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Roxatidine Suppresses Inflammatory Responses Via Inhibition of NF-κB and p38 MAPK Activation in LPS-Induced RAW 264.7 Macrophages

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ABSTRACT

Roxatidine is a novel, specific, competitive H_2-receptor antagonist that is used to treat gastric and duodenal ulcers, and which is known to suppress the growth of several tumors by reducing vascular endothelial growth factor (VEGF) expression. Nevertheless, it remains unclear whether roxatidine has anti-inflammatory effects. In this study, we the authors investigated the anti-inflammatory effect of roxatidine in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. It was found that roxatidine dose-dependently inhibited the productions of prostaglandin E_2 (PGE_2), nitric oxide (NO), and histamine, and the protein and mRNA expressions of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and histidine decarboxylase (HDC). In addition, roxatidine reduced the productions and expressions of VEGF-1 and pro-inflammatory cytokines, including those of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Electrophoretic mobility shift assays (EMSA) and reporter gene assays revealed that treatment with roxatidine attenuated the LPS-induced DNA-binding and transcriptional activity of nuclear factor kappa B (NF-κB). In addition, it was found that pretreatment with roxatidine significantly inhibited the nuclear translocations of the p65 and p50 subunits of NF-κB, and these inhibitions were not found to be associated with decreases in the phosphorylation or degradation of inhibitory kappa B-α (IκBα). Furthermore, roxatidine suppressed the phosphorylation of p38 MAP kinase, but not of IκB kinase-α/β (IKKα/β), c-Jun NH_2-terminal kinase (JNK), or extracellular signal-regulated kinase (ERK). Taken together, these results indicate that the anti-inflammatory properties of roxatidine in LPS-treated RAW 264.7 macrophages are mediated by the inhibition of NF-κB transcriptional activity and the p38 MAP kinase pathway. J. Cell. Biochem. 112: 3648–3659, 2011.

KEY WORDS: ROXATIDINE; INFLAMMATION; NF-κB; P38 MAP KINASE

Eu-Jin Cho and Hyo-Jin An contributed equally to this work.

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Inflammation is a response to injury caused by physical or chemical noxious stimuli or microbiological toxins, and occurs in multiple pathologies, such as, arthritis, asthma, multiple sclerosis, inflammatory bowel diseases, and atherosclerosis [Nathan, 2002; Guzik et al., 2003; Rankin, 2004]. During the inflammatory processes, large amounts of pro-inflammatory mediators, such as, nitric oxide (NO) and prostaglandin E_2 (PGE_2), are generated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [Kim et al., 2009]. nuclear factor kappa B (NF-κB) is a transcriptional factor that plays pivotal roles in immune and inflammatory responses via the regulation of genes that encode pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes like COX-2 and iNOS [Karin and Ben-Neriah, 2000; Tak and Firestein, 2001]. Under normal conditions, NF-κB is sequestered in the cytoplasm as an inactive complex, due to its binding with inhibitory kappa B (IκB) [Baeuerle and Baltimore, 1988]. In the classic pathway, activation of NF-κB, especially the most abundant form, p50/p65 heterodimer, depends on the phosphorylation of its endogenous inhibitor IκB, mainly by IκB kinases (IKKs) [Zandi et al., 1998; Nagai et al., 2002]. This phosphorylation leads to the ubiquitination and proteasomal degradation of IκB, and liberated NF-κB dimer then translocates to the nucleus, where it activates specific target genes [Palombella et al., 1994; Traenckner et al., 1994]. Several studies have reported that the activation of NF-κB is triggered by mitogen-activated protein kinases (MAPK), such as, extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun NH_2-terminal kinase (JNK) [Guha and Mackman, 2001]. However, other reports have shown a negative regulation between NF-κB and MAPK [Jung et al., 2005; Ahmed et al., 2006]. Accordingly, it appears that relationships between NF-κB and MAPKs are complex and depend on cell type and stimulus.

Histamine formed by histidine decarboxylase (HDC) from histidine can recruit major effecter cells into tissues and regulate immune and inflammatory cells, including monocytes, dendritic cells, and T and B cells [Akdis et al., 2008]. Mast cells and basophils constitutively express HDC, and the histamine so produced is stored in intracellular vesicles and secreted in response to extracellular signals. On the other hand, HDC is also expressed by extracellular ligands in macrophages, neutrophils, and T lymphocytes [Aoi et al., 1989; Shiraishi et al., 2000; Ghosh et al., 2002; Tanaka et al., 2004]. Since the excess secretion of histamine from macrophages may be particularly important for the induction of inflammation, the regulation of histamine release offers a potential immunopharmacologic strategy [Akdis and Simons, 2006]. Many studies have described the anti-inflammatory effects of histamine receptor antagonists. For example, the inflammatory effects of the selective histamine H_4-receptor antagonists, JNJ7777120 and VUF6002, have been reported to have anti-inflammatory effects in a rat model of carrageenan-induced paw edema [Coruzzi et al., 2007]. Furthermore, histamine H_1-receptor antagonists have been reported to suppress the activations of NF-κB and activator protein-1 (AP-1) in A549 lung epithelial cells overexpressing H_1-receptor [Roumestan et al., 2008], and to prevent NF-κB transcription and the productions and expressions of pro-inflammatory cytokines, such as, interleukin-6 (IL-6) and interleukin-8 (IL-8) in tumor necrosis factor-α (TNF-α) -stimulated human bronchial epithelial BEAS-2B cells [Holden et al., 2007].

Roxatidine is an H_2-receptor antagonist that is used clinically as an anti-ulcer agent. It is also known to increase gastric mucus [Tanioka et al., 1991; Ichikawa et al., 1997; Ichikawa et al., 1999], to inhibit gastric acid secretion, and to suppress gastric mucosal injury caused by diclofenac or aspirin [Simon et al., 1991; Muller et al., 1994]. In particular, roxatidine has also been reported to suppress histamine release, and thus, to inhibit proton secretion, and to inhibit the production of VEGF-1, an important marker of inflammation and angiogenesis [Tomita et al., 2003]. However, no information is available on the anti-inflammatory effects of roxatidine. Therefore, as a part of our on-going screening project to evaluate the anti-inflammatory potentials of compounds, we investigated the anti-inflammatory effects and its underlying molecular mechanisms of roxatidine in RAW 264.7 macrophages, which can be stimulated with lipopolysaccharide (LPS) to mimic infection and inflammation.

