation and degradation of IκB-α. *Arterioscler. Thromb. Vasc. Biol.* **18**, 1795–1802
17. Katsuyama, K., Shichiri, M., Kato, H., Imai, T., Marumo, F., and Hirata, Y. (1999) Differential inhibitory actions by glucocorticoid and aspirin on cytokine-induced nitric oxide production in vascular smooth muscle cells. *Endocrinology* **140**, 2183–2190
18. Eberhardt, W., Kunz, D., Hummel, R., and Pfeilschifter, J. (1996) Molecular cloning of the rat inducible nitric oxide synthase gene promoter. *Biochem. Biophys. Res. Commun.* **223**, 752–756
19. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159

20. Iwashina, M., Hirata, Y., Imai, T., Sato, K., and Marumo, F. (1996) Molecular cloning of endothelial, inducible nitric oxide synthase gene from rat aortic endothelial cell. *Eur. J. Biochem.* **237**, 668–673
21. Honda, M., Orii, F., Ayabe, T., Imai, S., Ashida, T., Obara, T., and Kohgo, Y. (2000) Expression of glucocorticoid receptor β in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. *Gastroenterology* **118**, 859–866
22. Katsuyama, K., Shichiri, M., Marumo, F., and Hirata, Y. (1998) Role of nuclear factor-κB activation in cytokine- and sphingomyelinase-stimulated inducible nitric oxide synthase gene expression in vascular smooth muscle cells. *Endocrinology* **139**, 4506–4512
23. Dinarello, C.A. (1994) Interleukin-1 in disease. *Keio J. Med.* **43**, 131–136
24. Cao, Z., Henzei, W.J., and Gao, X. (1996) IRAK: A kinase associated with the interleukin-1 receptor. *Science* **271**, 1128–1131
25. Malinin, N.L., Boldin, M.P., Kovalenko, A.V., and Wallach, D. (1997) MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature* **385**, 540–544
26. Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev.* **9**, 1586–1597
27. Didonato, J., Mercurio, F., Rosette, C., Wu, L.J., Suyang, H., Ghosh, S., and Karin, M. (1996) Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell Biol.* **16**, 1295–1304
28. Shackelford, R.E., Alford, P.B., Xue, Y., Thai, S., Adams, D.O., and Pizzo, S. (1997) Aspirin inhibits tumor necrosis factor-α gene expression in murine tissue macrophage. *Mol. Pharmacol.* **52**, 421–429
29. Farivar, R.S., Chobanian, A.V., and Brecher, P. (1996) Salicylate or aspirin inhibits the induction of the inducible nitric oxide synthase in rat cardiac fibroblasts. *Circ. Res.* **78**, 759–768
30. Lyß, G., Knorre, A., Schmidt, T.J., Pahl, H.L., and Merfort, I. (1998) The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-κB by directly targeting p65. *J. Biol. Chem.* **273**, 33508–33516
31. Chaturvedi, M.M., Kumar, A., Darnay, B.G., Chainy, G.B.N., Agarwal, S., and Aggarwal, B.B. (1997) Sanguinarine (pseudochelerythrine) is a potent inhibitor of NF-κB activation, IκBα phosphorylation, and degradation. *J. Biol. Chem.* **272**, 30129–30134
32. Yu, S.M., Wu, J.F., Lin, T.L., and Kuo, S.C. (1997) Inhibition of nitric oxide synthase expression by PPM-18, a novel anti-inflammatory agent, in vitro and in vivo. *Biochem. J.* **328**, 363–369
33. Schreck, R., Rieber, P., and Baeuerie, P.A. (1991) Reactive oxygen intermediates as apparently wide messengers in the activation of NF-κB transfactor and HIV-1. *EMBO J.* **10**, 2247–2258
34. Ling, L., Cao, Z., and Goeddel, D.V. (1998) NF-κB-inducing kinase activates IKK-α by phosphorylation. *Proc. Natl. Acad. Sci. USA* **95**, 3792–3797
35. Gannett, P.M., Ye, J., Ding, M., Powell, J., Zhang, Y., Darian, E., Daft, J., and Shi, X. (2000) Activation of AP-1 through the MAP kinase pathway: a potential mechanism of the carcinogenic effect of arenediazonium ions. *Chem. Res. Toxicol.* **13**, 1020–1027
36. Huwiler, A. and Pfeilschifter, J. (1999) Nitric oxide stimulates the stress-activated protein kinase p38 in rat mesangial cells. *J. Exp. Biol.* **202**, 655–660
37. Glauser, M.P., Zanetti, G., Baumgartner, J.D., and Cohen, J. (1991) Septic shock: pathogenesis. *Lancet* **338**, 732–736
38. Kawashima, S., Hayashi, M., Takii, T., Kimura, H., Zhang, H.L., Nagatsu, A., Sakakibara, J., Murata, K., Oomoto, Y., and Onozaki, K. (1998) Serotonin derivative, N-(p-coumaroyl) serotonin, inhibits the production of TNF-α, IL-1α, IL-1β, and IL-6 by endotoxin-stimulated human blood monocytes. *J. Interferon Cytokine Res.* **18**, 423–428
39. Christman, J.W., Lancaster, L.H., and Blackwell, T.S. (1998) Nuclear factor κB: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. *Intensive Care Med.* **24**, 1131–1138