Korean J Physiol Pharmacol
Vol 6: 319–325, December, 2002

Blockade of p38 Mitogen-activated Protein Kinase Pathway Inhibits Interleukin-6 Release and Expression in Primary Neonatal Cardiomyocytes

Han-Jung Chae, Hyun-Ki Kim, Wan-Ku Lee, and Soo-Wan Chae

Department of Pharmacology and Institute of Cardiovascular Research, Chonbuk National University Medical School, Jeonju 560–180, Korea

The induction of interleukin-6 (IL-6) using combined proinflammatory agents (LPS/IFN-γ or TNF-α/IFN-γ) was studied in relation to p38 mitogen-activated protein kinase (MAPK) and NF-κB transcriptional factor in primary neonatal cardiomyocytes. When added to cultures of cardiomyocytes, the combined agents (LPS/IFN-γ or TNF-α/IFN-γ) had stimulatory effect on the production of IL-6 and the elevation was significantly reduced by SB203580, a specific p38 MAPK inhibitor. SB203580 inhibited protein production and gene expression of IL-6 in a concentration-dependent manner. In this study, IFN-γ enhancement of TNF-α-induced NF-κB binding affinity as well as p38 MAP kinase activation was observed. However, a specific inhibitor of p38 MAPK, SB203580, had no effect on TNF-α/IFN-γ or LPS/IFN-γ-induced NF-κB activation. This study strongly suggests that these pathways about TNF-α/IFN-γ or LPS/IFN-γ-activated IL-6 release can be primarily dissociated in primary neonatal cardiomyocytes.

Key Words: Interleukin-6, p38 MAPK, NF-κB, SB203580

INTRODUCTION

Interleukin-6 (IL-6) is well known as a pleiotropic cytokine that has crucial physiological effects, such as promoting B cell differentiation and T cell activation and inducing acute phase proteins (Akira S et al, 1990; Van Snick J, 1990). IL-6 is produced by several kinds of cell lineages, such as macrophages (Horri Y et al, 1988), lymphocytes (Hirano T et al, 1988), endothelial cells (Jirik FR et al, 1989), and fibroblasts (Kohase M et al, 1987), that have important roles in inflammation. Elevated levels of serum IL-6 have been demonstrated not only in patients with inflammations resulting from bacterial or viral infections (Nijisten MWN et al, 1987), but also in patients with acute myocardial infarction (Ikeda U et al, 1992), suggesting that IL-6 may play an important role in the pathogenesis of ischemic heart disease.

Mitogen-activated protein kinases (MAPKs) have been proven to play a role in mediating intracellular signal transduction and regulating cytokine production by mononuclear cells in response to a variety of extracellular stimuli (Shapiro L et al, 1995; Shapiro L & Dinarello CA, 1997; Zu YL et al, 1998). In response to appropriate stimuli, the MAPK are activated by phosphorylation on both adjacent threonine and tyrosine residues that are separated by a single amino acid (Li Z et al, 1996; Kumar S et al, 1997; Ludwig S et al, 1998). For extracellularly regulated kinases (ERK), the best studied of the MAPK families, this intervening amino acid is glutamate; for the p38 MAPK family, it is glycine. While ERK has been classically associated with growth- and differentiation-inducing signals, p38 MAPK is involved in inflammatory cytokines and environmental stress inducers (Ludwig S et al, 1998).

In particular, p38 MAPK plays a role in the combined cytokines including TNF-α/IFN-γ-induced production of IL-6 (Beyaert R et al, 1996). In this study, SB203580, a specific p38 MAPK inhibitor, significantly inhibited combined agents-induced synthesis of interleukin-6 (IL-6) in primary neonatal cardiomyocyte. However neither the combined agents-induced DNA binding of NF-κB, nor IκBα degradation was modulated by SB203580, suggesting that NF-κB is not a direct target for the p38 MAP kinase pathway. Here, we report that the p38 MAP kinase pathway is activated in TNF-α/IFN-γ or LPS/IFN-γ-treated cells and demonstrates its crucial but remarkably selective role in the synthesis of IL-6 in primary neonatal cardiomyocytes.

METHODS

Materials

Rat interferon-γ and recombinant TNF-α were from R&D systems (Minneapolis, MN, U.S.A.). The concentrations

Corresponding to: Soo-Wan Chae, Department of Pharmacology and Institute of Cardiovascular Research, Chonbuk National University Medical School, Jeonju 560-180, Korea. (Tel) 82-63-270-3089, (Fax) 82-63-275-2855, (E-mail) soowan@moak.chonbuk.ac.kr

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; IL-6, interleukin-6; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; IFN, interferon.

used were described in units per milliliter with the specific activity for TNF-α as 10^7 U/mg protein. The value correspond to 100 ng/ml of TNF-α for 1000 U/ml. Lipopolysaccharide was obtained from Sigma Chemical (St. Louis, MO). Anti-phospho-specific p38 MAPK (Tyr^{182}) antibody was purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-total p38 MAPK and anti-IL-6 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). PD98059 (2-(2-amino-methoxyphenyl)-oxanaphalen-4-one) and SB203580 were obtained from New England Biolabs (Beverly, MA, U.S.A.). All cell culture media and reagents were obtained from Life Technologies, Inc.

