Triptolide suppresses interleukin-1ß-induced human ß-defensin-2 mRNA expression through inhibition of transcriptional activation of NF-κB in A549 cells

BYEONG-CHURL JANG[1], KI-JO LIM[1], IN-HAK CHOI[2], MIN-HO SUH[1], JONG-GU PARK[1], KYO-CHUL MUN[1], JAE-HOON BAE[1], DONG-HOON SHIN[1] and SEONG-IL SUH[1]

[1]Chronic Disease Research Center and Institute for Medical Science, Keimyung University School of Medicine, Daegu 700-712; [2]Department of Microbiology and Center for Viral Disease Research, Inje University College of Medicine, Busan 614-735, Korea

Received October 13, 2006; Accepted November 30, 2006

Abstract. The immunosuppressive effect of triptolide has been associated with suppression of T-cell activation. However, the immunosuppressive effects of triptolide on innate immunity in the epithelial barrier remain to be elucidated. Human ß-defensin (HBD)-2 is an inducible antimicrobial peptide and plays an important role in the innate immunity. We have previously demonstrated that IL-1ß induced HBD-2 mRNA expression in A549 cells through activation of nuclear factor-κB (NF-κB) transcriptional factor as well as p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), or phosphatidylinositol-3-kinase (PI3K). In this study, we investigated effects of triptolide on IL-1ß-induced HBD-2 mRNA expression in A549 cells. Triptolide inhibited IL-1ß-induced HBD-2 mRNA expression in a dose-dependent manner. Addition of triptolide did not suppress activation of p38 MAPK, JNK, or PI3K in response to IL-1ß. Triptolide inhibited IL-1ß-induced MAPK phosphatase-1 expression at the transcriptional level and resulted in sustained phosphorylation of JNK or p38 MAPK, explaining the little effect of triptolide on IL-1ß-induced phosphorylation of these kinases. Although triptolide partially suppressed IL-1ß-mediated degradation of IκB-α and nuclear translocation of p65 NF-κB, triptolide potently inhibited NF-κB promoter-driven luciferase activity in A549 cells. These results collectively suggest that the inhibitory effect of triptolide on IL-1ß-induced HBD-2 mRNA expression in A549 cells seems to be at least in part mediated through nuclear inhibition of NF-κB transcriptional activity, but not inhibition of p38 MAPK, JNK, or PI3K. This inhibition may explain the ability of triptolide to diminish innate immune response.

Correspondence to: Dr Seong-Il Suh, Chronic Disease Research Center and Institute for Medical Science, Keimyung University School of Medicine, #194 DongSan-Dong, Jung-Gu, Daegu 700-712, Korea
E-mail: seong@dsmc.or.kr

Key words: Triptolide, IL-1ß, Human-ß-defensin-2, MKP-1, NF-κB, A549 cells

Introduction

The defensin family of antimicrobial peptides is widely distributed among various species and participates primarily in the innate immune system against microorganisms (1). In addition to antimicrobial activity, defensins also possess chemotactic activity for immature dendritic cells, memory T-cells, or monocytes, and the stimulatory activity for cytokine production (2,3). These suggest that the human defensin family serves as a linker between the innate and acquired immune responses. In humans, based on the structural characteristics, defensins are classified as α- and ß-defensins. Human α-defensins are mainly expressed in granulocytes and intestinal Paneth cells whereas human ß-defensins (HBDs) are predominantly expressed by various epithelial cells (1,4-6). Six HBDs have been described so far. HBD-1 is constitutively expressed in epithelial cells and also induced in monocyte-derived macrophages by interferon-γ or lipopolysaccharide (7). HBD-2, -3 or -4 is also inducible by bacterial factors or pro-inflammatory stimuli (5,8,9). HBD-5 and -6 have been identified and known to be specially localized to the epididymis (6).

Epithelial cells are the initial barrier to bacterial infections and play an important role in inflammation by the production of cytokines and other mediators. It has been reported that HBD-2 expression is increased in response to bacterial infections or pro-inflammatory agonists in epithelial cells and is involved in host defense under inflammatory conditions (2,10-13). Evidence to date suggests that induction of HBD-2 is largely dependent of nuclear factor-κB (NF-κB) transcriptional factor and/or mitogen-activated protein kinases (MAPKs) (14-18).

Triptolide is an immunosuppressive and anti-inflammatory agent from Chinese herb *Tripterygium Wilfordii* Hook F (19,20). It has been shown that triptolide inhibits the activity of NF-κB (21-23), down-regulates the expression of NF-κB-

related inflammatory genes (24,25), and suppresses the expression of dual-specificity MAPK phosphatases-1 (MKP-1) (26-28). MAPKs play a critical role in the regulation of cell growth and differentiation in the control of cellular responses to stresses and cytokines. The activities of MAPKs are negatively regulated via dephosphorylation of the conserved tyrosine and threonine residues by a family of MKPs (29). MKP-1, the archetypal MKP, has been shown to preferentially dephosphorylate c-jun N-termian kinase (JNK) or p38 MAPK (26). In a previous report, we have shown that the pro-inflammatory cytokine, IL-1ß induces HBD-2 in human type II A549 pneumocyte cells through activation of NF-κB, phosphatidylinositol-3-kinase (PI3K), p38 MAPK, and JNK (14). However, the regulatory mechanism by which triptolide modulates HBD-2 mRNA expression in response to pro-inflammatory cytokine signals is not fully understood.

