p38 Mitogen-Activated Protein Kinase and Extracellular Signal-Regulated Kinase Regulate Nitric Oxide Production and Inflammatory Cytokine Expression in Raw Cells

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ABSTRACT

Background: p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signaling are thought to have critical role in lipopolysaccharide (LPS)-induced immune response but the molecular mechanism underlying the induction of these signaling are not clear. **Methods:** Specific inhibitors for p38, SB203580, and for ERK, PD98059 were used. Cells were stimulated by LPS with or without specific MAPK inhibitors. **Results:** LPS activated inducible nitric oxide synthase (iNOS), subsequent NO productions, and pro-inflammatory cytokine gene expressions (TNF-α, IL-1β, IL-6, and IL-12). Treatment of both SB203580 and PD98059 decreased LPS-induced NO productions. Concomitant decreases in the expression of iNOS mRNA and protein were detected. SB203580 and PD98059 decreased LPS-induced gene expression of IL-1β and IL-6. SB203580 increased LPS-induced expression of TNF-α and IL-12, and reactive oxygen species production, but PD98059 had no effect. **Conclusion:** These results indicate that both p38 and ERK pathways are involved in LPS-stimulated NO synthesis, and expression of IL-1β and IL-6. p38 signaling pathways are involved in LPS-induced TNF-α and IL-12, and reactive oxygen species plays an important role in these signaling in macrophage. **(Immune Network 2005;5(1):30-35)**

Key Words: p38, ERK, nitric oxide, cytokine, LPS, ROS

Introduction

Stimulation of macrophages by cytokines and endotoxin such as lipopolysaccharide (LPS) results in the induction and release of several immune effector elements such as nitric oxide (NO) and various inflammatory cytokine genes. NO is a small, relatively stable gas that readily diffuse into cells and cell membranes which reacts with molecular targets. In macrophage, NO is generated by inducible NO synthase (iNOS), and modulates immune response and hose defense against pathogens and tumor cells (1,2).

The mitogen-activated protein kinases (MAPKs) are important serine/threonine signaling kinase that modulated by phosphorylation result in mediation of cellular response to extracelluar signals with gene induction in the nucleus (3). In mammalian cells, three major groups of MAPK identified include extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 kinase (4).

LPS is a toxic component of gram negative bacteria and activate macrophages and monocytes by forming a complex with serum LPS-binding protein and leading to the secretion of variety of immunomodulatory molecules such as NO and pro-inflammatory cytokines (5). LPS is a potent macrophage activator and stimulate a various signal transduction elements especially MAPKs in a many cellular systems, and activate all three MAPKs in murine macrophages (6). p38 regulates LPS-induced production of NO (7), and synthesis of tumor necrosis factor-α (TNF-α) (8), interleukin-1 (IL-1) (9), IL-6 (10), and IL-12 (11) in murine macrophages. Also, ERK is known to regulate the LPS-induced production of NO (12), and

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pro-inflammatory cytokines (13) in murine macrophages. However, there exists many contradictory reports about the role of p38 and ERK on LPS-induced iNOS expression, subsequence NO production, and cytokine synthesis in macrophages.

In this report, we hypothesized that both the p38 and ERK MAPKs are essential to LPS-induced iNOS gene expression, NO production, and cytokine gene expression in macrophages. Recently, highly selective pharmacologic inhibitors of the p38 and ERK are developed; SB203085 for p38 and PD98059 for ERK. By using specific inhibitors, we studied the importance of each pathway in the mediation of LPS-induced macrophage activation.

Materials and Methods

Cell cultures. Raw cells from American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in RPMI 1640 (2 mM glutamine, 100 units/ml penicillin, 100μg/ml streptomycin) with 10% fetal bovine serum in 5% CO_2 at 37°C. Cells were exposed to the specific concentration of either SB203580, or PD98059 1 h prior to stimulation of LPS and then incubated for additional 6～24 h before collection of supernatants and/or cell lysates.