Isolation and cultures of primary neonatal cardiomyocytes

Primary cultures of cardiac myocytes were prepared from the ventricles of 1~2 day-old Wistar rats essentially according to the methods of Yamazaki et al (1998). Ventricles were separated from atrial tissue and washed briefly in digestion solution (116 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$ [pH 7.35], collagenase [95 U/ml], and pancreatin [0.6 mg/ml]). The myocytes were dissociated in fresh digestion buffer and collected by centrifugation. The isolated cells, which were a mixture of myocytes and nonmyocytes, were suspended in plating media (Dulbecco's modified Eagle's medium) and plated onto 150-mm-diameter non-coated culture dishes for 1 h to reduce contaminated cardiac fibroblasts. After 1 h incubation, unattached cells were collected and more than 95% of the cells were myocytes determined by cell morphology and anti-sarcomeric actin staining. Myocytes were purified by Percoll gradient, replated at a density of 1×10^5 in 25-mm-diameter etched coverslips pre-coated with 1% collagen and grown in plating media.

Assay for IL-6

Primary neonatal cardiomyocytes were stimulated by various agents in 1 ml of D-MEM containing 10% FBS for the indicated periods. The conditioned medium was collected, and IL-6 in the medium from neonatal rat cardiomyocytes was measured by a rat IL-6 ELISA kit (Kozawa O et al, 1999).

Northern blot analysis of IL-6 mRNA expression

Total cellular RNA was prepared with TRIzol reagent (Gibco BRL, Gaiterberg, MD) according to the manufacturer's instructions. RNA (20 μg) was subjected to electrophoresis in 1% formaldehyde agarose gels, transferred to a nylon membrane by capillary blotting and fixed by UV irradiation. Hybridization was carried out 42°C in 50 mM Tris-HCl, pH 7.4, 40% foramide, 4×SSC (15 mM sodium citrate, 150 mM NaCl). 10× Denhardt's solution, 0.1% $Na_4P_2O_7$, 1% SDS and 200 μg/ml herring sperm DNA. The blots were washed to a stringency of 2 ×SSC and 0.1% SDS at 42°C, and then exposed to an X-ray film at −70°C. A 655 bp fragment of IL-6 cDNA was labeled with ^{32}P, using random primers (Boehringer, Mannheim. Germany), and used as a hybridization probe.

Immunoblotting

Immunoblotting was performed by modification of the procedure previously described (Hibi M et al, 1993). Briefly, phosphorylation of p38 was determined by immunoblot analysis of 100 μg of cell extracts, using a phospho-specific p38 MAPK (Thr^{180}/Tyr^{182}) antibody (New England Biolabs). For IκBα immunoblotting, cytosolic extracts were prepared as described in EMSA. Cytosolic proteins (50 μg) were resolved on 10% polyacrylamide gels and blotted onto nitrocellulose. Equal loading was confirmed by Ponceau S staining. Filters were incubated overnight at 4°C with IκBα-Ab (1 : 500). After washing, the filters were incubated with peroxidase-conjugated antibody for 1 h before detection of immunolabelled bands by ECL (Amersham, Brauschweig, Germany).

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytosolic extracts (10 μg of protein) were used for EMSA. The detailed procedures of EMSA were described in our previous report (Chae HJ et al, 1999). The cells were washed twice with ice-cold PBS and lysed with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol, 10 μg/ml aprotinin, 20 μM pepstatin A, 100 μM leupeptin). After centrifugation at 1000×g, the nuclear pellets were resuspended in extraction buffer (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.4 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol) and incubated on ice for 10 min. The nuclear proteins in the supernatant were recovered after centrifugation at 15,000×g, quantified by using a BCA protein assay kit (Sigma Co., Saint Louis, MO) and used to carry out EMSA. To measure the activation of NF-κB transcription factor, the oligonucleotide probe of NF-κB containing the IgG chain binding site (NF-κB: 5'-CCG GTT AAC AGA GGG GGC TTT CCG AG-3') was used. Two complementary strands of the oligonucleotide were annealed and labeled with [α-^{32}P]-dCTP using a random primer labeling kit (rediprime, Amersham Life Science, England). Nuclear extracts (5 μg) were reacted with 2~5 ng of the radiolabelled NF-κB probe (50,000~100,000 cpm/ng). The reaction was performed in the presence of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 4% glycerol (final volume: 25 μl) at room temperature for 30 min. The reaction products were subjected onto 4% polyacrylamide gel electrophoresis in 0.5× TBE buffer (50 mM Tris-HCl, pH 8.5, 50 mM borate, and 1 mM EDTA). Gels were dried under a vacuum for 1 h. DNA binding activity for NF-κB was measured by using PhospholImager analyzer (BAS, Fuji Co, Japan).

