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Involvement of p38 mitogen-activated protein kinase in the induction of interleukin-12 p40 production in mouse macrophages by berberine, a benzodioxoloquinolizine alkaloid

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Abstract

Interleukin (IL)-12 plays a pivotal role in the development of T helper type 1 (Th1)-immune response, which may have therapeutic effects on diseases associated with pathologic Th2 responses such as allergic disorders and asthma. In this study, we investigated the effects of berberine, a benzodioxoloquinolizine alkaloid with anti-microbial and anti-tumor activities, on the production of IL-12 p40, an inducible subunit of IL-12, in mouse macrophages. Berberine-induced IL-12 p40 production and activation of p38 mitogen-activated protein kinase (MAPK) in dose-dependent manners, which were significantly inhibited by p38 MAPK inhibitors and yohimbine, indicating that p38 MAPK and α_2-adrenergic receptor were involved in the induction of IL-12 p40 production in mouse macrophages by berberine. Furthermore, berberine significantly enhanced IL-12 p40 production in mouse macrophages when combined with lipopolysaccharide, a well-known inducer of IL-12 production. These findings may explain some of the known biological effects of berberine and suggests berberine as an immunotherapeutic compound for induction of IL-12, which is potentially applicable for tumors, infectious disease, and airway inflammation. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Berberine; Interleukin-12; p38 MAPK; Macrophage; Lipopolysaccharide; T helper

1. Introduction

Interleukin (IL)-12 is a heterodimeric cytokine comprised of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes. Secretion of p40 or p70 is limited to cells of the macrophage/monocyte lineage and occurs only after activation of these cells [1]. In contrast, the IL-12 p35 chain is produced by a number of cell types and constitutively expressed. Thus, p40 expression governs the production of the bioactive p70, which induces the production of IFN-γ and, in turn, drives the production of a number of inflammatory cytokines. Additionally, IL-12-induced IFN-γ can direct activated $CD4^+$ T lymphocytes to differentiate into T helper type 1 (Th1) cells [2]. Adequate production of IL-12 is essential for the maintenance of normal host defense mechanisms and this key role has raised considerable interest in the mechanisms involved in the regulation of IL-12 biosynthesis [3,4]. Inducible expression of IL-12 has been documented in macrophages and dendritic cells after stimulation by microbial antigens or *via* CD40–CD40L interaction [5,6]. In lipopolysaccharide (LPS)- and IFN-γ-treated monocytes, the expression of IL-12 p40 has been shown to be primarily regulated at the transcriptional level, which involves functional synergy with transcription factors, C/EBP or Ets families with NF-κB [7–10].

Berberine is a benzodioxoloquinolizine alkaloid that has been isolated from *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). Its extracts and decoctions have demonstrated significant anti-microbial activity against a variety of organisms including bacteria, viruses,

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Abbreviations: IL, interleukin; Th1, T helper type 1; MAPK, mitogen-activated protein kinase; MKK3, MAPK kinase 3; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase-polymerase chain reaction.

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fungi, protozoans, helminths, and chlamydia [11]. Furthermore, berberine has shown a number of beneficial effects, including immunostimulation *via* increased blood flow to the spleen, macrophage activation, elevation of platelet counts in cases of primary and secondary thrombocytopenia. In addition, berberine may possess anti-tumor promoting properties as evidenced by inhibition of cyclooxygenase-2 transcription and *N*-acetyltransferase activity in colon and bladder cancer cell lines, and transient, but marked, inhibitory action on the growth of mouse sarcoma cells in culture [12].

p38 mitogen-activated protein kinase (MAPK) pathway is activated by environmental perturbation (e.g. osmotic changes, heat shock) and by inflammatory cytokines including TNF-α and IL-1 [13]. This pathway has been proposed to function in the regulation of cytokine production [14,15], B cell and T cell proliferation and differentiation [16–18], the innate immune response [19], cell cycle control [20,21] and apoptosis [22,23]. Recent studies have shown that the p38 MAPK, activated through MAPK kinase 3 (MKK3), may be involved in the production of proinflammatory cytokines by both antigen-presenting cells and $CD4^+$ T cells [24–26].