In the present study, we have investigated the effect of triptolide on IL-1ß-induced HBD-2 mRNA expression in A549 cells. We showed that triptolide suppressed IL-1ß-induced HBD-2 mRNA expression in A549 cells, and the suppression seemed to be in part associated with inhibition of NF-κB transcriptional activation, but not through modulation of JNK, p38 MAPK, or PI3K activity in response to IL-1ß signal.

Materials and methods

Cell culture. A549 cells (ATCC, CCL 185), a human alveolar type II epithelial cell-like adenocarcinoma cell line, were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Typically, $3x10^5$/ml cells were seeded, and maintained in the tissue culture incubator for 12-16 h before the addition of IL-1ß or other reagents.

Drugs and materials. Antibodies against phospho-p70S6 kinase (p-p70S6K), phospho-AKT (p-AKT), AKT, phospho-extracellular signal-regulated kinase (p-ERK), ERK, phospho-p38 MAPK (p-p38 MAPK), p38 MAPK, phospho-JNK (p-JNK), and JNK were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against MKP-1, inhibitory κB-α (IκB-α), p65 NF-κB, ß-actin, and ß-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human IL-1ß was obtained from R&D Systems (Minneapolis, MN, USA). (Benzyloxycarbonyl)-Leu-Leu-phenylalaninal (ZLLF), MG132, and triptolide were obtained from Calbiochem (La Jolla, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). The expression values of HBD-2 and MKP-1 were quantified by semi-quantitative RT-PCR analysis, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Total cellular RNA was isolated from cells and reverse transcribed using a random hexadeoxynucleotide primer (Perkin Elmer, Branchburg, NJ, USA), and reverse transcriptase (Perkin Elmer) in 20 μl volume. PCR amplification was performed by using 0.25 μl of AmpliTaq DNA polymerase (Perkin Elmer), 1 μl of each 10 mM deoxyribonucleotide triphosphate, 6.25 μl of GeneAmp 10X PCR buffer II (Perkin Elmer), 4 μl of 25 mM $MgCl_2$, 1 μl of each specific sense and anti-sense primer at 25 μM, and water with the hot-start method to enhance the sensitivity and specificity of amplification. The PCR products were analyzed on 1.5% agarose gel. The primer sequences and product sizes were as follows: HBD-2 forward 5'-CCA GCC ATC AGC CAT GAG GGT-3', reverse 5'-GGA GCC CTT TCT GAA TCC GCA-3', 254 bp; MKP-1 forward 5'-GCT GTG CAG CAA ACA GTC GA-3', reverse 5'-CGA TTA GTC CTC ATA AGG TA-3', 450 bp; and GAPDH forward 5'-CGT CTT CAC CAC CAT GGA GA-3', reverse 5'-CGG CCA TCA CGC CAC AGT TT-3', 300 bp.

Plasmids. pNF-κB-Luc reporter vector was obtained from Stratagene (La Jolla, CA, USA). The MKP-1 luciferase DNA was kindly provided by Dr Yusen Liu (30). The HBD-2 promoter was amplified by PCR using sense: 5'-TTGGTACCGCATCAACAGCTAACATCACAC-3' and anti-sense: 5'-TTCTCGAGTACAAGACCCTCATGGCTGA-3'. Both primers were designed based on GeneBank number AF071216, additional restriction sites are indicated by underlining. The synthesized 2320-bp fragments were cloned into TA-vector (Promega, Madison, WI, USA). The 2320-bp fragments of the HBD-2 promoter with the *Kpn*I and *Xho*I sites were subcloned into the promoterless firefly luciferase vector pGL3-Basic (Promega, Madison, WI, USA).

Transfection and luciferase assay. A549 cells were seeded into 35-mm plates 1 day before transfection at the density of $1x10^5$ cells/plate. Cells were transiently transfected with 1 μg reporter constructs and 20 ng Renilla luciferase expression vector pRL-TK (Promega) using Lipofectamine (Promega), according to the protocol provided by the company. Following 24-h post-transfection, cells were exposed to IL-1ß (1 ng/ml) for an additional 6 h. After incubation, cells were harvested, washed and lysed in 200 μl Dual-Luciferase reporter assay reagent (Promega). Firefly and Renilla luciferase activities were measured using a Dual-Luciferase reporter assay kit (Promega) and luminometer. Promoter activity was expressed as relative luciferase activity (fold) normalized to Renilla luciferase activity. Statistical analysis was performed using paired or unpaired Student's t-test. The accepted level of significance was p-value <0.05.