MATERIALS AND METHODS

CHEMICALS

Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY). Roxatidine (98.6% purity) was obtained from DAE HWA Pharm. Co., Ltd. (Kyunggi-Do, Korea). iNOS, COX-2, HDC, p65, phospho-IκBα, phospho-IKKα/β, phospho-ERK, phospho-p38, phospho-JNK, IκB, IKKα, IKKβ, ERK, p38, JNK, PARP, α-tubulin, β-actin monoclonal antibodies, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The enzyme immunoassay (EIA) kits for PGE_2, TNF-α, IL-1β, IL-6, and VEGF-1 were obtained from R&D Systems (Minneapolis, MN, USA). Histamine EIA kit was purchased from Cayman Chemical (Ann Arbor, MI). Random oligonucleotide primers and M-MLV reverse transcriptase were purchased from Promega (Madison, WI). dNTP Mix and ex Taq were obtained from TaKaRa (Seoul, Korea). iNOS, COX-2, HDC, TNF-α, IL-1β, IL-6, VEGF-1, and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). SN50 was obtained from Calbiochem (San Diego, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, dithiothreitol, L-N6-(1-iminoethyl)lysine (L-NIL), NS-398, LPS (*Escherichia coli*, serotype 0111:B4), Triton X-100, pyrrolidine dithiocarbamate (PDTC), SB203580, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

CELL CULTURE AND SAMPLE TREATMENT

The RAW 264.7 murine macrophage cell line and THP-1 human monocyte cell line were obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM or RPMI medium supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with roxatidine at concentrations of 40, 80, and 120 μM, or with positive controls (L-NIL or NS-398), and then stimulated with LPS 1 μg/ml for the indicated time. Various

concentrations of test compounds dissolved in DMSO were added together with LPS. Cells were either treated with 0.05% DMSO as vehicle control.

MTT ASSAY FOR CELL VIABILITY

RAW 264.7 cells were plated at a density of 1×10^5 cells/well in 96-well plates. To determine the appropriate concentration of roxatidine, which has no effect on cell viability, cytotoxicity studies were performed at 24 h following treatment of cells with various concentrations of roxatidine. Viabilities were determined using colorimetric MTT assays, as described previously [Choi et al., 2008].

NITRITE DETERMINATION

RAW 264.7 cells were plated at 1×10^5 cells/well in 24 well-plates and then pretreated with various concentrations (40, 80, and 120 μM) of roxatidine for 1 h. After 1 h, the cells were stimulated with LPS (1 μg/ml) for 24 h. Nitrite levels in culture media were determined using the Griess reaction assay and presumed to reflect NO levels [Choi et al., 2008]. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin Elmer Cetus, Foster City, CA). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the serial dilution standard curve of sodium nitrite.

PGE_2, TNF-α, IL-1β, IL-6, VEGF-1, AND HISTAMINE ASSAY

RAW 264.7 cells were pretreated with roxatidine for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Levels of PGE_2, TNF-α, IL-1β, IL-6, and VEGF-1 in the culture media were quantified using EIA kits (R&D Systems), and the levels of histamine was analyzed by using a commercial enzyme immunoassay kit (Cayman Chemical).

WESTERN BLOT ANALYSIS

RAW 264.7 cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride, and 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated with 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's instruction. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a PVDF membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, UK).

RNA PREPARATION AND REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM deoxyribonucleotide triphosphate (dNTP), and oligo (dT_{12-18}) 0.5 μg/μl. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, HDC, TNF-α, IL-1β, IL-6, VEGF-1, and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus). Reactions were carried out in a volume of 25 μl containing; 1 unit of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5′ and 3′primers. After an initial denaturation for 2 min at 95°C, 26 or 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min 72°C extension), COX-2 (1 min of 94°C, 1 min of 60°C, and 1 min 72°C), HDC(1 min of 94°C, 1 min of 53°C, and 1 min 72°C), TNF-α (1 min of 94°C, 1 min of 55°C, and 1 min 72°C), IL-1β (1 min of 94 °C, 1 min of 60 °C, and 1 min 72°C), IL-6 (1 min of 94°C, 1 min of 56°C, and 1 min 72°C), and VEGF-1 (20 s of 95°C denaturation, 10 s of 55°C annealing, and 30 s 72°C extension). The PCR primers used in this study are listed Supplementary Table I, and were purchased from Bioneer. After amplification, the PCR reactions were electrophoresised on 1% agarose gel and visualized by ethidium bromide staining and UV irradiation.

NUCLEAR EXTRACTION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY

RAW 264.7 macrophages cells were plated in 100-mm dishes (1×10^6 cells/ml), and treated with roxatidine (40, 80, and 120 μM) for 1 h, and then stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously [Choi et al., 2008]. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at $12{,}000 \times g$ for 1 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extracts (10 μg) were mixed with double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACT-TTCCCAGGC3′ end-labeled with [γ-^{32}P] dCTP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric or heterodimeric complex). Binding reactions were performed at 37°C for 30 min in 30 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC), and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × Tris Boric acid EDTA (TBE)

buffer. Gels were vacuum-dried for 1 h at 60°C and exposed to X-ray film at −70°C for 24 h.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

RAW 264.7 cells were transfected with NF-κB-Luc reporter plasmid (Clontech, Shiga, Japan) using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) as instructed by the manufacturers. Cells were incubated for 2 h before the addition of 5 ml of DMEM containing of 10% FBS. Following transfection for 48 h, cells were pretreated with roxatidine for 1 h and then stimulated with LPS (1 μg/ml). After 24 h of stimulation, cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega) and luminometer (Perkin Elmer Cetus).

PHOSPHO-IKK-β ACTIVITY ASSAY

THP-1 human monocyte cell line was cultured for phospho-IKK-β activity assay. THP-1 cells are differentiated into the macrophage cells by Phorbol-12-myristate-13-acetate (PMA). After 24 h of incubation with PMA, THP-1 cells were pretreated with roxatidine for 1 h and then stimulated with LPS (1 μg/ml). After 10 min of stimulation, cells were lysed and the phospho-IKK-β activity was determined using the PathScan® Phospho-IKKβ Sandwich ELISA kit (Cell Signaling Technology, Inc., MA).

STATISTICAL ANALYSIS

Results are expressed as the mean ± SD of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett's post hoc test, and *P*-values of less than 0.05 were considered statistically significant.

ROXATIDINE SUPPRESSED LPS-INDUCED PGE_2, NO, AND HISTAMINE PRODUCTION AND COX-2, iNOS, AND HDC EXPRESSIONS IN RAW 264.7 CELLS

To examine the inhibitory effects of roxatidine on LPS-induced productions of PGE_2, NO, and histamine in RAW 264.7, cells were treated with the different concentrations of roxatidine (40, 80, or 120 μM) for 1 h before being exposed to 1 μg/ml LPS for 24 h. As shown in Figure 1A–C, pretreatment with roxatidine significantly