In this report, we have demonstrated that berberine significantly induced IL-12 p40 production in mouse macrophages and, furthermore, enhanced IL-12 p40 production in macrophages when combined with LPS. Berberine-activated p38 MAPK in macrophages and p38 MAPK inhibitors significantly suppressed this berberine-induced IL-12 p40 production, indicating that p38 MAPK pathway may be involved in the berberine-mediated induction of IL-12 p40 production from mouse macrophages.

2. Materials and methods

2.1. Mice, monoclonal antibodies, cytokines, and reagents

Female DBA/2 mice were obtained from the Japan SLC, Inc. and used at 6–10 weeks of age. Anti-IL-12 p40 mAbs C17.8 and C15.6 were purified from ascitic fluid by ammonium sulfate precipitation followed by DEAE–Sephagel chromatography (Sigma Chemical Co). Anti-p38 MAPK mAb and anti-phosphotyrosine mAb were purchased from Santa Cruz Biotechnology, Inc. Recombinant murine IL-12 was generously provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). LPS (from *Escherichia coli* 0111:B4), berberine, cycloheximide, yohimbine, and protein A were purchased from the Sigma. The α_2-adrenergic agonist, clonidine and the p38 MAPK inhibitors, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole] and SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole], and the control chemical, SB202474 [4-ethyl-2-(4-acetylphenyl)-5-(4-pyridyl)-imidazole], were purchased from Calbiochem–Novabiochem Co.

2.2. Preparation of splenic macrophages

Spleen cells were cultured at 10^6 cells/mL for approximately 3 hr at 37°. The non-adherent cells were removed by washing with warm Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and antibiotics (Life Technologies, Inc.) until visual inspection revealed a lack of lymphocytes (>98% of the cell population). The adherent cells were removed from plates by incubating for 15 min with ice-cold phosphate-buffered saline solution with 5 mM EDTA and rinsing repeatedly. The isolated adherent cell population was treated in the absence or presence of berberine, clonidine, yohimbine, and/or p38 MAPK inhibitors. In some experiments, the cells were treated with berberine in the presence of LPS.

2.3. Cytokine assay

The quantities of IL-12 p40 in culture supernatants were determined by a sandwich ELISA using mAbs specific for IL-12 p40, as previously described [27]. The mAb for coating the plates and the biotinylated second mAb were as follows: C17.8 and C15.6. Standard curve was generated using recombinant IL-12 p40 and the lower limit of detection was 30 pg/mL.

2.4. Preparation of cell lysates

The stimulated cells were washed twice with ice-cold phosphate-buffered saline solution and harvested with a plastic scraper. The cells were lysed in lysis buffer (50 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/mL each of leupeptin, aprotinin, and PMSF) by incubation on ice for 30 min. Lysates were then centrifuged at 13,000 *g* at 4° for 10 min, and the supernatants were transferred to fresh tubes and stored at −70° until required. Protein concentrations of the lysates were determined using the Coomassie protein assay reagent (Bio-Rad).

2.5. Immunoprecipitation and Western blot analysis

The cell lysates were incubated with protein A coupled with anti-phosphotyrosine mAb for overnight and resolved by 12% SDS–polyacrylamide gel electrophoresis before transfer to PVDF membrane using a Semi-Phor (Hoefer Scientific Instrument). The membrane was then incubated with washing buffer (phosphate-buffered saline solution containing 0.1% Tween 20) containing 2% bovine serum albumin for at least 1 hr to block nonspecific protein binding. Primary mAb was diluted up to 1:1000 in washing buffer and applied to the membrane for 1 hr at room