Western blot analysis. Whole cell extracts were prepared in the lysis buffer as described previously (31). For phospho-protein detection, cells were washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 1 mM NaF, and lysed in a buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. To isolate cytosolic and nuclear proteins, cells were homogenized in ice-cold hypotonic buffer (10 mM HEPES, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 1 mM DTT, 200 nM aprotinin) for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a cytosolic fraction. The pellets were homogenized in ice-cold nuclear extract buffer [10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF,

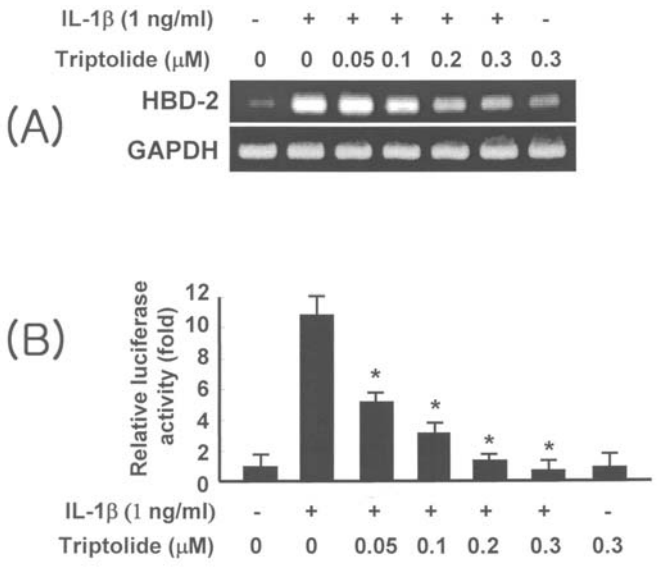

Figure 1. Concentration-dependent down-regulation of IL-1ß-induced HBD-2 mRNA expression and luciferase activity by triptolide in A549 cells. (A) Cells were pretreated for 1 h with the indicated concentrations of triptolide and then cells were treated with IL-1ß (1 ng/ml) for 6 h. Total RNA was prepared and used for HBD-2 or GAPDH RT-PCR. (B) Effect of triptolide on HBD-2 promoter activity induced by IL-1ß. Cells were co-transfected with 1 μg of the HBD-2 promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Cells were pretreated for 1 h with the indicated concentrations of triptolide and then treated with IL-1ß (1 ng/ml) for 6 h. Cells were harvested and assayed for luciferase activity as described in Materials and methods. Data represent mean ± SD (n=3). *p<0.05 vs. IL-1ß.

200 nM aprotinin] for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a nuclear fraction. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as p-ERK (1:2000), ERK (1:2000), p-JNK (1:1000), JNK (1:2000), p-p38 MAPK (1:1000), p38 MAPK (1:2000), p-p70S6K (1:1000), p-AKT (1:1000), AKT (1:2000), MKP-1(1:2000), p65 NF-κB (1:2000), IκB-α (1:2000), ß-actin (1:10000), and ß-tubulin (1:5000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Results

Triptolide suppresses IL-1β-induced HBD-2 mRNA expression at the transcriptional level. We previously reported that IL-1ß induced HBD-2 mRNA expression in A549 cells and its induction was due to increased transcription (14). In order to determine whether triptolide could modulate IL-1ß-induced HBD-2 mRNA expression in A549 cells, cells were pretreated for 1 h with different concentrations of triptolide and then treated with IL-1ß for an additional 6 h. As shown in Fig. 1A, pretreatment with triptolide suppressed IL-1ß-induced HBD-2 mRNA expression in a concentration-dependent manner. To determine whether the down-regulation of IL-1ß-induced HBD-2 mRNA expression by triptolide was due to decreased HBD-2 transcription, HBD-2 reporter gene construct was transiently transfected to A549 cells, and cells were then exposed to different concentrations of triptolide for 1 h, following measurement of the luciferase activity. As shown in Fig. 1A and B, a significant correlation between HBD-2 mRNA expression and luciferase activity was observed. Triptolide suppressed IL-1ß-induced luciferase expression in a concentration-dependent manner (Fig. 1B). These results suggest that down-regulation of IL-1ß-induced HBD-2 mRNA expression by triptolide is associated with HBD-2 transcriptional suppression.

Effects of triptolide on IL-1β-induced activation of JNK, p38 MAPK, and PI3K. In a previous report, we have also shown that activation of p38, JNK, or PI3K is critical for IL-1ß-induced HBD-2 mRNA expression in A549 cells (14). Therefore, we determined the effect of triptolide on IL-1ß-induced activation or phosphorylation of these kinases in A549 cells. As shown in Fig. 2A, treatment with 1 ng/ml IL-1ß resulted in phosphorylation of JNK, p38 MAPK, AKT, and