Reagents. LPS (from E.coli 026: B6, Sigma, St. Louis, MO, USA) was dissolved in the culture medium. SB203580 and PD98059 (Calbiochem, La Jolla, CA, USA) was reconstituted in DMSO at stock concentrations of 10 mM, and added into cultures at the indicated concentrations.

Nitric oxide assay. The amount of stable nitrite, the end product of NO generation by activated macrophages was determined by a colorimetric assay as previously described (14). Briefly, 50μl of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrocholoride, 2.5% H_3PO_4), and incubated at room temperature for 10 min. The absorbance at 540 nm was read on an SLT microplate reader (Quest Scientific, Duluth, GA, USA). Nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Analysis of mRNA expression. Total cellular RNA was isolated from macrophages using TRI reagent LS (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's protocol. The first strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY, USA). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for iNOS, TNF-α, IL-6, IL-1β, IL-12, and β-actin (internal control). The condition for reverse transcription and PCR steps were performed as previously reported (15). Optimization of cycle number was performed to ensure that product accumulation was in the linear range. Amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide. The gels were documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). Band intensities for the genes of interest were normalized to that of β-actin in the same sample.

Western blot analysis. Cell lysates were prepared as described (16). Protein concentrations in the cell lysates were determined by Bio-Rad (Richmond, CA, USA) protein assay. Western blot analyses were performed on 5μg of proteins as previously described (17). Briefly, samples were mixed with 2X sample buffer, heated to 95°C for 5min, separated electrophoretically using 8% SDS-PAGE and then transferred to nitrocellulose paper. The iNOS was assayed by anti-iNOS antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ, USA).

Reactive oxygen species (ROS) generation. The production of ROS was measured by detecting the fluorescence intensity of oxidant-sensitive probe dihydrorhodamine 123 (DHR, 10μM, Molecular Probes, Eugene, OR, USA), which is a cell permeable non-fluorescent molecule (18). Upon interaction with free radicals, DHR is oxidized resulting in the liberation of rhodamine, a highly fluorescent mitochondrial specific marker. The cells were preincubated for 1h with SB203580 or PD98059, followed by 18 h with LPS, and the fluorescent intensity was recorded using Spectramax Germini® fluorescence plate reader (Molecular Devices, Irvine, CA, USA). The DHR fluorescence was detected at excitation of 507 nm and emission 529 nm. The fluorescence values were digitized using SoftMax Pro Version 3.1.1 (Molecular Devices). The results were similar in at least three independent replications and data from a representative experiment (n=5 wells) have been presented.

Data presentation and statistical analysis. All experiments were repeated 2 to 3 times with similar trends; however, data from a representative trial are depicted in the results. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

Results

Nitric oxide production. We previously reported that exposed of LPS to macrophages produced NO in time and concentration manner (15,19). The cells

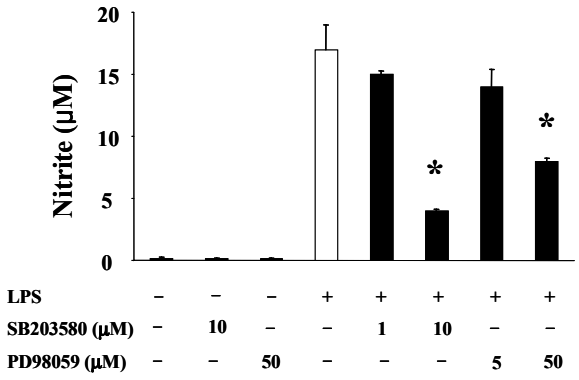

Figure 1. p38 inhibitor SB203580 and ERK inhibitor PD98059 inhibited NO production. Representative experiments were done by adding inhibitors at indicated concentrations 1 h prior to cell stimulation with LPS (30 ng/ml) for 24 h. Supernatants were collected and the amount of NO as measured. Results are expressed as mean±SE (n=4). *Significantly different from the LPS treatment group at $p < 0.05$.