Statistical treatment of data

Statistical differences were evaluated by analysis of variance (ANOVA) in dose-response experiments and by two-tailed Student's t-tests. In each case, the statistical test used is indicated, and the number of experiments is stated individually in the legend of each figure.

RESULTS

LPS or TNF-α stimulates the production of IL-6 in the presence of IFN-γ in primary neonatal cardiomyocytes

We tested whether primary neonatal cardiomyocytes produce IL-6. Cardiomyocytes were stimulated with IFN-γ

(100 IU/ml), LPS (1 μg/ml) and TNF-α (10 ng/ml) alone or in combinations (LPS/IFN-γ or TNF-α/IFN-γ) for 24 h. After stimulation of cells with inflammatory agents, IL-6 released into culture medium was determined by ELISA using an IL-6 neutralization antibody. As shown in Fig. 1A, the individual agents, IFN-γ, TNF-α and LPS had only a slight stimulatory effect on the production of IL-6. However, LPS and TNF-α synergies with IFN-γ to induce IL-6 synthesis. To determine the expression of IL-6 in inflammatory agents-treated cultures, we carried out western blot using antibodies specific for IL-6. The data illustrated in Fig. 1B confirm the induction of IL-6 synthesis in response to combined LPS/IFN-γ or TNF-α/IFN-γ. The cultures treated with LPS and TNF-α individually did not contain detectable immunoreactive IL-6 protein. However, IFN-γ had a relatively stimulatory effect on the expression of IL-6, compared with other individual agents.

SB203580 inhibits IL-6 production in primary neonatal cardiomyocytes

We examined the effect of a highly specific inhibitor of p38 MAP kinase, SB203580, on IL-6 production by the treatment of TNF-α (10 ng/ml), IFN-γ (100 U/ml), LPS (1 μg/ml), TNF-α/IFN-γ, or LPS/IFN-γ in primary neonatal cardiomyocytes. Fig. 2A has showed that the inclusion of SB203580 in the presence of LPS/IFN-γ, or TNF-α/IFN-γ suppressed the induction of IL-6 production. The block of IL-6 synthesis correlated with marked inhibition of the expression of IL-6 protein as determined by western blot analysis (Fig. 2B). SB203580 at the concentration used in these experiments did not affect the viability of cells after 24 h of culture, thereby excluding nonspecific toxicity of the agent (data not shown).

Furthermore, SB203580-induced inhibitory effect on IL-6 secretion was determined in neonatal cardiomyocytes that had been pre-incubated for 1 h with various concentrations of p38 MAPK inhibitor (0.5~20 μM) before TNF-α/IFN-γ or LPS/IFN-γ, clearly indicating that SB203580 directly acts on cardiomyocytes to suppress IL-6 synthesis (Fig. 2C). Essentially the same results were obtained using other specific p38 inhibitor such as SB 202190 (data not shown).

SB203580 inhibits IL-6 mRNA expression in primary neonatal cardiomyocytes

Northern blot analysis was performed to determine whether SB203580 affected the IL-6 mRNA steady-state level. As shown in Fig. 3, SB203580 (20 μM) inhibited IL-6 mRNA expression induced by TNF-α/IFN-γ, or LPS/IFN-γ in primary neonatal cardiomyocytes. Furthermore, we performed experiments using an inhibitor of translation, cycloheximide (1 μM), to analyze whether the SB203580 effect was dependent on de novo protein synthesis. Cycloheximide alone had no effect on the TNF-α/IFN-γ or LPS/IFN-γ-induced IL-6 mRNA expression. The concurrent presence of cycloheximide abolished the SB203580-induced inhibitory effect on IL-6 release (data not shown), but had no effect on IL-6 mRNA expression, clearly demonstrating that the suppressive effect of SB203580 on TNF-α/IFN-γ or LPS/IFN-γ-stimulated IL-6 mRNA expression on de novo protein synthesis.

TNF-α or LPS in the presence of IFN-γ potentiates p38 MAP Kinase activity in primary neonatal cardiomyocytes

p38 MAP kinase is activated by the dual specificity kinases that phosphorylate the threonine and tyrosine residues. The phosphorylation state of p38 MAP kinase antibody means the fully phosphorylated p38 MAP kinase in this system. There was rather a strong activation of p38 MAPK by LPS or TNF-α, which was enhanced further by IFN-γ, as confirmed by western data (Fig. 4B), whereas the protein level of p38 MAP kinase was not significantly affected.