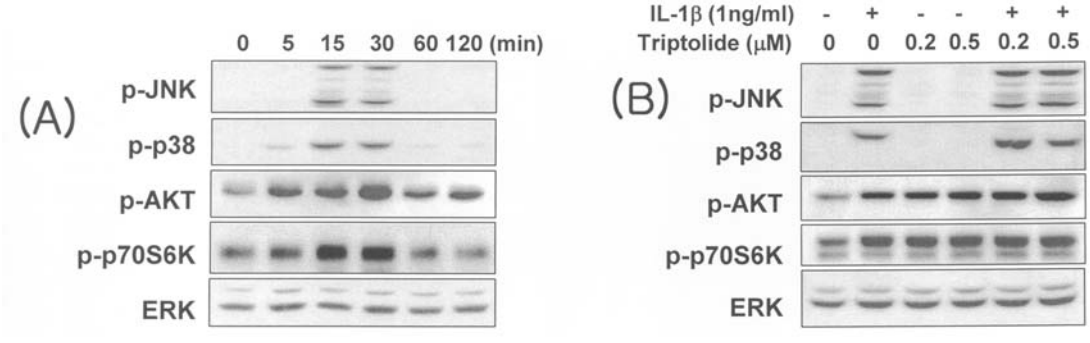

Figure 2. Effects of triptolide on IL-1ß-induced activation of JNK, p38 MAPK, AKT, and p70S6K. (A) A549 cells were treated with IL-1ß (1 ng/ml) for the indicated times. At each time, whole cell lysates were prepared and used for ERK, p-p38 MAPK, p-JNK, p-AKT, or p-p70S6K Western blotting with respective antibodies. (B) Cells were pretreated for 1 h with the indicated concentrations of triptolide and then treated with IL-1ß (1 ng/ml) for 15 min. Whole cell lysates were prepared and used for ERK, p-p38 MAPK, p-JNK, p-AKT, or p-p70S6K Western blotting with respective antibodies.

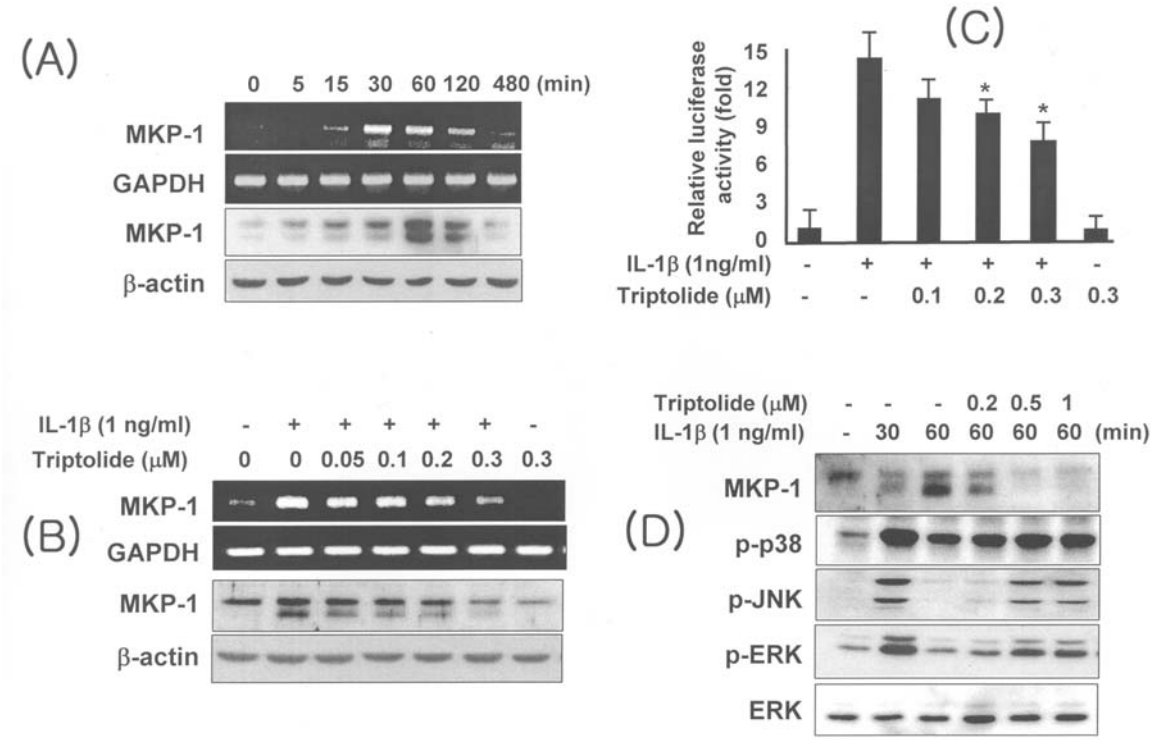

Figure 3. IL-1ß induces MKP-1 expression and its induction is inhibited by triptolide at the transcriptional level. (A) Time-dependent increase of IL-1ß-induced MKP-1 mRNA and protein expression. A549 cells were treated with IL-1ß (1 ng/ml) for the indicated time intervals. Total RNA and whole cell lysates were prepared and analyzed for MKP-1 or GAPDH RT-PCR and MKP-1 or ß-actin Western blotting, respectively. (B) Cells were pretreated with the indicated concentrations of triptolide and then treated with IL-1ß (1 ng/ml) for an additional 1 h. Total RNA and whole cell lysates were prepared and analyzed for MKP-1 or GAPDH RT-PCR and MKP-1 or ß-actin Western blotting, respectively. (C) Effect of triptolide on MKP-1 promoter activity induced by IL-1ß in A549 cells. A549 cells were co-transfected with 1 μg of the MKP-1 promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Cells were pretreated for 1 h with the indicated concentrations of triptolide and then treated with IL-1ß (1 ng/ml) for 60 min. Cells were harvested and assayed for luciferase activity as described in Materials and methods. Data represent mean ± SD (n=3). $^{*}p<0.05$ vs. IL-1ß. (D) Cells were pretreated with the indicated concentrations of triptolide and then treated with IL-1ß (1 ng/ml) for an additional 30 or 60 min. Whole cell lysates were prepared and used for MKP-1, p-ERK, ERK, p-p38 MAPK, or p-JNK Western blotting with respective antibodies.