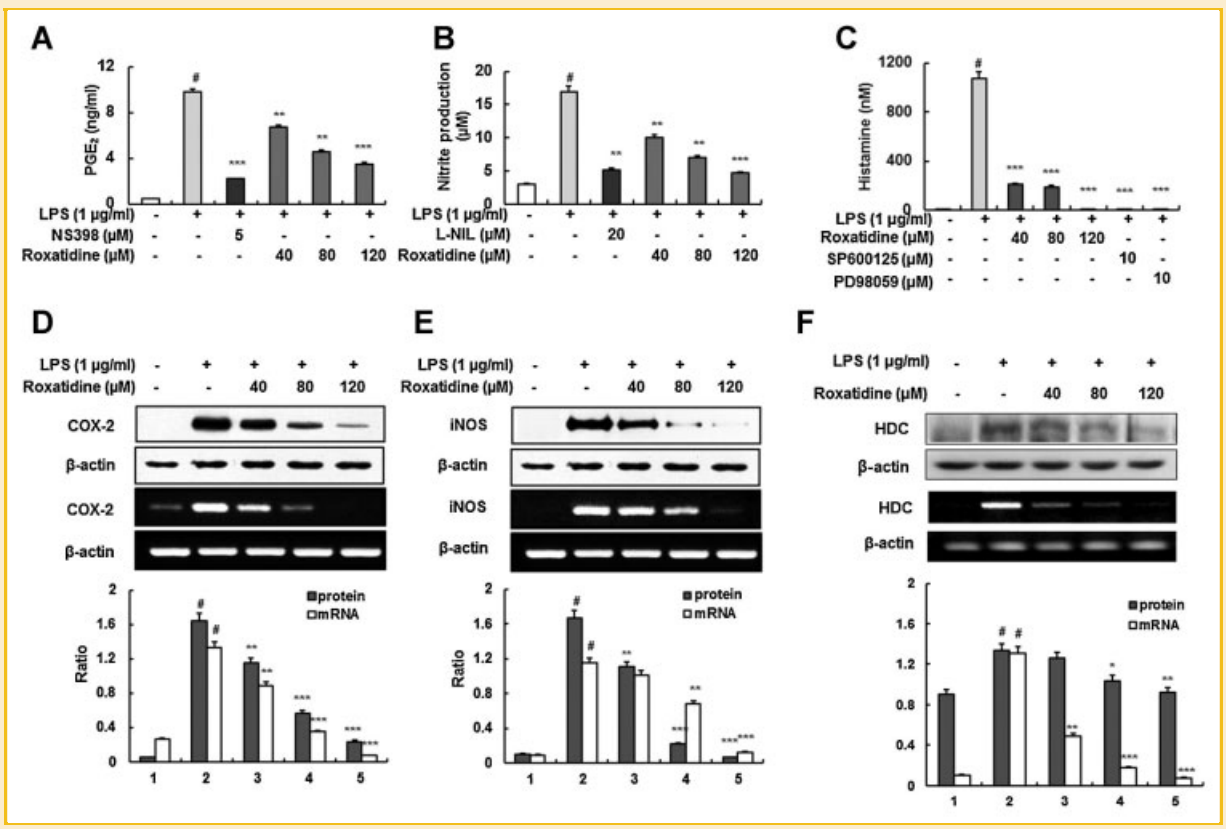

Fig. 1. The effects of roxatidine on LPS-induced production of PGE_2 (A), Nitrite (B), and histamine (C) and the protein and mRNA expressions of COX-2 (D), iNOS (E), and HDC (F) in RAW 264.7 macrophage cells. Following pretreatment with roxatidine (40, 80, and 120 μM) for 1 h, the cells were treated with LPS (1 μg/ml) for 24 h. NS-398 (5 μM), L-NIL (20 μM), SP600125 (10 μM), and PD980959 (10 μM) were used as positive control in the assay (A,B). Lysates were prepared from control or 24 h LPS (1 μg/ml)-stimulated cells alone or LPS plus with different concentration (40, 80, and 120 μM) of roxatidine. Western blot analysis was performed as described in Materials and Methods section. A representative immunoblot of three separate experiments is shown. Total RNA was prepared for the RT-PCR analysis of COX-2, iNOS, and HDC gene expression from RAW 264.7 macrophages stimulated with LPS (1 μg/ml) with/without different concentration of roxatidine for 4 h. The above experiment was repeated three times with similar results (D–F). Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus the LPS-treated group; significant differences between groups were determined using ANOVA and Dunnett's post hoc test.

inhibited the productions of LPS-induced PGE_2, NO, and histamine in a concentration-dependent manner. Furthermore, these inhibitory effects of roxatidine could not be attributed to its cytotoxicity, because roxatidine concentrations that suppressed LPS-induced NO and PGE_2 production did not affect cell viability as determined by MTT assays (Supplementary Fig. 1). We used NS398 (5 μM) and L-NIL (10 μM) as positive controls to inhibit the productions of PGE_2 and NO, respectively. In addition, the JNK inhibitor SP600125 and the ERK inhibitor PD98059 were also used as positive controls to inhibit histamine production in RAW macrophages. Furthermore, to investigate whether these effects of roxatidine correlated with the expressions of corresponding genes, the protein and mRNA levels of COX-2, iNOS, and HDC were determined using Western blot and RT-PCR, respectively. It was found that roxatidine dose-dependently inhibited the LPS-induced expressions of COX-2, iNOS, and HDC at both the protein and mRNA levels (Fig. 1D–F).

ROXATIDINE INHIBITED THE LPS-INDUCED PRODUCTIONS AND EXPRESSIONS OF TNF-α, IL-1β, IL-6, AND VEGF-1 IN RAW 264.7 CELLS

Enzyme immunoassays and RT-PCR were used to examine the effects of roxatidine on the productions of pro-inflammatory cytokines induced by LPS. Pretreatment of cells with 80 μM roxatidine for 1 h considerably reduced LPS-induced productions of TNF-α, IL-1β, and IL-6 by 63.2%, 43.8%, and 48.3%, respectively (Fig. 2A). Furthermore, roxatidine also dose-dependently reduced the LPS-induced mRNA expressions of these entities (Fig. 2B). These results suggest that the inhibitory effects of roxatidine on the LPS-induced production of pro-inflammatory cytokines take place at the transcription level.

Since it is known LPS induces significant NF-κB-dependent VEGF-1 expression in human macrophages [Kiriakidis et al., 2003],