temperature. Following washing, the blots were incubated with the appropriate HRP-conjugated secondary mAb (diluted up to 1:3000 in washing buffer) for 1 hr at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the cells and reverse-transcribed into cDNA, and then PCR amplification of the cDNA was preformed. The sequences of PCR primers used in the experiments are as follows: mouse IL-12 p40 (sense, 5′-CAGAAGCTAACCATCTCCTGGTTTG-3′; antisense, 5′-TCCGGAGTAATTTGGTGCTTCACAC-3′), IL-12 p35 (sense, 5′-TCAGCGTTCCAACAGCCTC-3′; antisense, 5′-CGCAGAGTCTCGCCATTATG-3′), TNF-α (sense, 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′; antisense, 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′), IFN-γ (sense, 5′-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC-3′; antisense, 5′-TGGACCTGTGGGTTGTTGACCTCAAACTTGGC-3′), IL-6 (sense, 5′-TGAACAACGATGATGCACTT-3′; antisense, 5′-CGTAGAGAACAACATAAGTC-3′), and β-actin (sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; antisense, 5′-TAAAACGCAGCTCAG TAACAGTCCG-3′). The PCR reactions were run for 35 cycles for 94° (30 s), 58° (45 s), 72° (30 s) using an MJ Thermal Cycler. After the amplification, the RT-PCR products were separated in 1.5% (w/v) agarose gels and stained with ethidium bromide.

2.7. Transient transfection

For transfection, RAW264.7 monocytic cells were grown in 24-well plates with DMEM supplemented with 10% FBS for 24 hr and transfected with the indicated plasmid in the presence of Superfectam according to the manufacturer's protocol (Qiagen). After 24 hr, cells were washed and refed with DMEM containing 10% FBS. Cells were harvested 24 hr later, luciferase activity was assayed as previously described [28], and the results were normalized to the *LacZ* expression. For IL-12 p40 promoter constructs, the −689/+98 fragments of the murine IL-12 p40 promoter from pXP2 [7] was subcloned into the *Kpn*/*Xho*I sites of the pGS3-basic luciferase vector (Promega Co).

2.8. Electrophoretic mobility shift assay

The nuclear extracts were prepared from RAW264.7 cells, as previously described [29]. An oligonucleotide containing an NF-κB-binding site within the Igk-chain (5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′) was used as a probe. Labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature, along with 10 mg of nuclear extracts, in 20 mL of binding buffer (10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI–dC), and 1 mM dithiothreitol). The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris–borate buffer. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

2.9. Statistical analysis

The Student's *t*-test and one-way analysis of variance were used to determine the statistical differences between the values of various experimental and control groups. A P value of <0.01 was considered as significant.

3. Results

3.1. Berberine induces IL-12 p40 production from splenic macrophages

To assess the effect of berberine on IL-12 production, splenic macrophages were treated with berberine (0.1–1 μg/mL) for 48 hr and the levels of IL-12 p40 protein in the culture supernatants were determined by a sandwich ELISA for IL-12 p40. An IL-12 p40 subunit was known as the highly inducible and tightly regulated component of IL-12 [30]. Berberine strongly induced IL-12 p40 production in a dose-dependent manner (Fig. 1). Treatment of macrophages with 1 μg/mL berberine induced approximately similar levels of IL-12 p40 production compared with that treated with 5 μg/mL LPS, a well-known inducer of IL-12 p40. Macrophages' viability remained constant throughout the incubation period in the presence of berberine

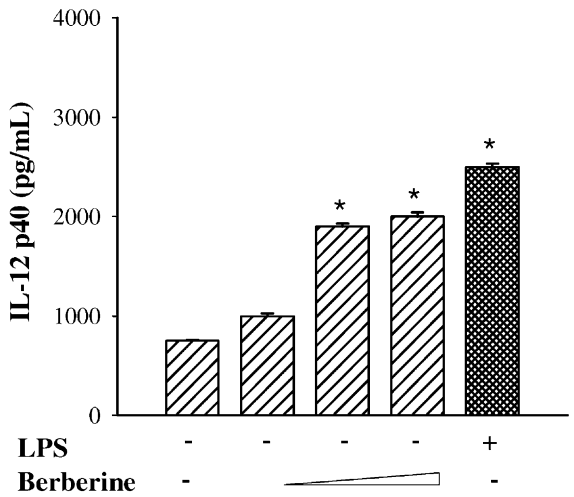

Fig. 1. Induction of IL-12 p40 production in primary mouse macrophages by berberine. Splenic macrophages were incubated in the absence or presence of berberine (0.1, 0.5 and 1 μg/mL) or LPS (5 μg/mL) for 48 hr, and the levels of IL-12 p40 protein in the culture supernatants were determined by a sandwich ELISA and the results are presented as the mean ± SEM ($n = 4$). (*) $P < 0.001$ vs. unstimulated control.