p70S6K. Activation of these kinases became apparent at 5 or 15 min. In order to link the inhibitory effect of triptolide on IL-1ß-induced HBD-2 mRNA expression to JNK, p38 MAPK, or PI3K pathway, we next determined the effect of triptolide on IL-1ß-induced activation of these kinases. Pretreatment with triptolide had little effect on IL-1ß-induced activation of JNK, p38 MAPK, AKT, or p70S6K (Fig. 2B), suggesting that triptolide-mediated inhibition of IL-1ß-induced HBD-2 mRNA expression is not through JNK, p38 MAPK, or PI3K pathway.

Triptolide down-regulates IL-1ß-induced MKP-1 expression. MAPKs could be rapidly inactivated through dephosphorylation by MKPs. In particular, among MKPs, MKP-1 plays an important role in regulating the activity of MAPKs (27,29). Interestingly, triptolide has been known to suppress MKP-1 expression at the transcriptional and protein level (26-28). Suppression of MKP-1 expression by triptolide can sustain MAPK activation. As shown in Fig. 3A, treatment with IL-1ß in A549 cells time-dependently induced MKP-1 protein expression that was associated with increased MKP-1 mRNA levels. High expression of MKP-1 mRNA and protein were observed at 30 min and 1 h after IL-1ß stimulation, respectively. We next determined whether triptolide inhibited IL-1ß-induced MKP-1 expression in A549 cells. Pretreatment with triptolide for 1 h attenuated IL-1ß-induced MKP-1 protein and mRNA expression in a dose-dependent manner (Fig. 3B). The luciferase data further demonstrated that triptolide suppressed IL-1ß-induced MKP-1 promoter-driven luciferase expression (Fig. 3C), suggesting that triptolide attenuates in part MKP-1 expression at the transcriptional level. We thus asked whether no inhibitory effect of triptolide on IL-1ß-induced phosphorylation of MAPKs as shown in Fig. 2A was due to triptolide-mediated suppression of MKP-1 protein expression. As shown in Fig. 3D, in the absence of triptolide, IL-1ß treatment for 30 min led to strong phosphorylation of ERKs, p38 MAPK, and JNK, while it did not affect expression of MKP-1 protein in A549 cells. On the other hand, in the absence of triptolide, there was high expression of MKP-1 protein and low levels of phosphorylation of ERK, p38 MAPK, or JNK in A549 cells after IL-1ß treatment for 60 min. Interestingly, treatment with triptolide dose-dependently down-regulated IL-1ß-mediated induction of MKP-1 protein that was associated with sustained phosphorylation of these MAPKs in A549 cells (Fig. 3D), suggesting a link between expression level of MKP-1 protein and degree of phosphorylation of these MAPKs in response to IL-1ß. Nevertheless, these results suggest that the inhibitory effect of triptolide on IL-1ß-induced HBD-2 mRNA expression is not associated with inactivation of JNK or p38 MAPK.

Triptolide inhibits IL-1ß-induced NF-κB activation. NF-κB signaling pathway has been implicated in the expression of HBD-2 (14,15,17). Consistent with this, we have previously