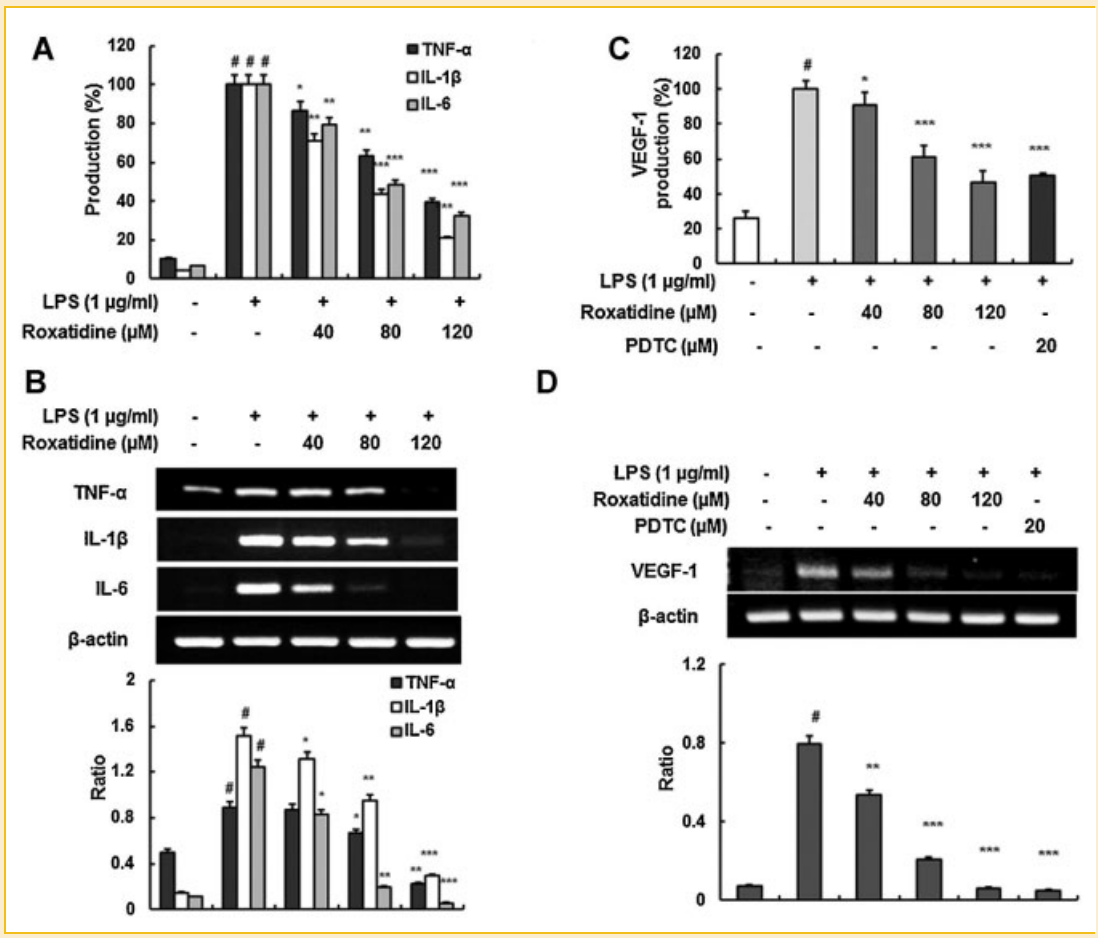

Fig. 2. The effects of roxatidine on the production (A) and mRNA expression (B) of TNF-α, IL-1β, and IL-6 in RAW 264.7 macrophages. The effects of roxatidine on LPS-induced production (C) and mRNA expression (D) of VEGF-1 in RAW 264.7 macrophages. Following pretreatment with roxatidine (40, 80, 120 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 24 h. Cytokines and VEGF-1 were detected as described in Materials and Methods section (A,C). Cells were treated with LPS (1 μg/ml) for 4 h in the presence or absence of roxatidine, and the expressions of the mRNAs of TNF-α, IL-1β, IL-6, and VEGF-1 were determined by RT-PCR (B,D). The data shown are representative of three independent experiments. Data are presented as means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ versus LPS-treated group; the significances of differences were determined using ANOVA and Dunnett's post hoc test.

and that roxatidine suppresses VEGF-1 levels in tumor tissues from mice implanted colon cancer [Tomita et al., 2003], we also examined whether roxatidine inhibits the LPS-induced VEGF-1 production and mRNA levels in RAW 264.7 cells by performing immunoassays and RT-PCR, respectively. Cells were treated with roxatidine (40, 80, or 120 μM) for 1 h before exposing them to 1 μg/ml of LPS for 24 h. We found that roxatidine strongly and dose-dependently inhibited LPS-induced VEGF-1 production (Fig. 2C) and mRNA expression (Fig. 2D). PDTC (20 μM; a NF-κB inhibitor) was used as a positive control. The observed inhibitory effects of roxatidine on pro-inflammatory mediator production were not likely to have been caused by its cytotoxicity, because the roxatidine concentration that suppressed these pro-inflammatory mediators did not affect cell viability, as determined by the MTT assay (Supplementary Fig. 1).

ROXATIDINE REDUCED LPS-INDUCED DNA BINDING AND THE TRANSCRIPTIONAL ACTIVITY OF NF-κB IN LPS-INDUCED RAW 264.7 CELLS

Accumulated evidence indicates that NF-κB is a major transcription factor that modulates the expressions of pro-inflammatory proteins, such as, iNOS, COX-2, and HDC, and those of pro-inflammatory cytokines, such as, TNF-α, IL-1β, IL-6, and VEGF-1, induced by LPS [Bonizzi and Karin, 2004]. Thus, we hypothesized that the NF-κB signaling pathway is involved in the roxatidine-mediated inhibitions of COX-2, iNOS, and pro-inflammatory cytokines. To explore this possibility, we performed electrophoretic mobility shift assays (EMSA) and luciferase reporter gene assays in LPS-stimulated RAW 264.7 macrophages. To examine the DNA-binding activities of NF-κB, nuclear extracts isolated from LPS-stimulated RAW 264.7 cells pretreated or not with roxatidine were reacted with NF-κB specific ^{32}P-labeled oligonucleotides. As shown in Figure 3A, the DNA-binding activity of NF-κB was markedly increased after treatment with LPS alone, but this binding was significantly and dose-dependently reduced in the presence of roxatidine. PDTC (20 μM) was used as a positive control. The specific interaction between DNA and NF-κB was demonstrated by competitive inhibition using excess unlabelled NF-κB oligonucleotides, as treatment with an 80-fold excess of unlabeled NF-κB oligonucleotides was found to block DNA to NF-κB binding completely. To examine the effect of roxatidine on the transcriptional activity of NF-κB, a reporter gene assay was performed using a pNF-κB-luc plasmid, which was generated by inserting four spaced NF-κB binding sites into pLuc-promoter vector. Accordingly, pNF-κB-luc plasmid was transiently transfected into RAW 264.7 cells, which were then stimulated with LPS either in the presence or in the absence of roxatidine. Upon exposure to LPS alone, luciferase activity in transfected cells increased by up to 10-fold that of the basal level, but pretreatment with roxatidine significantly and dose-dependently inhibited LPS-induced luciferase activity (39% inhibition at 40 μM, 67% at 80 μM, and 77% at 120 μM) (Fig. 3B). However, roxatidine had no effect on the activation of LPS-induced AP-1, another representative transcriptional factor associated with inflammatory responses (data not shown).