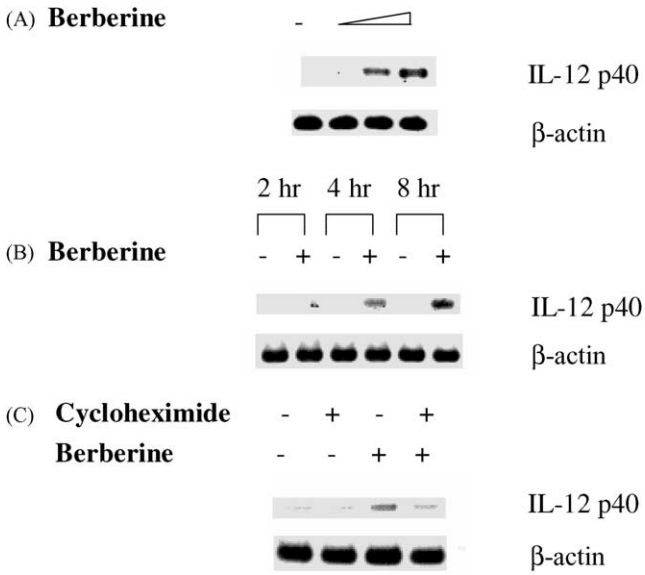

Fig. 2. Increased IL-12 p40 mRNA expression by berberine. Splenic macrophages were incubated with varying concentrations of berberine for 8 hr (A) or 1 μg/mL berberine for varying treatment times (B), or pretreated with 10 μg/mL cycloheximide for 1 hr, followed by treatment with 1 μg/mL berberine for 6 hr (C). RT-PCR products were analyzed in 1.5% agarose gels.

concentrations used in the experiments, as demonstrated by trypan blue exclusion test (unpublished observation).

To examine whether the IL-12 p40 induction by berberine at the protein level is associated with IL-12 p40 mRNA expression, RT-PCR for IL-12 *p40* gene was performed in the berberine-treated mouse macrophages. The levels of RT-PCR product for IL-12 *p40* gene were significantly increased in a dose-dependent manner when macrophages were treated with berberine (0.1–1 μg/mL) for 8 hr, indicating that the induced IL-12 p40 production by berberine might occur at the mRNA level (Fig. 2A). Treatment with berberine did not influence β-actin mRNA expression in macrophages, suggesting that the inductive effect on IL-12 p40 production by berberine was not the result of general phenomena of cellular activation. To further investigate the kinetics of the response, macrophages were cultured with 1 μg/mL of berberine for varying periods. As shown in Fig. 2B, IL-12 p40 RT-PCR product was detectable at 4 hr and increased in a time-dependent manner. The increased levels of IL-12 p40 mRNA were sustained at 24 hr after treatment (unpublished observation). Such kinetics of berberine-induced IL-12 p40 mRNA expression are similar to those reported for LPS-induced IL-12 p40 mRNA expression [31]. Treatment of macrophages with cyclohexamide before berberine addition inhibited the IL-12 p40 mRNA expression induced by berberine (Fig. 2C).

3.2. Induced IL-12 p40 production by berberine is not mediated through NF-κB

The transcription factor NF-κB is essential for IL-12 p40 production [32]. The inducible promoter activity of the *p40* gene was first localized to a novel sequence for binding of the NF-κB/Rel family and, subsequently, the C/EBP and Ets elements were known to exhibit functional synergy with the NF-κB site [7–10]. To address the mechanism by which berberine induces IL-12 p40 production in mouse macrophages, we investigated whether berberine mediates its effect on IL-12 production through modulating NF-κB activity. We used an IL-12 p40 promoter-luciferase construct to test the ability of berberine to activate the IL-12 p40 promoter containing NF-κB sites. We transiently transfected RAW264.7 monocytic cells with a −689/+98 fragment of the p40 promoter plasmid containing the binding site for NF-κB. Berberine did not induce IL-12 *p40* gene promoter activity while LPS significantly stimulated the activity (Fig. 3A). Next, we tested whether the induced IL-12 p40 production by berberine is mediated