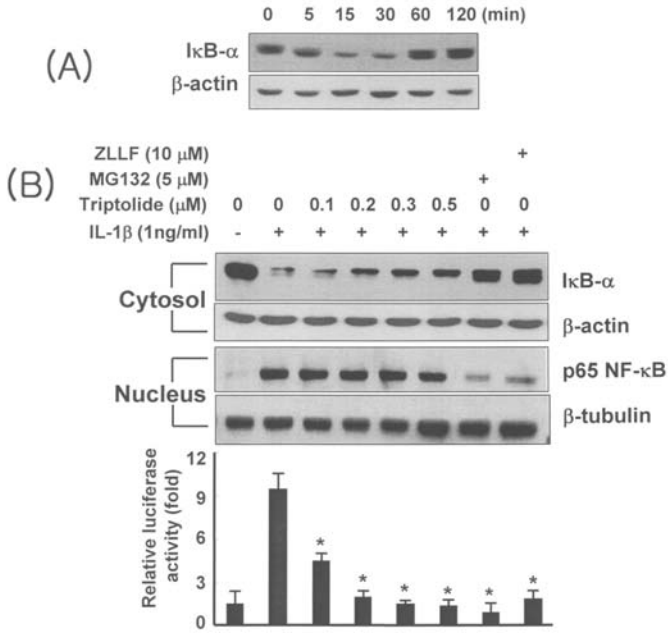

Figure 4. Triptolide inhibits p65 NF-κB transcriptional activity in IL-1ß-treated A549 cells. (A) Cells were treated with IL-1ß for the indicated times. At each time, whole cell lysates were prepared and used for IκB-α or ß-actin Western blotting. (B) Cells were pretreated with MG132 (5 μM), ZLLF (10 μM), or the indicated concentration for triptolide and then treated with IL-1ß (1 ng/ml) for an additional 15 min. Nuclear and cytosolic fractions were prepared and used for p65 NF-κB, IκB-α, ß-tubulin, or ß-actin Western blotting. In addition, cells were co-transfected with 1 μg of pNF-κB-Luc DNA along with 20 ng of control pRL-TK DNA for 24 h. Cells were pretreated with MG132 (5 μM), ZLLF (10 μM), or the indicated concentration of triptolide for 1 h and then treated with IL-1ß (1 ng/ml) for 15 min. After treatment with IL-1ß, cells were harvested and assayed for luciferase activity as described in Materials and methods. Data represent the mean ± SD (n=3). *p<0.05 vs. IL-1ß.

reported that pretreatment with NF-κB inhibitors effectively suppresses IL-1ß-induced HBD-2 mRNA expression (14). Therefore, we next determined whether the inhibitory effect of triptolide on IL-1ß-induced HBD-2 mRNA expression was associated with modulation of NF-κB activity. As shown in Fig. 4A, IL-1ß resulted in the marked degradation of IκB-α, an inhibitory protein of NF-κB, which occurred within 15-30 min. Treatment with IL-1ß also resulted in the marked nuclear translocation of p65 NF-κB and concomitant degradation of IκB-α in the cytosol (Fig. 4B, upper panel). MG132 and ZLLF, the NF-κB inhibitors, effectively inhibited not only nuclear translocation of p65 NF-κB but also NF-κB promoter-driven luciferase activity following IL-1ß treatment (Fig. 4B, upper panel). Interestingly, treatment with triptolide partially suppressed IL-1ß-induced degradation of IκB-α and nuclear translocation of p65 NF-κB in a concentration-dependent manner. Triptolide has been shown to inhibit NF-κB transcriptional activation without inhibiting NF-κB DNA binding activity (21,22). This led us to test the effect of triptolide on NF-κB promoter-driven luciferase activity following treatment with IL-1ß. Indeed, data of the luciferase transfection studies demonstrated that triptolide potently inhibited NF-κB promoter-driven luciferase activity in a dose-dependent manner (Fig. 4B, lower panel). These results suggest that the inhibitory effect of triptolide on IL-1ß-induced HBD-2 mRNA expression in A549 cells may be, in part, due to inhibition of NF-κB pathway.

Discussion

HBD-2, a cationic peptide with antimicrobial and chemotactic properties, is induced by mainly epithelial cells in response to bacterial infection or pro-inflammatory cytokines and plays an important role in innate immunity. The immunosuppressive effect of triptolide has been associated with suppression of T-cell activation (19,20). Treatment of patients with immunosuppressive agents such as corticosteroids could lead to vulnerability to infection by down-regulation of the expression of antimicrobial peptides or impaired leukocyte migration. However, the immunosuppressive effects of triptolide on innate immunity in the epithelial barrier remain to be elucidated. In this study, we show for the first time that triptolide significantly decreases IL-1ß-induced HBD-2 mRNA expression in A549 cells by NF-κB-dependent HBD-2 transcriptional suppression.

MAPKs play an important role in the control of cellular responses to cytokines. Moreover, MAPKs are linked to the expression of HBD-2 induced by various stimuli including IL-1ß (16,18,32). In support of this, we previously demonstrated that p38 MAPK and JNK, but not ERK, involved in IL-1ß-induced HBD-2 mRNA expression in A549 cells (14). It has been shown that triptolide and (5R)-5-Hydroxytriptolide (LLDT-8) decrease the LPS-induced activation of ERK, JNK, or p38 MAPK in RAW 264.7 cells (33,34). However, in this study, we observed that triptolide had little effect on IL-1ß-induced phosphorylation of JNK and p38 MAPK. MKP-1 plays an important role in the activity of MAPKs (29). Interestingly, triptolide is shown to suppress MKP-1 expression and such inhibition of MKP-1 induction by triptolide can delay the dephosphorylation of MAPKs (26). Consistently, we demonstrated that triptolide inhibited IL-1ß-induced MKP-1 expression at the transcriptional level and resulted in sustained phosphorylation of JNK and p38 MAPK in A549 cells, explaining the little effect of triptolide on IL-1ß-induced phosphorylation of these kinases in this study. Nevertheless, these results clearly indicated that the suppressive effect of triptolide on IL-1ß-induced HBD-2 mRNA expression was not associated with inactivation of JNK and p38 MAPK. Involvement of PI3K signaling pathway in IL-1ß-induced HBD-2 mRNA expression has been reported (14,35). PI3K comprises a family of lipid signaling enzymes that promote phosphorylation of phosphoinositides. Miyata *et al* (36) reported that triptolide-induced inhibitory signal for tumor cell proliferation was initiated by the decrease in PI3K activity. In this study, however, triptolide had little effect on IL-1ß-induced activation or phosphorylation of PI3K downstream effectors, AKT and p70S6K, excluding the possibility that triptolide inhibits IL-1ß-induced HBD-2 mRNA expression through PI3K pathway.