TNF-α or LPS in the presence of IFN-γ induces NF-κB transcription factor activation in primary neonatal cardiomyocytes

Activation of the transcription factor NF-κB has previously been shown to be indispensable for IL-6 gene induction by various cytokines (Awane M et al, 1999; Ahn YS et al, 1999). NF-κB is not present in the nucleus of non-stimulated cells, but becomes activated after cytokine stimuli and then translocates to the nucleus. In addition, activation of NF-κB is controlled by an inhibitory subunit, IκB, which retains NF-κB in the cytoplasm. NF-κB activation requires sequential activation, phosphorylation, ubiquitination, and degradation of IκB as well as consequent exposure of a nuclear localization signal on NF-κB. Therefore, the measurement of IκB levels offers information regarding the mechanism of NF-κB activation. In this study, we performed an experiment on NF-κB DNA binding activity and rapid and important degradation of IκB protein expression in various agents-exposed cardiomyocytes. Cells were treated with 100 U/ml IFN-γ, 1 μg/ml

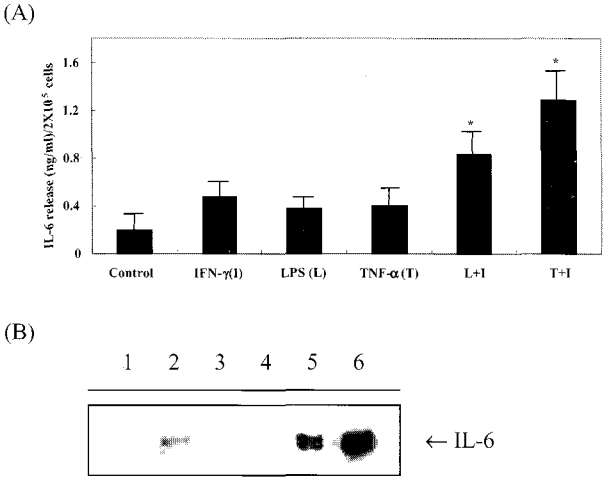

Fig. 1. LPS or TNF-α stimulates IL-6 release in the presence of IFN-γ in primary neonatal cardiomyocytes. A. Cells were treated with IFN-γ (100 U/ml), LPS (1 μg/ml), and TNF-α (10 ng/ml) individually and in different combinations (LPS/IFN-γ or TNF-α/IFN-γ) for 24 h. A. The culture media were analyzed for IL-6 levels using an IL-6-specific ELISA (Materials and Methods). Data expressed the mean±S.E. of four experiments. *Significantly different from control, $p<0.05$. B. Cell extracts were subjected to western blot analysis using antibodies specific IL-6. 1: Control, 2: 100 U/ml IFN-γ, 3: 1 μg/ml LPS, 4: 10 ng/ml TNF-α, 5: LPS + IFN-γ, 6: TNF-α + IFN-γ.