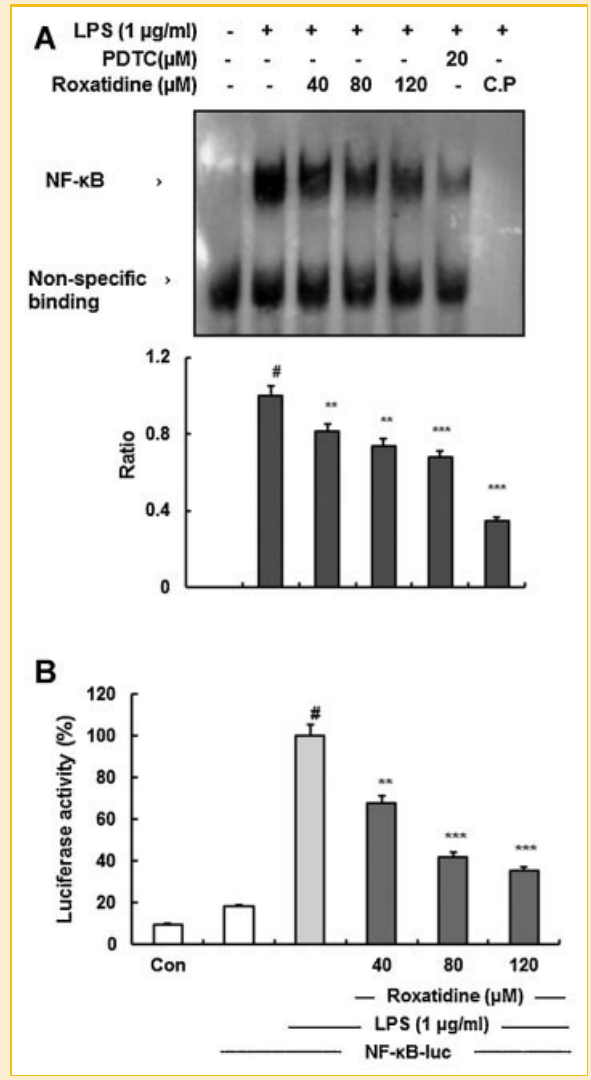

Fig. 3. Effects of roxatidine on LPS-induced NF-κB-DNA binding (A) and transcriptional activity (B) in RAW 264.7 macrophages. Following pretreatment with roxatidine (40, 80, and 120 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 1 h. Nuclear extracts from cells were prepared and used for analysis of NF-κB-DNA binding using EMSA. The arrow indicates the position of NF-κB-DNA binding. The data shown are representative of three independent experiments (A). Cells were transiently transfected with pNF-κB-luc reporter and then pretreated with roxatidine (40, 80, and 120 μM) for 1 h. LPS (1 μg/ml) was then added and cells were further incubated for 24 h. Control (CON) values were obtained in the absence of LPS and roxatidine. Cells were then harvested and luciferase activities were determined using a Promega luciferase assay system and a luminometer (B). Data are presented as means ± SD of three independent experiments; $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$ $^{***}P < 0.001$ versus LPS-treated group; the significances of differences were determined using ANOVA and Dunnett's post hoc test.

ROXATIDINE PREVENTED THE LPS-INDUCED NUCLEAR TRANSLOCATION OF NF-κB IN RAW 264.7 CELLS

RAW 264.7 cells were pretreated with roxatidine for 1 h, stimulated with LPS (1 μg/ml) for 1 h, and nuclear and cytosolic fractions were then separated. As shown in Figure 4A, roxatidine significantly and

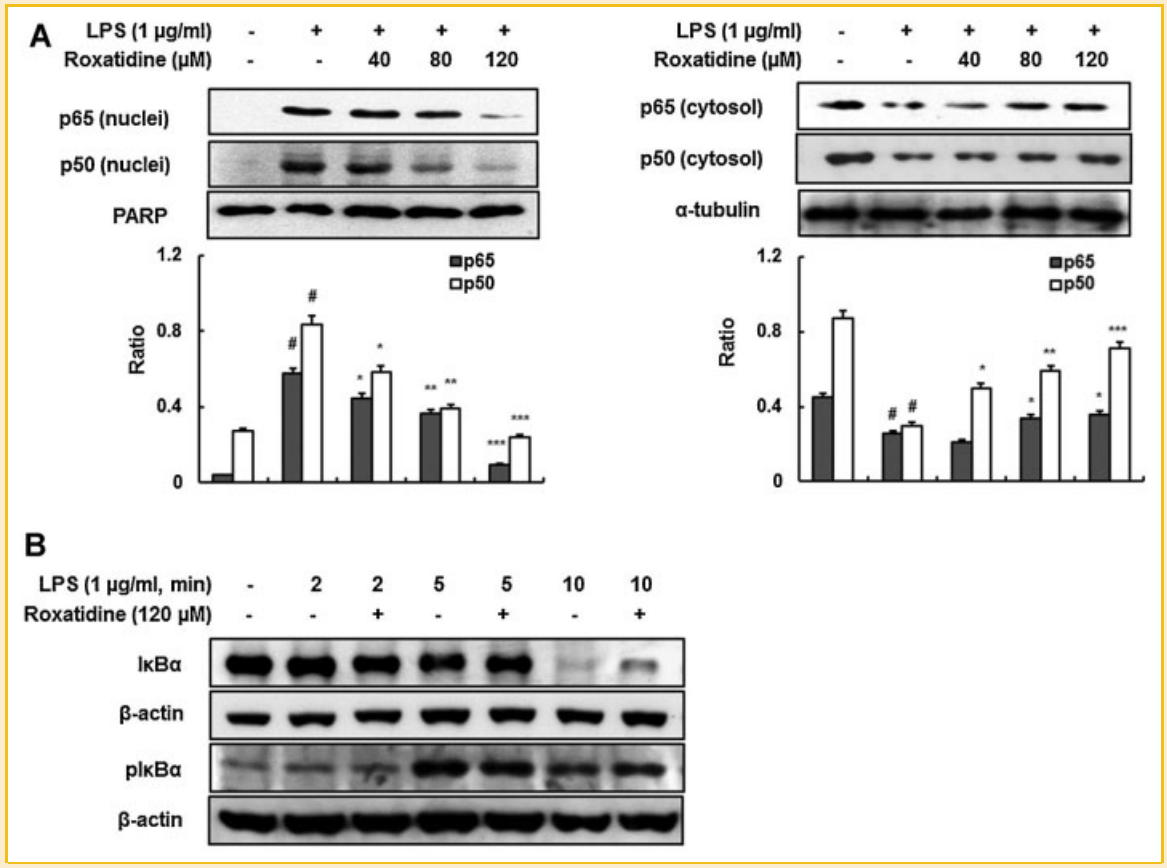

Fig. 4. The effects of roxatidine on LPS-induced NF-κB translocation (A) and IκBα activation (B) in RAW 264.7 macrophages. Cells were treated with LPS (1 μg/ml) alone or in combination with roxatidine (40, 80, and 120 μM) for 1 h. Nuclear extracts were prepared for the Western blot analysis of NF-κB protein using specific anti-p65 and anti-p50 monoclonal antibodies in nuclear and cytosolic fractions, respectively, and PARP and α-tubulin were used as internal control for nuclear and cytosolic fraction, respectively (A). Following pretreatment with roxatidine (120 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 2–10 min. Total proteins were prepared and Western blotting was performed using specific IκBα or p-IκBα antibodies. The data shown are representative of three independent experiments (B). Data are presented as the means ± SD of three independent experiments; $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ versus LPS-treated group; the significances of differences were determined using ANOVA and Dunnett's post hoc test.

concentration-dependently attenuated the nuclear translocations of p65 and p50 (subunits of NF-κB) versus LPS only treated cells. In addition, we investigated whether roxatidine could inhibit the LPS-induced degradation and phosphorylation of IκBα by Western blotting. LPS-stimulated RAW 264.7 cells were pretreated with 120 μM roxatidine (shown by our previous work to be an effective concentration) for 1 h and then stimulated with LPS (1 μg/ml) for 2–10 min. It was found that cytoplasmic IκBα levels were significantly degraded at 10 min after adding LPS and that the phosphorylation of IκBα was increased at 5–10 min. However, roxatidine did not affect the degradation or phosphorylation of IκBα at these times after LPS treatment (Fig. 4B). These findings suggest that roxatidine suppresses the LPS-induced nuclear translocation of NF-κB without affecting IκB degradation or phosphorylation.