Previously we have shown that activation of NF-κB is essential for IL-1ß-induced HBD-2 mRNA expression in A549 cells (14). Triptolide has been regarded as a specific inhibitor of NF-κB and reported to inhibit the expression of NF-κB-regulated genes (20,23-25,33). Nuclear translocation and DNA binding of NF-κB are preceded by the phosphorylation and degradation of IκB-α. We here showed that treatment

with IL-1ß induced the marked degradation of IκB-α and nuclear translocation of p65 NF-κB. It has been shown that triptolide inhibits NF-κB transcriptional activation even in the presence of enhanced nuclear NF-κB DNA binding activity (21,22). Qui *et al* (21) have also demonstrated that the effect of triptolide in inhibiting transcriptional activation in the nucleus after specific binding of NF-κB to DNA might involve interference with recruitment of co-activators that modulate chromatin conformation or inhibition of interaction between p65 NF-κB and RNA polymerase. In the present study, we found that triptolide potently inhibited NF-κB promoter-driven luciferase activity in A549 cells, whereas triptolide partially suppressed IL-1ß-mediated degradation of IκB-α and nuclear translocation of p65 NF-κB. Therefore, it is likely that the inhibitory effect of triptolide on IL-1ß-induced HBD-2 mRNA expression seems to be in part through inhibition of nuclear transcriptional activation of NF-κB.

In conclusion, findings of the present study demonstrate that triptolide down-regulates IL-1ß-induced HBD-2 mRNA expression in A549 cells, and this effect seems to be at least in part mediated through inhibition of transcriptional activation of NF-κB, but not p38 MAPK, JNK, or PI3K. Considering that HBD-2 is an inducible antimicrobial peptide and plays an important role in the innate immune system, the suppression of IL-1ß-induced HBD-2 mRNA expression by triptolide may explain its ability to diminish innate immunity.

Acknowledgments

This study was supported by grant no. R13-2002-028-02001-0 from Basic Research Program of Korea Science and Engineering Foundation (KOSEF) to Chronic Disease Research (CDR) center at Keimyung University.