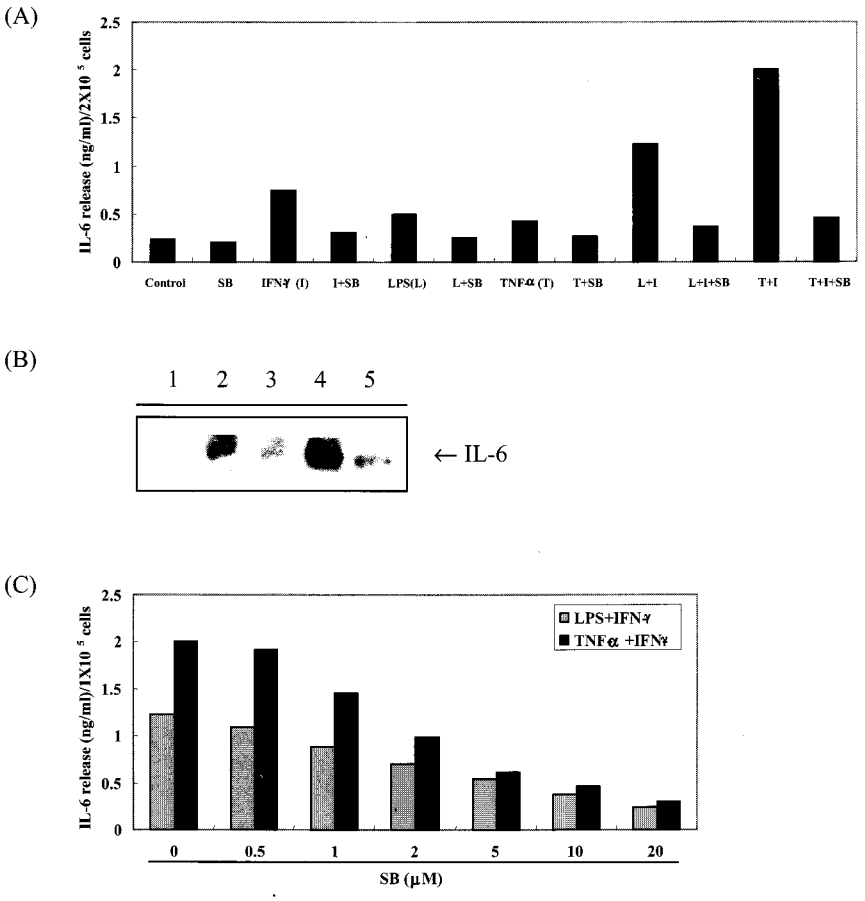

Fig. 2. SB203580 inhibits IL-6 release and expression in primary neonatal cardiomyocytes. A. Cells were untreated or pretreated for 1 h with 20 μM SB203580. The cells were then treated for 24 h with 100 U/ml IFN-γ, 1 μg/ml LPS, 10 ng/ml TNF-α, LPS/IFN-γ or TNF-α/IFN-γ in the continuous presence of SB203580. The culture media were analyzed for IL-6 levels using an IL-6-specific ELISA (Materials and Methods). A representative experiment is shown. B. Cells were treated as indicated as panel A. The cell extracts were subjected to western blot analysis using antibodies specific IL-6. 1: Control, 2: LPS + IFN-γ, 3: LPS + IFN-γ + SB203580, 4: TNF-α + IFN-γ, 5: TNF-α + IFN-γ + SB203580. C. IL-6 secretion is inhibited after 24 h of LPS/IFN-γ or TNF-α/IFN-γ treatment in the presence of various concentration of SB203580 (0.5~20 μM). The culture media were analyzed for IL-6 levels using an IL-6-specific ELISA (Materials and Methods). A representative experiment is shown.

LPS, 10 ng/ml TNF-α, LPS/IFN-γ or TNF-α/IFN-γ for various periods of time and followed by isolation of nuclear extract. Our data shows that LPS/IFN-γ or TNF-α/IFN-γ markedly activated the binding activity of nuclear extract to oligonucleotide probe of NF-κB at 30 and 60 min (Fig. 5A). LPS and TNF-α each increased NF-κB activity relatively low, compared with each the agent in the presence of IFN-γ. As well, when the time course of IκB levels was determined in cardiomyocytes stimulated with LPS or TNF-α in the presence of IFN-γ, a rapid and important degradation of IκB protein was observed (Fig. 5B). The addition of TNF-α and IFN-γ together greatly potentiated NF-κB activation to a great extent than the addition of either alone. The stimulation of NF-κB binding affinity paralleled the loss of IκBα protein from the cytoplasm in the combined agents-treated cardiomyocytes.

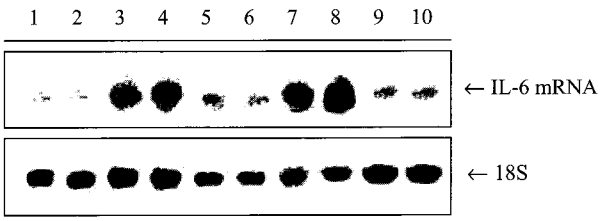

Fig. 3. SB203580 inhibits TNF-α/IFN-γ or LPS/IFN-γ-stimulated IL-6 mRNA expression on de novo protein synthesis. Cardiomyocytes were stimulated with 1 μg/ml LPS or 10 ng/ml TNF-α in the presence of 100 U/ml IFN-γ for 18 h. Some cultures were preincubated with cycloheximide (1 μM) and SB203580 alone or in combinations for 1 h before the treatment of LPS/IFN-γ or TNF-α /IFN-γ. Total RNA (20 μg) was fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N-positive nylon membranes and then hybridized to the appropriate cDNA probes. Results shown are a representative of two experiments. 1: control, 2: SB203580, 3: LPS+ IFN-γ, 4: LPS+IFN-γ+cycloheximide, 5: LPS+IFN-γ+SB203580, 6: LPS+IFN-γ+SB203580+cycloheximide, 7: TNF-α+IFN-γ, 8: TNF-α+IFN-γ+cycloheximide, 9: TNF-α+IFN-γ+SB203580, 10: TNF-α+IFN-γ+SB203580+cycloheximide.