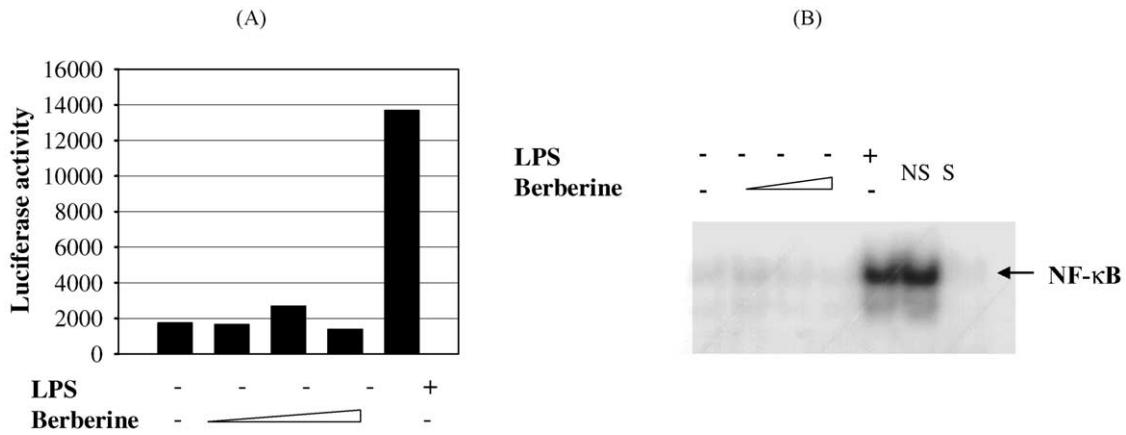

Fig. 3. Induced IL-12 p40 production by berberine is not mediated through NF-κB. (A) Effect of berberine on IL-12 p40 promoter activity. RAW264.7 cells were transiently transfected with pGL3 or IL-12 p40 promoter construct (pGL/p40), followed by incubation with berberine (0.1, 0.5 and 1 μg/mL) or LPS (5 μg/mL). Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions. The data are the means of three independent experiments. (B) Effect of berberine on NF-κB binding activity to κB sites. Nuclear extracts from RAW264.7 cells treated with berberine (0.1, 0.5 and 1 μg/mL) or LPS (5 μg/mL) were examined for κB binding activity in the electrophoretic mobility shift assay using a labeled oligonuleotide containing a consensus Ig-κB site. 'S' and 'NS' indicate the presence of an unlabeled identical oligonucleotide and nonspecific oligonucleotide, respectively.

through binding of NF-κB complexes to the κB sites. We analyzed the κB binding activity present in nuclear extract stimulated with LPS or berberine. Nuclear extracts from LPS-stimulated macrophages exhibited strong κB binding activity in the electrophoretic mobility shift assay using a labeled oligonucleotide containing a consensus Ig-κB site [33]. This binding was specific as it was competed with an unlabeled, identical oligonulceotide, but not with unrelated, nonspecific oligonucleotide. In contrast, the κB binding activities were not affected in the berberine-treated macrophages (Fig. 3B), suggesting that berberine may not modulate the NF-κB–DNA interactions.

3.3. Berberine activates p38 MAPK in mouse macrophages

Although IL-12 p40 induction by berberine occurred at the transcriptional level (Fig. 2), it was not mediated through modulation of NF-κB (Fig. 3). Therefore, we investigated whether berberine modulates IL-12 p40 production by activating p38 MAPK in macrophages. Splenic macrophages were incubated for 45 min with berberine and the activation of p38 MAPK was examined by detecting the phosphorylated form of p38 MAPK. We found that berberine stimulated the phosphorylation of p38 MAPK in a dose-dependent manner (Fig. 4A). The berberine-induced activation of p38 MAPK in mouse macrophages was significantly inhibited by SB203580, a selective inhibitor of p38 MAPK, but not by SB202474, a chemical used as a negative control (Fig. 4B). Interestingly, the phosphorylation of p38 MAPK by berberine was inhibited by yohimbine, an α_2-adrenergic receptor antagonist. Berberine was reported to interact with α_2-adrenergic receptor and to have similar pharmacological effects with clonidine, an α_2-adrenergic receptor agonist [34].