ROXATIDINE INHIBITED THE LPS-INDUCED PHOSPHORYLATION OF P38 MAP KINASE IN RAW 264.7 CELLS

In order to investigate whether the inhibitions of inflammatory responses by roxatidine are mediated through a MAPK pathway, we examined the effect of roxatidine on the LPS-induced phosphorylations of MAPKs in LPS-stimulated RAW 264.7 cells by Western blotting. As shown in Figure 5A, LPS significantly induced the phosphorylations of ERK 1/2, JNK, and p38 MAP kinase within 20 min, and roxatidine pretreatment suppressed the LPS-induced activation of p38 MAP kinase, but did not affect the phosphorylations of ERK 1/2 or JNK. Furthermore, total ERK 1/2, JNK, and p38 MAP kinase levels were unaffected by LPS or by LPS plus roxatidine. MAPK-activated protein kinase 2 (MAPKAPK2, MK2) is a downstream target of p38 MAPK and is essentially required for LPS-induced inflammation [Kotlyarov et al., 1999]. Furthermore, the major post-transcriptional effects of p38 MAPK are medicated by MK2 [Kotlyarov and Gaestel, 2002]. Therefore, we hypothesized that the effect of roxatidine on the p38 MAPK/MK2 signaling pathway might be involved in the phosphorylation of p38 MAPK induced by LPS. Accordingly, we examined the effect of roxatidine on MK-2 phosphorylation in LPS-stimulated RAW macrophages, and found that roxatidine suppressed the p38 MAPK/MK2 signaling pathway in LPS-induced macrophages.

Since the activation of IKK is a key step in the modulation of NF-κB activation via the phosphorylation of IκB [Mercurio et al., 1997; Zandi

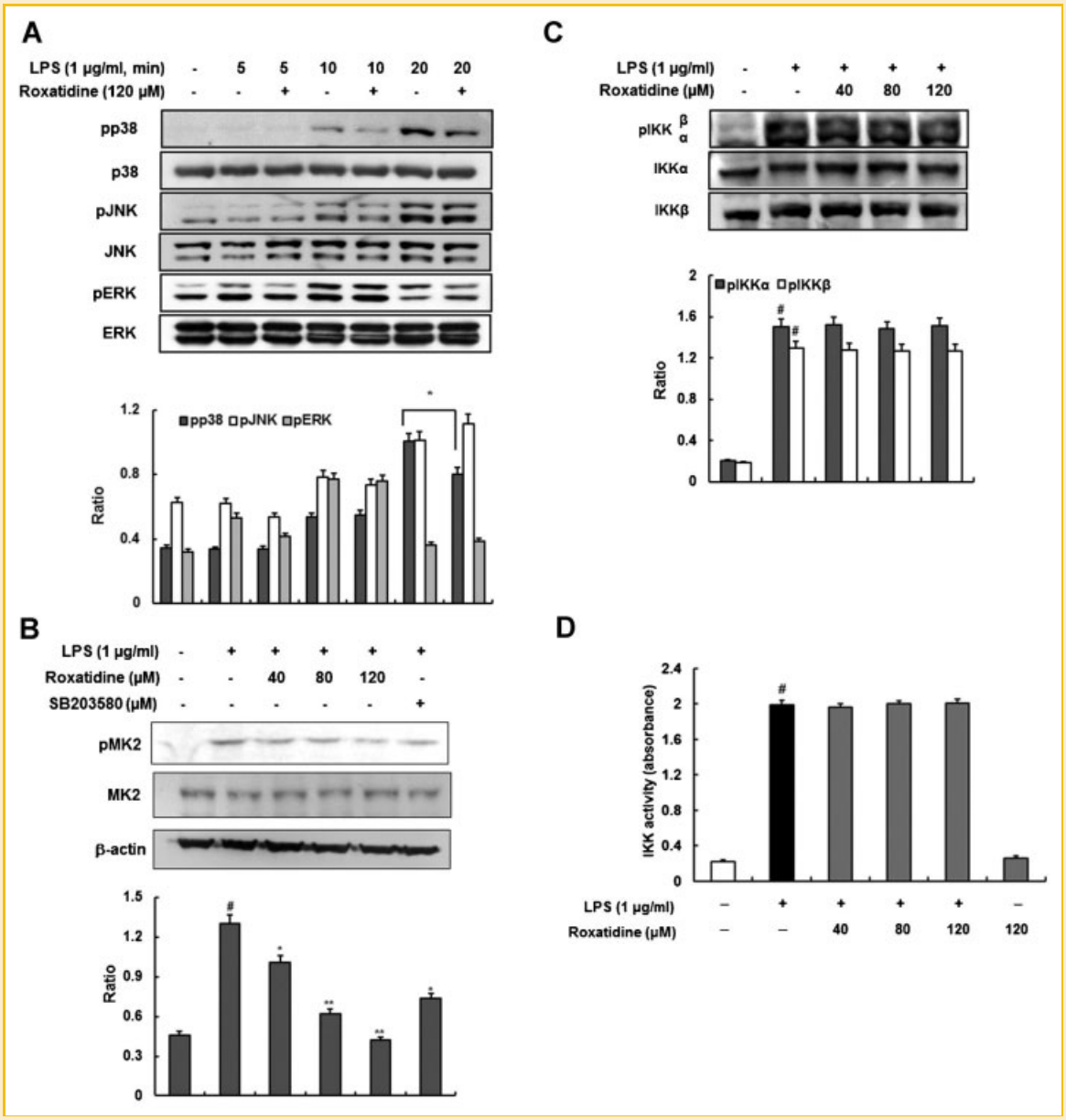

Fig. 5. The effects of roxatidine on LPS-induced MAPK (A), MK2 phosphorylation (B), IKK phosphorylation (C) in RAW 264.7 macrophages, and pIKK-β activation (D) in THP-1 macrophages. Following pretreatment with roxatidine (40, 80, and 120 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 5–20 min. Whole cell lysates were analyzed by Western blotting using antibodies against activated MAPK (A). Western blotting using antibodies against activated MK2 (B). Western blotting using antibodies against activated IKK-α, -β (C). After 24 h of incubation with PMA, THP-1 cells were pretreated with roxatidine for 1 h and then stimulated with LPS (1 μg/ml). After 10 min of stimulation, Cells were lysed and the phospho-IKK-β activity was determined using the PathScan® Phospho-IKKβ Sandwich ELISA kit (D). The data shown are representative of three independent experiments. Data are presented as the means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ versus LPS-treated group; the significances of differences were determined using ANOVA and Dunnett's post hoc test.

et al., 1997; Yamaoka et al., 1998], we examined whether roxatidine inhibits LPS-induced IKK activation by Western blotting using IKK-α, -β, and phosphorylated IKK-α/β antibodies. In addition, a pIKK-β sandwich ELISA kit was used to measure IKK-β activities using differentiated THP-1 cells induced by PMA. Briefly, cells were pre-treated with roxatidine (40, 80, or 120 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 10 min. As shown in Figure 5C,D, LPS was found to induce IKK-α/β phosphorylation and pIKK-β activity strongly, whereas roxatidine did not affect the LPS-induced phosphorylation of IKK-α/β, total IKK-α or IKK-β levels, or pIKKβ activity.