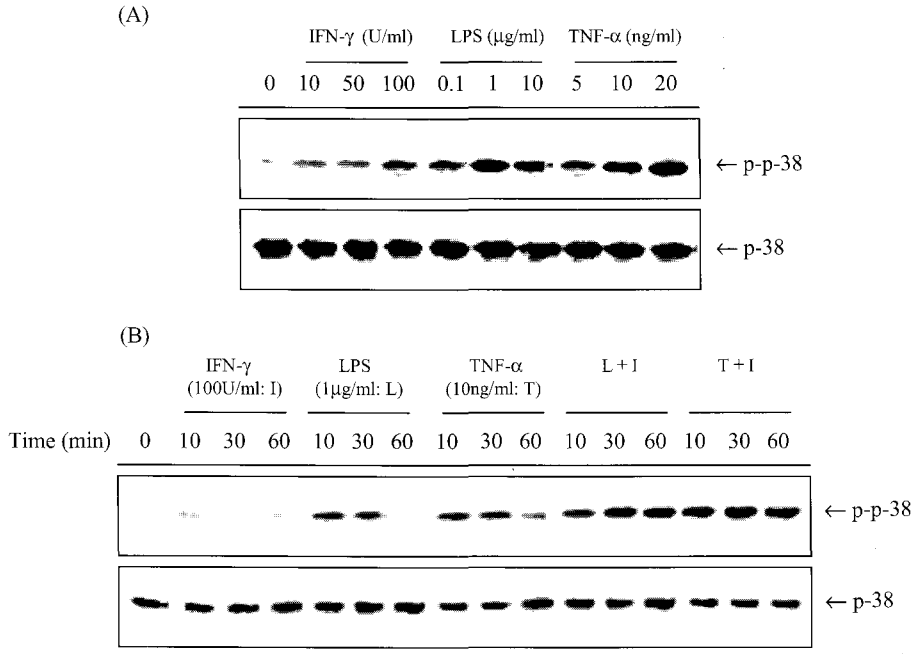

Fig. 4. TNF-α or LPS in the presence of IFN-γ potentiates p38 MAP Kinase activity in primary neonatal cardiomyocytes. A. Cells were treated for 30 min with various concentrations of IFN-γ (10, 50 or 100 U/ml), LPS (0.1, 1 or 10 μg/ml) or TNF-α (5, 10 or 20 ng/ml). B. Cells were also treated with 1 μg/ml LPS or 10 ng/ml TNF-α in the presence or absence of 100 U/ml IFN-γ for various time intervals (0, 30, 60 or 120 min). Aliquots of the cell lysates were then subjected to immunoblot analysis using antibodies specific for the active (phosphorylated) form of p38 MAP Kinase. Parallel blots run with anti-total p38 MAPK antibodies served as control. Results shown are a representative of three experiments.

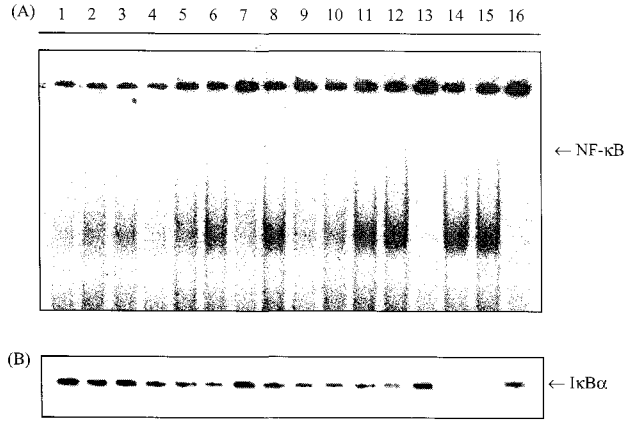

Fig. 5. TNF-α or LPS in the presence of IFN-γ potentiates NF-κB activity in primary neonatal cardiomyocytes. Cells were also exposed to 100 U/ml IFN-γ, 1 μg/ml LPS and 10 ng/ml TNF-α in the presence or absence of 100 U/ml IFN-γ for various time intervals (0, 30, 60 or 120 min). A. Nuclear proteins were analyzed in an EMSA with a α-^{32}P-labelled oligonucleotide encompassing the NF-κB binding site of the IL-6 promoter. B. And then the cytosolic extracts were examined by protein immunoblotting for IκB-α degradation. Results shown are a representative of two experiments. 1: Control, 2: IFN-γ (30 min), 3: IFN-γ (60 min), 4: IFN-γ (120 min), 5: LPS (30 min), 6: LPS (60 min), 7: LPS (120 min), 8: TNF-α (30 min), 9: TNF-α (60 min), 10: TNF-α (120 min), 11: LPS+IFN-γ (30 min), 12: LPS+IFN-γ (60 min), 13: LPS+IFN-γ (120 min), 14: TNF-α+IFN-γ (30 min), 15: TNF-α+IFN-γ (60 min), 16: TNF-α+IFN-γ (120 min).

p38 MAP kinase and NF-κB pathways are dissociated with IL-6 release in primary neonatal cardiomyocytes

To explore the possible role of the p38 MAP kinase pathway in the combined agents-induced activation of NF-κB, we performed a NF-κB binding assay in TNF-α/IFN-γ or LPS/IFN-γ-stimulated neonatal cardiomyocytes in the presence or absence of SB203580. However, the pretreatment of 20 μM SB203580 revealed no significant decrease of TNF-α-induced NF-κB DNA biding, indicating that the p38 MAP kinase pathway does not interfere with release of NF-κB from IκB, nor with its nuclear translocation and DNA binding (Fig. 6A). In consistent with above results, the pretreatment of SB203580 had no regulatory effect on the combined agents-induced IκBα degradation (Fig. 6B). These data suggest that these two pathways can be dissociated in combined agents-induced IL-6 release in neonatal cardiomyocytes.

DISCUSSION

Cellular elements of the immune system have been suggested to play a role in mediating the global shock, cardiac allograft rejection, ischemic heart disease, and some forms of idiograft rejection, ischemic heart disease, and some forms of idiopathic cardiomyopathy. However, recent reports have pointed out the importance of proinflammatory cytokines, which have direct effects on the contractility of the mammalian heart and on neutrophil-myocyte adhesion. Interleukin-6 is secreted by various