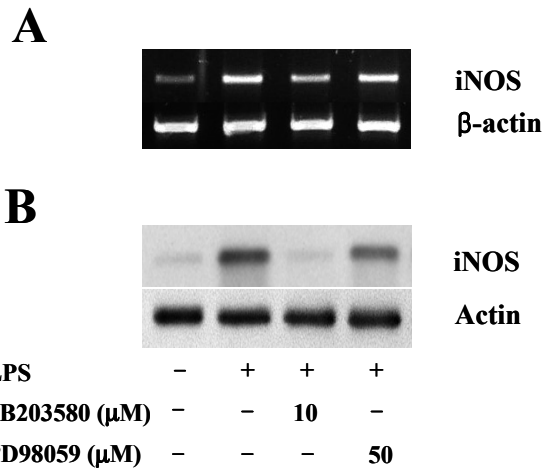

Figure 2. p38 inhibitor SB203580 and ERK inhibitor PD98059 inhibited iNOS mRNA expression (A) and production (B). Representative experiments were done by adding inhibitors at indicated concentrations 1 h prior to cell stimulation with LPS (30 ng/ml). Extraction and analysis of mRNA and protein performed as described in Materials and Methods.

were stimulated with LPS (30 ng/ml) for 24 h. Pretreatment of high concentration of both SB203580 and PD98059 decreased LPS-induced NO production (Fig. 1). The concentrations of inhibitors used in these studies did not showed cytotoxicity as determined by MTT assay (data not shown). The p38 inhibitor SB203580 and ERK inhibitor PD98059 alone had no effects to LPS-induced NO productions. Both the SB203580 and PD98059 showed dose-related inhibition of LPS-induced NO production.

Inhibition of p38 and ERK attenuate LPS-induced iNOS.

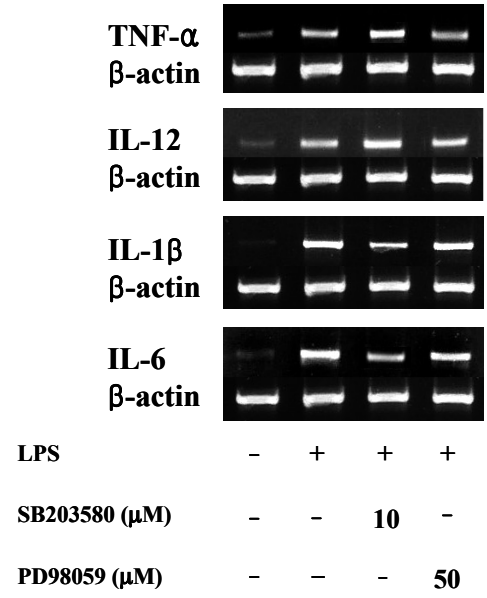

Figure 3. p38 inhibitor SB203580 and ERK inhibitor PD98059 differentially altered pro-inflammatory cytokine gene expression. Representative experiments were done by adding inhibitors at indicated concentrations 1 h prior to cell stimulation with LPS (30 ng/ml). Extraction and analysis of mRNA performed as described in Materials and Methods. Levels of TNF-α, IL-1β, IL-6, and IL-12 were quantified by RT-PCR and normalized against β-actin.

In macrophages, iNOS is induced by LPS and resulting in NO formation. To assay the correlation between decreased NO production by inhibitors and iNOS, we assayed iNOS expression and production by RT-PCR and western blotting, respectively. iNOS mRNA was increased following 6 h treatment with LPS, both SB203580 and PD98059 decreased LPS-induced iNOS gene expression (Fig. 2A). Treatment with both SB203580 and PD98059 also significantly decreased LPS-induced iNOS production (Fig. 2B).