EFFECTS OF SIGNALING INHIBITORS ON LPS-INDUCED INFLAMMATORY RESPONSES IN RAW 264.7 CELLS

To confirm whether the inhibitory effects of roxatidine on LPS-induced inflammatory mediators are due to its influence on the

activations of NF-κB and p38 MAP kinase signaling, we evaluated the effects of co-treatments of LPS with various signaling inhibitors (PDTC, SN50, or SB203580) on the NF-κB signaling pathway and on the productions of NO and PGE_2 in RAW 264.7 cells. It was found that the broad NF-κB inhibitor PDTC suppressed both the translocation of the NF-κB subunit p65 and the degradation of IκBα, whereas the specific NF-κB translocation inhibitor SN50 only inhibited the translocation of p65 (Fig. 6A). In accord with previous reports, SB203580 inhibited NF-κB transcriptional activity without affecting NF-κB translocation or IκBα degradation (Fig. 6A,C) [Wesselborg et al., 1997; Vermeulen et al., 2002]. However, all three signaling inhibitors examined significantly down-regulated the productions of NO and PGE_2 in LPS-stimulated RAW 264.7 cells (Fig. 6B). Furthermore, the transcriptional activity of NF-κB was significantly inhibited by SB 203580 (Fig. 6). We then confirmed p65 phosphorylation by Western blotting. Roxatidine was found to have an inhibitory effect on p65 phosphorylation, and SB 203580 inhibited p65 phosphorylation but did not inhibit p65 translocation. The results of this experiment correspond with previous experiment, in which SB203580 blocked LPS-induced NF-κB activation and tyrosine phosphorylation of p65 NF-κB in RAW 264.7 macrophages [Kim et al., 2006]. These results suggest that the inhibitions of NO,

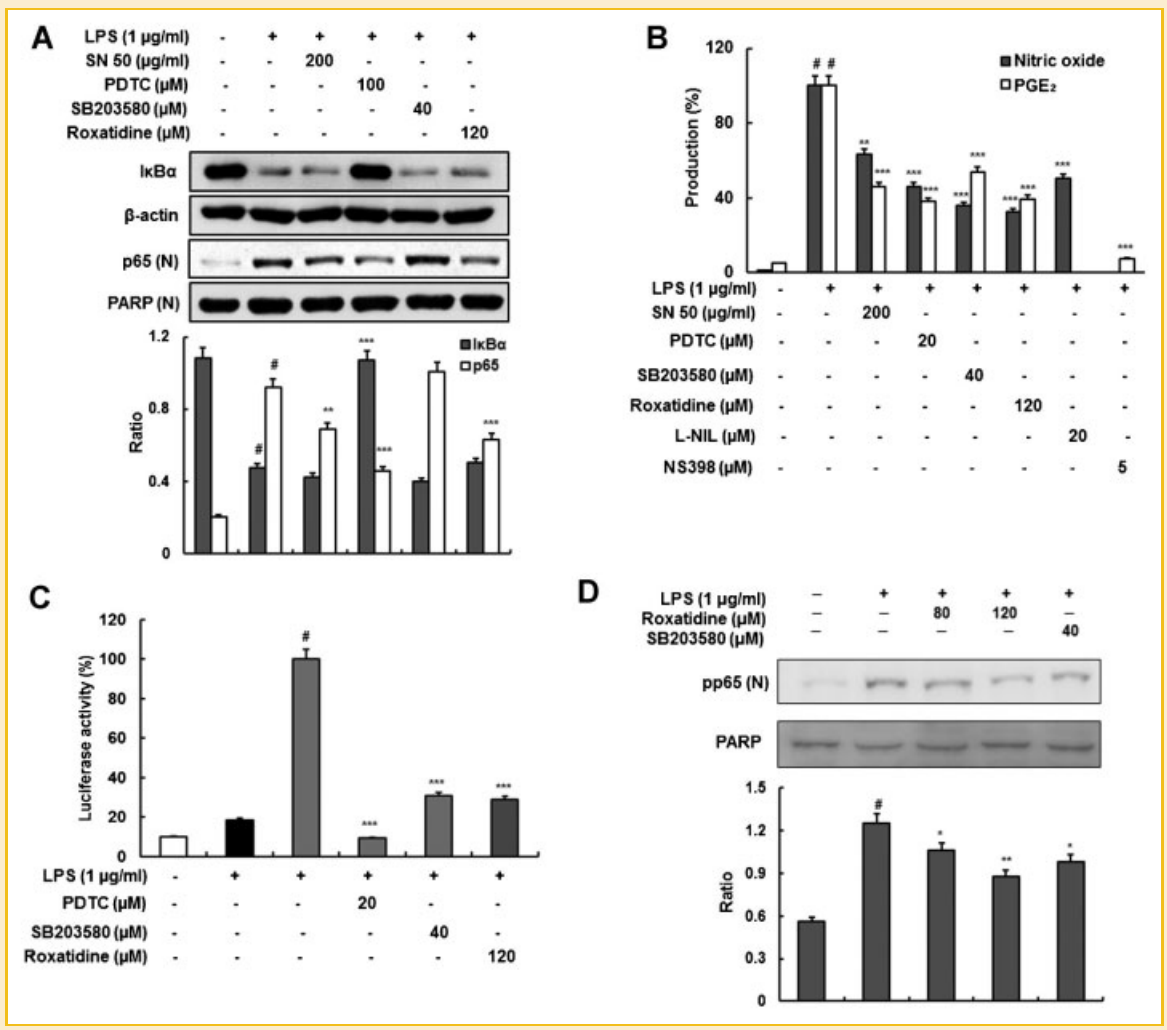

Fig. 6. The effects of signaling inhibitors on LPS-induced NF-κB translocation (A), on the production of NO and PGE_2 (B), NF-κB activation (C), and NF-κB phosphorylation (D) in RAW 264.7 macrophages. Cells were treated with LPS (1 μg/ml) alone or in combination with SN 50 (200 μg/ml), PDTC (100 μM), SB203580 (40 μM), or roxatidine (120 μM) for 1 h. Proteins were prepared and Western blot was performed using specific IκBα and p65 antibodies (A). After pretreatment with SN 50 (200 μg/ml), PDTC (20 μM), SB203580 (40 μM), or roxatidine (120 μM) for 1 h, cells were treated with LPS (1 μg/ml) for 24 h. NS-398 (5 μM) and L-NIL (20 μM) were used as positive controls (B). Cells were transiently transfected with pNF-κB-luc reporter and then pretreated with PDTC (20 μM), SB203580 (40 μM), or roxatidine (120 μM) for 1 h. LPS (1 μg/ml) was then added and cells were further incubated for 24 h. Control (CON) values were obtained in the absence of LPS and roxatidine. Cells were then harvested and luciferase activities were determined using a Promega luciferase assay system and a luminometer (C). Cells were treated with LPS (1 μg/ml) alone or in combination with roxatidine (80 and 120 μM), or SB203580 (40 μM) for 1 h. Nuclear extracts were prepared for the Western blot analysis of p65 phosphorylation using specific anti-pp65 monoclonal antibodies in nuclear, and PARP were used as internal control for nuclear fraction (D). Data are presented as the means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus LPS-treated group; significances were determined using ANOVA and Dunnett's post hoc test.

PGE_2, and histamine production by roxatidine are mediated by the suppression of p38 MAP kinase/MK2 phosphorylation and NF-κB activation via the inhibition of the nuclear translocation of p65.