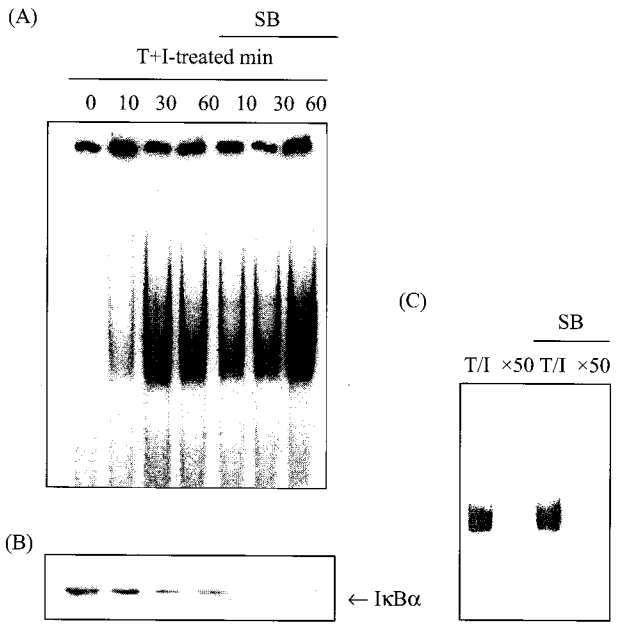

Fig. 6. p38 MAP kinase and NF-κB pathways are dissociated with IL-6 release in primary neonatal cardiomyocytes. A. Cells were untreated or pretreated for 1 h with 20 μM SB203580. The cells were then treated for 30 min with 100 U/ml IFN-γ, 1 μg/ml LPS, 10 ng/ml TNF-α, LPS/IFN-γ or TNF-α/IFN-γ in the continuous presence of SB203580. The nuclear proteins were analyzed in an EMSA with a -^{32}P-labelled oligonucleotide encompassing the NF-κB binding site of the IL-6 promoter. B. And then the cytosolic extracts were examined by protein immunoblotting for IκB- degradation. Results shown are a representative of two experiments. 1: Control, 2: SB203580, 3: LPS+IFN-γ, 4: LPS+IFN-γ+SB203580, 5: TNF-α+IFN-γ, 6: TNF-α+IFN-γ+SB203580. C. Cells were untreated or pretreated for 1 h with 20 μM SB203580. The cells were then treated for 30 min with 100 U/ml IFN-γ and 10 ng/ml TNF-α. The nuclear proteins were analyzed in an EMSA with α-^{32}P-labelled or unlabelled (50 folded) NF-κB probe.

kinds of cells such as endothelial cells (Jirik FR et al, 1989), vascular smooth muscle cells (Loppnow H & Libby P., 1990), and activated monocytes and macrophages (Horri Y., 1988), which have important roles in inflammation.

Our present study clearly demonstrated that SB203580, a pyridinylimidazole inhibitor specific for p38 MAP kinase, concentration- and time-dependently suppressed protein production and mRNA expression of IL-6 induced by TNF-α or LPS in the presence of IFN-γ (Fig. 2, 3). The inhibition of transcriptional regulation of IL-6 and the subsequent production of IL-6 in neonatal myocytes by SB203580 indicate that the pyridinylimidazole compound may be useful for therapy of disease states in which IL-6 has been shown to play a pro-inflammatory role.

Because IL-6 also engages NF-κB signaling, experiments were performed to determine whether these two pathways run on the same axis or in parallel. We have found that inhibition of p38 MAPK through the highly specific inhibitor, SB203580 did not affect the combined agents-induced NF-κB binding activity (Fig. 6A). These results suggest that these two pathways can be primarily dissociated. This is in agreement with reports demonstrating NF-κB and p38 signal divergence in response to cytokines including TNF-α in other cells (Hernandez M et al, 1999; Yamakawa T et al, 1999).

IκBα levels in the cytoplasm of neonatal cardiomyocytes were rapidly decreased in response to TNF-α/IFN-γ or LPS/IFN-γ for 30~60 min but gradually restored after 120 min of the combined agents. Degradation and subsequent re-synthesis of IκBα have commonly been observed in myeloid, epithelial and fibroblast cells, stimulated with cytokine, phorbol myristate acetate (PMA), and LPS. The rate of IκBα degradation in cytoplasm varied among different types of cells, but translocation of the released NF-κB to the nucleus paralleled the loss of IκBα protein from the cytoplasm. On the basis of the reports that IκBα may be involved in the regulation of the multiple NF-κB-dependent gene expressions, degradation of IκBα coupled with the activation of NF-κB in cardiomyocytes could play an important role in the activation and regulation of inflammatory cytokine genes expressions.

Although the effects of IL-6 on the cardiovascular system are not well known, Finkel et al (1992) reported that several recombinant cytokines, including TNF-α, IL-2 and IL-6, had a negative inotropic effect on hamster papillary muscle. The production of IL-6 induced by combined agents such as TNF-α/IFN-γ or LPS/IFN-γ in cardiomyocytes may also be involved in contractile dysfunction in inflammatory heart diseases. The association between cytokine stresses and augmented production of IL-6 as a potential mediator in the progression of myocardial dysfunction provided in inflammatory state. Thus, these findings lead to speculations that pathological inflammation such as sepsis might result from failure in the regulatory system of p38 MAP kinase and NF-κB in cardiomyocytes. Further investigation would be required to clarify the exact role of p38 MAP kinase in heart metabolism.