Inhibition of p38 and ERK differentially regulate pro-inflammatory cytokines expression. p38 and ERK are known to regulate pro-inflammatory cytokine expression. To determine which cytokines are regulated by p38 and ERK, we measured the gene expression of TNF-α, IL-1β, IL-6 and IL-12 (Fig. 3). Stimulation with LPS to cells increased all 4 types of cytokine expression. Treatment of SB203580 potentiated LPS-induced TNF-α and IL-12 expression. PD98059 had no effect on LPS-induced TNF-α and IL-12 expression.

Inhibition of p38 potentiates LPS-induced ROS production. LPS increase the cellular oxidative stress and produced ROS regulate MAPK pathways (20). Generation of ROS during LPS treatment caused by not short-term but long-term treatment to cells (21). We pretreated SB203580 or PD98059 and stimulated LPS

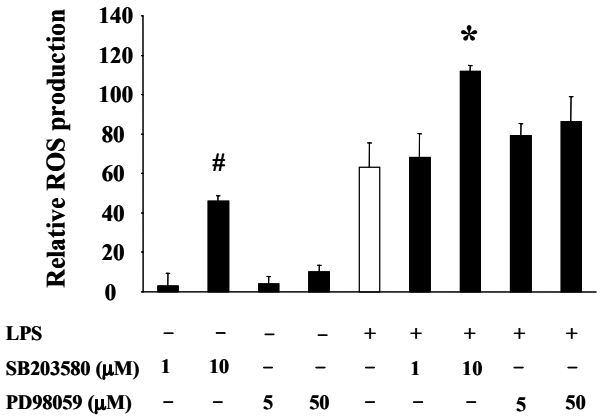

Figure 4. p38 inhibitor SB203580 increased ROS production. Representative experiments were done by adding inhibitors at indicated concentrations 1 h prior to cell stimulation with LPS (30 ng/ml) for 18 h. Results from a representative experiment of a replicates are expressed as mean±SE (n=6). *Significantly different from the LPS treatment group at $p < 0.05$. #Significantly different from the control group at $p < 0.05$.

for 18 h to induce ROS in the presence of DHR as previously reported (14). Relatively high concentration of SB203580 increased oxidation of DHR and additively increased LPS-induced DHR oxidation, which indicate that generation of ROS (Fig. 4). Relatively low concentration of SB203580 and PD98059 did not induce ROS production.

Discussion

We studied the effects of well-characterized MAPK inhibitors on the ability of activated macrophages to produce NO and pro-inflammatory cytokines. SB203580 inhibits the p38 MAPK pathways, and PD98059 inhibits the ERK pathway and these inhibitors are highly specific for these cascades (22). We found that both inhibitors decreased LPS-activated iNOS expression and production, and concomitant NO production. These results indicated that both p38 and ERK pathways play an important role in the up-regulation of iNOS during macrophage activation. Several studies reported blocking of p38 and ERK inhibitors on LPS-stimulated NO production in macrophages (23, 24). However, other studies showed different results. It has been reported that SB203580 but not PD98059 mediated LPS-induced NO production in murine macrophages due to the differential regulation to NF-κB-DNA binding (7). Activation of p38 but not ERK by LPS resulted in the stimulation of NF-κB-DNA binding and subsequent expression of iNOS and NO release. Monier et al., showed that SB203580 but not PD98059 inhibited gram-positive bacteria component activated iNOS and NO production in macrophages (25). These reports suggest that p38 and ERK pathways are important on macrophage activation by bacterial products but they regulate different macrophage responses in a distinct manner. In our study, that relatively low level of inhibitors (1μM of SB203580, 5μM of PD98059) did not inhibit LPS-induced iNOS and NO production. Only relatively high concentration (10μM of SB203580, 50μM of PD98059) of inhibitors blocked LPS-induced iNOS and NO production. Our data are consistent with the report implicating p38 and ERK pathways are involved in the up-regulation of macrophage iNOS production in concentration and time dependent manner. However, our data conflict with recent report showing bi-directional effect of SB203580 on iNOS and NO production in macrophages (26). Low concentration (0.5～1μM) of SB203580 stimulated NO production. In contrast, high concentration of SB203580 inhibited LPS-induced NO production and the inhibitory effect is unrelated to inhibition of p38 MAPK. Reasons for these discrepancies are currently unknown, but may be explained by difference of experiment conditions such as, concentration of inhibitors, incubation time, and types of cells.