DISCUSSION

Peptic ulcers are the most commonly encountered type of ulcer, and are caused by *Helicobacter pylori* infections or by the excessive administration of NSAIDs [Huang et al., 1998; Uemura et al., 2001]. The pathogenesis of gastric ulceration is a multifactorial process that involves inflammation, acid-induced necrosis, oxidative damage, apoptosis, and the loss of gastroprotection [Atherton, 2006; Naito and Yoshikawa, 2006; Solmaz et al., 2009]. Roxatidine is widely used to treat gastric and duodenal ulcers, because it inhibits gastric acid secretion by acting as a H_2-receptor antagonist. In addition, roxatidine is known to possess anti-oxidant properties [Kirkova et al., 2006], and to have a suppressive effect on the growth of colon cancer implants in mice, because it reduces VEGF-1 expression and thus, inhibits angiogenesis [Tomita et al., 2003]. However, the molecular targets and the mechanisms underlying its anti-inflammatory activity have not been well characterized. The present study demonstrates for the first time that the anti-inflammatory properties of roxatidine are due to the negative regulations of the LPS-stimulated p38 MAP kinase/MK-2 and NF-κB pathways in RAW 264.7 macrophages.

LPS stimulates macrophages to produce iNOS, COX-2, HDC, and pro-inflammatory cytokines, such as, TNF-α, IL-1β, and IL-6 (Janeway and Medzhitov, 2002). Furthermore, these pro-inflammatory mediators play key roles in the pathogenesis of various acute and chronic inflammatory diseases. Thus, blocking the effects of pro-inflammatory mediators offers an attractive therapeutic strategy. In the present study, we tested the possibility that roxatidine acts as an anti-inflammatory agent by attenuating the productions of VEGF-1 and pro-inflammatory mediators, such as, PGE_2, NO, histamine, TNF-α, IL-1β, and IL-6. Our findings provide evidence that roxatidine significantly inhibits COX-2, iNOS, and HDC expressions by downregulating their transcriptions in LPS-stimulated RAW 264.7 macrophages. In this regard, a number of anti-inflammatory drugs are known to target NF-κB and MAPK and to control the transcriptions of COX-2, iNOS, HDC, and pro-inflammatory cytokines, since the promoters of these pro-inflammatory genes possess an active NF-κB binding site, and because the p38 MAP kinase pathway is associated with NF-κB-dependent gene regulation [Carter et al., 1999; Yamamoto and Gaynor, 2001; Kaminska, 2005]. Furthermore, it has been reported that VEGF-1 is regulated by NF-κB in LPS-stimulated human primary macrophages [Kiriakidis et al., 2003]. Therefore, in the present study, we evaluated the effect of roxatidine on NF-κB activation. EMSA for DNA-binding activity and reporter gene assays conducted to determine the transcription activity of NF-κB revealed that roxatidine prevents the activation of NF-κB. In addition, pretreatment with roxatidine dose-dependently inhibited the translocations of the activated NF-κB subunits, p65 and p50, to the nucleus. As mentioned above, the activation of NF-κB results in the phosphorylation, ubiquitination, and proteasome-mediated degradation of IκB, and the subsequent translocation of NF-κB to the nucleus [Karin, 1999]. However, roxatidine was found to have no effect on the LPS-induced degradation or phosphorylation of IκBα. Furthermore, because IKK complex activation is required for IκBα ubiquitination, which phosphorylates IκBα at Ser 32 and Ser 36 [Chen et al., 1996], we investigated the inhibitory effect of roxatidine on the phosphorylation of IKK in LPS-stimulated cells. However, roxatidine pretreatment did not inhibit IKK-α/β phosphorylation.

In stimulated macrophages, MAPK phosphorylation is a prerequisite of NO and pro-inflammatory cytokine production [Schindler et al., 2007; Choi et al., 2008]. Moreover, several studies have reported that the activation of NF-κB is triggered by MAPK [Rao et al., 2002; Schindler et al., 2007]. In particular, the p38 MAP kinase pathway is required for the productions and activities of multiple pro-inflammatory cytokines, and for the inductions of key inflammatory enzymes like COX-2 and iNOS [Ajizian et al., 1999; Dean et al., 1999]. Thus, we examined the effects of roxatidine on the LPS-induced phosphorylations of MAPK in RAW 264.7 cells. We found that roxatidine prevented the LPS-induced phosphorylation of p38 MAP kinase, but that it had no effect on the phosphorylations of ERK and JNK.

To confirm that whether the inhibitory effects of roxatidine on LPS-induced inflammatory mediators are due to its influence on the activations of NF-κB and p38 MAP kinase signaling, we examined its effects on LPS-induced inflammatory response using the specific inhibitors (SN50, PDTC, and SB203580). The cell permeable synthetic peptide SN50 carrying the NLS, has been reported to specifically inhibit the nuclear translocation of NF-κB in response to LPS and TNF-α [Lin et al., 1995]. Roxatidine or SN50 suppressed the translocation of NF-κB and the subsequent productions of NO and PGE_2, but failed to affect the degradation of IκBα. Furthermore, SB203580, a p38 MAP kinase specific inhibitor, significantly suppressed the productions of NO, PGE_2, and NF-κB transcriptional activity, but did not effect on LPS-induced IκBα degradation and or NF-κB translocation. These observations suggest that p38 MAP kinase regulates NF-κB transcriptional activity by phosphorylating and activating p300 (a coactivator of NF-κB after its translocation into the nucleus) without affecting the translocation and DNA-binding activities of NF-κB [Vermeulen et al., 2002; Saha et al., 2007]. In addition, Schindler et al. found that p38 MAP kinase mediates the stabilizations of the mRNAs of several inflammatory genes by interacting with MK2, a critical p38 MAP kinase substrate during the mediation of pro-inflammatory response [Schindler et al., 2007]. These findings suggest that roxatidine suppresses the LPS-induced phosphorylation of p38 MAP kinase and the nuclear translocation of NF-κB without influencing IκB degradation.

Since roxatidine inhibited NF-κB transcriptional activity and the phosphorylation p65 without affecting NF-κB translocation or IκBα degradation (as did SB203580, a p38 MAP kinase inhibitor), its ability to inhibit the productions of inflammatory mediators could be the result of the inhibition of p38 MAP kinase/MK2 and of the inhibition of NF-κB translocation and phosphorylation. Accumulated data also showed that although p38 MAP kinase and NF-κB activation are mediated by separate pathways, convergence may occur further downstream in the cell nucleus [Wesselborg et al.,

1997]. Our data showed that the inhibition of p38 MAP kinase may contribute to NF-κB inactivation via the MK2 pathway.

To determine whether the inhibitory effect of roxatidine on the nuclear translocation of NF-κB is related to H_2 receptor, we examined the effects of various H_2 receptor antagonists on LPS-induced NF-κB translocation in RAW 264.7 macrophages. Each antagonist was found to have inhibitory effects on NF-κB transcription in LPS-induced RAW macrophages (Supplementary Fig. 2). Further work is required to determine whether the anti-inflammatory effects of H_2 receptor antagonists involve the NF-κB pathway.

In summary, the present study shows that roxatidine attenuates the LPS-induced productions of inflammatory mediators, such as, COX-2, iNOS, and histamine, and of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and VEGF-1) in LPS-stimulated RAW 264.7 cells by suppressing the activations of NF-κB and p38 MAP kinase. These findings provide a partial molecular explanation for the anti-inflammatory properties of roxatidine.

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