ACKNOWLEDGEMENTS

This work was supported by Korea Research Foundation Grant (KRF-2002-EX0001) and research fund of Chonbuk National University.

REFERENCES

Ahn YS, Kim CH, Kim JH. Role of protein kinases on NF-κB activation and cell death in bovine cerebral endothelial cells. *Korean J Physiol Pharmacol* 3: 11–18, 1999

Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J* 4(11): 2860–2867, 1990

Awane M, Andres PG, Li DJ, Reinecker HC. NF-κB-inducing kinase is a common mediator of IL-17-, TNF-α-, and IL-1β-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* 162: 5337–5344, 1999

Beyaert R, Cuenda A, Vanden Berghe W, Plaisance S, Lee JC, Haegeman G, Cohen P, Fiers W. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J* 15: 1914–1923, 1996

Chae HJ, Chae SW, Weon KH, Kang IS, Kim HR. Signal transduction of thapsigargin-induced apoptosis in osteoblast. *Bone* 24: 453–458, 1999

Finkel MS, Oddis CV, Jacob TD, Watkins SC, Halter BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 257: 387–389, 1992

Hernandez M, Bayon Y, Sanchez Crespo M, Nieto ML. Signaling mechanisms involved in the activation of arachidonic acid metabolism in human astrocytoma cells by tumor necrosis factor-alpha: phosphorylation of cytosolic phospholipase A2 and transactivation of cyclooxygenase-2. *J Neurochem* 73: 1641–1649, 1999

Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* 7: 2135–2148, 1993

Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A, Tsunasawa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T, Kishimoto T. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 324(6092): 73–76, 1986

Horri Y, Muraguchi A, Suematsu S, Matsuda T, Yoshizaki K, Hirano T, Kishomoto T. Regulation of BSF-2/IL-6 production by human mononuclear cells: macrophage-dependent synthesis of BSF-2/IL-6 by T cells. *Journal of Immunology* 141: 1529–1535, 1988

Ikeda U,Ohkawa F, Seino Y, Yamamoto K, Hidaka Y, Kasahara T, Kawai T, Shimada K. Serum interleukin 6 levels become elevated in acute myocardial infarction. *J Mol Cell Cardiol* 24: 579–584, 1992

Jirik FR, Podor TJ, Hirano T, Kishimoto T, Loskutoff DJ, Carson DA, Lotz M. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. *J Immunol* 142(1): 144–147, 1989

Kohase M, May LT, Tamm I, Vilcek J, Sehgal PB. A cytokine network in human diploid fibroblasts: interactions of beta-interferons, tumor necrosis factor, platelet-derived growth factor, and interleukin-1. *Mol Cell Biol* 7(1): 273–280, 1987

Kozawa O, Tokuda H, Matsuno H, Uematsu T. Involvement of p38 mitogen-activated protein kinase in basic fibroblast growth factor-induced interleukin-6 synthesis in osteoblasts. *J Cell Biochem* 74: 479–485, 1999

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR. Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* 235: 533–538, 1997

Li Z, Jiang Y, Ulevitch RJ, Han, J. The primary structure of p38 γ: a new member of p38 group of MAP kinases. *Biochem Biophys Res Commun* 228: 334–340, 1996

Loppnow H, Libby P. Proliferating or interleukin 1-activated human vascular smooth muscle cells secrete copious interleukin 6. *J Clin Invest* 85: 731–738, 1990

Ludwig S, Hoffmeyer A, Goebeler M, Kilian K, Hafner H, Neufeld B, Han J, Rapp UR. The stress inducer arsenite activates mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 via a MAPK kinase 6/p38-dependent pathway. *J Biol Chem* 273: 1917–1922, 1998

Nijisten MWN, De Groot ER, Ten Duis HJ, Hack CE, Aarden LA. Serum levels of interleukin-6 and acute phase response. *Lancet* 2: 921, 1987

Shapiro L, Dinarello CA. Osmotic regulation of cytokine synthesis in vitro. *Proc Natl Acad Sci USA* 92: 12230–12234, 1995

Shapiro L, Dinarello CA. Hyperosmotic stress as a stimulant for proinflammatory cytokine production. *Exp Cell Res* 231: 354–362, 1997

Van Snick J. Interleukin-6: an overview. *Annu Rev Immunol* 8: 253–278, 1990

Yamakawa T, Eguchi S, Matsumoto T, Yamakawa Y, Numaguchi K, Reynolds CM, Motley ED, Inagami T. Intracellular signaling in rat cultured vascular smooth muscle cells: roles of nuclear factor-κB and p38 mitogen-activated protein kinase on tumor necrosis factor-α production. *Endocrinology* 140: 3562–3572, 1999

Yamazaki T, Komuro I, Zou Y, Kudoh S, Shiojima I, Hiroi Y, Mizuno T, Aikawa R, Takano H, Yazaki Y. Norepinephrine induces the raf-1 kinase/mitogen-activated protein kinase cascade through both α1- and β-adrenoreceptors. *Circulation* 95: 1260–1268, 1998

Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, Huang CK, Sha'afi RI. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. *J Immunol* 160: 1982–1989, 1998