MAPKs are important mediator of cytokine gene expression. In particular, p38 and ERK are known to play a key role in LPS-induced signal transduction pathways leading to cytokine synthesis. These effects have been investigated by using specific inhibitors in monocytes and macrophages (9,16,23,27-29). Our data showed that both SB203580 and PD98059 inhibited LPS-induced IL-1β and IL-6 expression. However, treatment of SB203580 increased LPS-induced TNF-α and IL-12 expression but not PD98059. Previous studies indicated that p38 MAPK functions by regulating processes that control translation of cytokine mRNA (16,29). In addition, the role of p38 in the cytokine synthesis is depending on the nature of assayed cytokine. Thus, the control of cytokine synthesis by p38 can function at different levels, even within the same cell. Our data showing inhibitory effect of SB203580 and PD98059 on LPS-induced IL-1β and IL-6 expression are consistent with previous reports in monocytes and macrophages (16,29,30). Marriott et al. (27) reported that p38 has a suppressive effect on IL-12 expression indicated by increasing effect of SB203580 on LPS-induced IL-12 in peripheral blood mononuclear cells. This report supports our data showing increasing effect of SB203580 on LPS-induced IL-12 expression in macrophages.

Surprisingly, pretreatment of SB203580 increased LPS-induced TNF-α expression. Because p38 pathway is known to mediate TNF-α production indicated by using SB203580 in macrophages (23), and other types of specific p38 inhibitor, SB202190, in monocytes (29), our data are unexpected result de-

spite SB203580 had no effect on the TNF-α expression in murine macrophages (16). In addition, treatment of PD98059 did not change TNF-α expression, although Valledor et al. (30) showed the decreasing effect of PD98059 in LPS-induced TNF-α expression in macrophages. To verify the effect of both inhibitors on TNF-α expression, we measured ROS production. Oxidative stress and ROS regulate MAPK pathways and downstream cytokine transcription (20,31). We hypothesized that if high concentration of SB203580 activates ROS production, produced ROS may stimulate expression of TNF-α. Our data clearly showed that only high concentration of SB203580 activated ROS production and additively increased LPS-induced ROS production. This data support our hypothesis that produced ROS by SB203580 may stimulate TNF-α transcription. We should rule out the possibility that the activation of ROS by SB203580 may result from the direct inhibition of antioxidant enzymes such as superoxide dismutase, catalase, or glutathione synthetase. To confirm this possibility, we treated SB203580 or PD98059 to macrophages and assayed the expression level of three enzymes. Both inhibitors did not change gene expressions of all three enzymes (data not shown). It has been reported that redox-sensitive transcription factor NF-κB transactivates promoters of many inflammatory cytokines such as TNF-α in macrophages (32). SB203580 but not PD98059 inhibit the LPS-induced stimulation of NF-κB-DNA binding (7). These data may suggest that treatment of SB203580 induces ROS production and may activate downstream ROS-sensitive NF-κB, and resulted in transcription of TNF-α. The involvement of NF-κB on differential effect of SB203580 and PD98059 should be further investigated.

Recently, it has been suggested that the use of specific inhibitors of MAPKs for the treatment of inflammatory disorders due to the inhibitory effect of iNOS and NO production (23). It may the useful strategy in point view of overexpressed NO regulation, however, due to the cell type and stimulation dependent effect of inhibitors, and the new finding, bi-directional effect on NO production (26) and stimulating effect of ROS, the commercial use of SB203580 should be more studied.

In conclusion, both p38 and ERK pathways mediated iNOS and NO production in macrophages. Both p38 and ERK regulate IL-1β and IL-6, however, only p38 regulate IL-12 and TNF-α. ROS are involved in increasing effect of SB203580 on TNF-α.

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