References

1. Huttner KM and Bevins CL: Antimicrobial peptides as mediators of epithelial host defense. Pediatr Res 45: 785-794, 1999.
2. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schroder JM, Wang JM, Howard OM and Oppenheim JJ: Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286: 525-528, 1999.
3. Territo MC, Ganz T, Selsted ME and Lehrer R: Monocyte-chemotactic activity of defensins from human neutrophils. J Clin Invest 84: 2017-2020, 1989.
4. Kaiser V and Diamond G: Expression of mammalian defensin genes. J Leukoc Biol 68: 779-784, 2000.
5. Garcia JR, Krause A, Schulz S, Rodriguez-Jimenez FJ, Kluver E, Adermann K, Forssmann U, Frimpong-Boateng A, Bals R and Forssmann WG: Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. FASEB J 15: 1819-1821, 2001.
6. Yamaguchi Y, Nagase T, Makita R, Fukuhara S, Tomita T, Tominaga T, Kurihara H and Ouchi Y: Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice. J Immunol 169: 2516-2523, 2002.
7. Duits LA, Ravensbergen B, Rademaker M, Hiemstra PS and Nibbering PH: Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. Immunology 106: 517-525, 2002.
8. Harder J, Bartels J, Christophers E and Schroder JM: Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 276: 5707-5713, 2001.
9. Hao HN, Zhao J, Lotoczky G, Grever WE and Lyman WD: Induction of human beta-defensin-2 expression in human astrocytes by lipopolysaccharide and cytokines. J Neurochem 77: 1027-1035, 2001.
10. Singh PK, Jia HP, Wiles K, Hesselberth J, Liu L, Conway BA, Greenberg EP, Valore EV, Welsh MJ, Ganz T, Tack BF and McCray PB Jr: Production of beta-defensins by human airway epithelia. Proc Natl Acad Sci USA 95: 14961-14966, 1998.
11. Bals R, Wang X, Wu Z, Freeman T, Bafna V, Zasloff M and Wilson JM: Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. J Clin Invest 102: 874-880, 1998.
12. Hiratsuka T, Nakazato M, Date Y, Ashitani J, Minematsu T, Chino N and Matsukura S: Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. Biochem Biophys Res Commun 249: 943-947, 1998.
13. Ohara T, Morishita T, Suzuki H, Masaoka T, Nagata H and Hibi T: Pathophysiological role of human beta-defensins 2 in gastric mucosa. Int J Mol Med 14: 1023-1027, 2004.
14. Jang BC, Lim KJ, Paik JH, Kwon YK, Shin SW, Kim SC, Jung TY, Kwon TK, Cho JW, Baek WK, Kim SP, Suh MH and Suh SI: Up-regulation of human beta-defensin 2 by interleukin-1beta in A549 cells: involvement of PI3K, PKC, p38 MAPK, JNK, and NF-kappaB. Biochem Biophys Res Commun 320: 1026-1033, 2004.
15. Tsutsumi-Ishii Y and Nagaoka I: NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation. J Leukoc Biol 71: 154-162, 2002.
16. Moon SK, Lee HY, Li JD, Nagura M, Kang SH, Chun YM, Linthicum FH, Ganz T, Andalibi A and Lim DJ: Activation of a Src-dependent Raf-MEK1/2-ERK signaling pathway is required for IL-1alpha-induced upregulation of beta-defensin 2 in human middle ear epithelial cells. Biochim Biophys Acta 1590: 41-51, 2002.
17. Krisanaprakornkit S, Kimball JR and Dale BA: Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. J Immunol 168: 316-324, 2002.
18. McDermott AM, Redfern RL, Zhang B, Pei Y, Huang L and Proske RJ: Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. Invest Ophthalmol Vis Sci 44: 1859-1865, 2003.
19. Chen BJ: Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb Tripterygium wilfordii Hook F. Leuk Lymphoma 42: 253-265, 2001.
20. Qiu D and Kao PN: Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb Tripterygium wilfordii Hook. f. Drugs Res Dev 4: 1-18, 2003.
21. Qiu D, Zhao G, Aoki Y, Shi L, Uyei A, Nazarian S, Ng JC and Kao PN: Immunosuppressant PG490 (triptolide) inhibits T-cell interleukin-2 expression at the level of purine-box/nuclear factor of activated T-cells and NF-kappaB transcriptional activation. J Biol Chem 274: 13443-13450, 1999.
22. Zhao G, Vaszar LT, Qiu D, Shi L and Kao PN: Anti-inflammatory effects of triptolide in human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 279: L958-L966, 2000.
23. Wu Y, Cui J, Bao X, Chan S, Young DO, Liu D and Shen P: Triptolide attenuates oxidative stress, NF-kappaB activation and multiple cytokine gene expression in murine peritoneal macrophage. Int J Mol Med 17: 141-150, 2006.
24. Lin N, Sato T and Ito A: Triptolide, a novel diterpenoid triepoxide from Tripterygium wilfordii Hook. f., suppresses the production and gene expression of pro-matrix metalloproteinases 1 and 3 and augments those of tissue inhibitors of metalloproteinases 1 and 2 in human synovial fibroblasts. Arthritis Rheum 44: 2193-2200, 2001.
25. Zhou HF, Niu DB, Xue B, Li FQ, Liu XY, He QH, Wang XH and Wang XM: Triptolide inhibits TNF-alpha, IL-1 beta and NO production in primary microglial cultures. Neuroreport 14: 1091-1095, 2003.
26. Chen P, Li J, Barnes J, Kokkonen GC, Lee JC and Liu Y: Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. J Immunol 169: 6408-6416, 2002.
27. Zhao Q, Shepherd EG, Manson ME, Nelin LD, Sorokin A and Liu Y: The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide: attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. J Biol Chem 280: 8101-8108, 2005.

28. Shepherd EG, Zhao Q, Welty SE, Hansen TN, Smith CV and Liu Y: The function of mitogen-activated protein kinase phosphatase-1 in peptidoglycan-stimulated macrophages. J Biol Chem 279: 54023-54031, 2004.
29. Keyse SM: Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr Opin Cell Biol 12: 186-192, 2000.
30. Li J, Gorospe M, Hutter D, Barnes J, Keyse SM and Liu Y: Transcriptional induction of MKP-1 in response to stress is associated with histone H3 phosphorylation-acetylation. Mol Cell Biol 21: 8213-8224, 2001.
31. Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ and Kato JY: D-type cyclin-dependent kinase activity in mammalian cells. Mol Cell Biol 14: 2066-2076, 1994.
32. Slomiany BL and Slomiany A: Role of ERK and p38 mitogen-activated protein kinase cascades in gastric mucosal inflammatory responses to Helicobacter pylori lipopolysaccharide. IUBMB Life 51: 315-320, 2001.
33. Kim YH, Lee SH, Lee JY, Choi SW, Park JW and Kwon TK: Triptolide inhibits murine-inducible nitric oxide synthase expression by down-regulating lipopolysaccharide-induced activity of nuclear factor-kappa B and c-Jun NH2-terminal kinase. Eur J Pharmacol 494: 1-9, 2004.
34. Zhou R, Zheng SX, Tang W, He PL, Li XY, Yang YF, Li YC, Geng JG and Zuo JP: Inhibition of inducible nitric-oxide synthase expression by (5R)-5-hydroxytriptolide in interferon-gamma- and bacterial lipopolysaccharide-stimulated macrophages. J Pharmacol Exp Ther 316: 121-128, 2006.
35. Kao CY, Huang F, Chen Y, Thai P, Wachi S, Kim C, Tam L and Wu R: Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. J Immunol 175: 6676-6685, 2005.
36. Miyata Y, Sato T and Ito A: Triptolide, a diterpenoid triepoxide, induces antitumor proliferation via activation of c-Jun NH2-terminal kinase 1 by decreasing phosphatidylinositol 3-kinase activity in human tumor cells. Biochem Biophys Res Commun 336: 1081-1086, 2005.