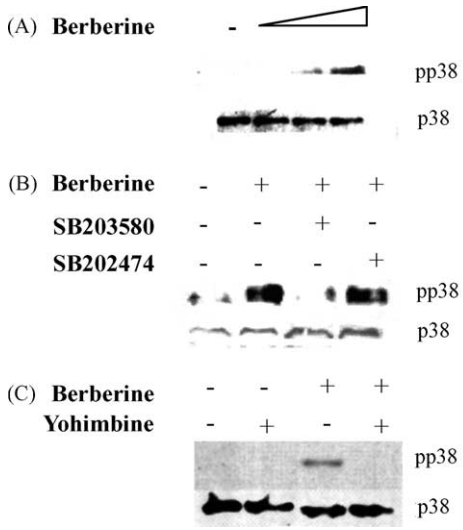

Fig. 4. Berberine-mediated activation of p38 MAPK in mouse macrophages. Splenic macrophages were cultured with varying concentrations of berberine for 45 min (A), or pretreated with 10 μM SB203580, 10 μM SB202474 (B) or 100 μM yohimbine (C) for 2 hr and then incubated with 1 μg/mL berberine for 45 min. The cell lysates were immunoprecipitated using anti-phosphorylated tyrosine mAb and blotted with anti-p38 MAPK mAb (pp38), or the cell lysates were directly probed with anti-p38 MAPK mAb (p38).

3.4. Selective p38 MAPK inhibitors suppress the berberine-induced IL-12 p40 production

To further investigate whether p38 MAPK activation is required for IL-12 p40 induction by berberine, we investigated the effect of p38 MAPK inhibitors on berberine-induced IL-12 p40 production in mouse macrophages. SB203580 inhibited the berberine-induced IL-12 p40 production in a dose-dependent manner (Fig. 5A). Moreover, the berberine-induced IL-12 production was also inhibited

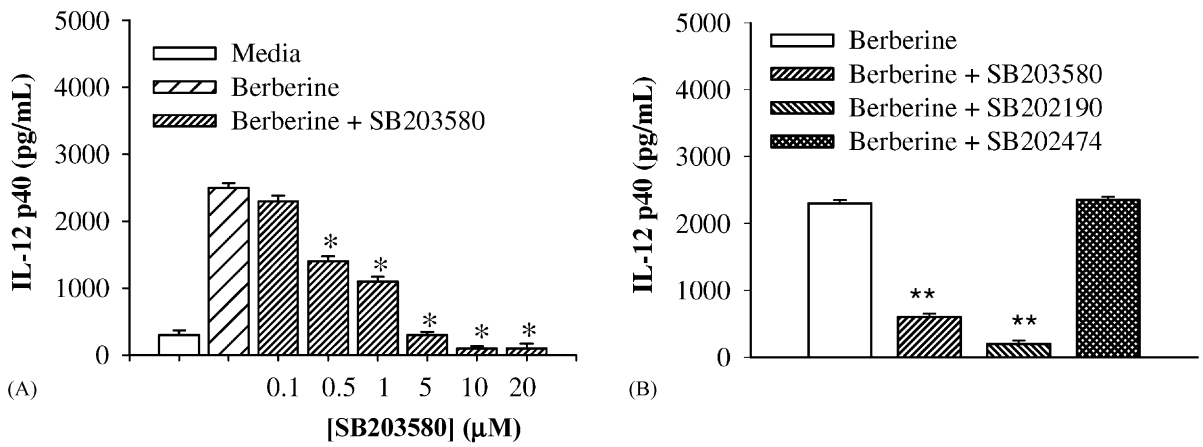

Fig. 5. Suppression of berberine-induced IL-12 p40 production by p38 MAPK inhibitors. (A) Splenic macrophages were cultured in the absence or presence of varying concentrations of SB203580 (0.1–20 μM) (A), or SB203580 (10 μM), SB202190 (10 μM) or SB202474 (10 μM) for 2 hr (B), and then treated with 1 μg/mL berberine. After 48 hr culture, the levels of IL-12 p40 protein in the cell supernatants were evaluated by a sandwich ELISA. The results are presented as the mean ± SEM ($n = 3$). (*) $P < 0.01$ vs. berberine-treated group in the absence of SB203580. (**) $P < 0.01$ vs. berberine-treated group in the presence of SB202474.

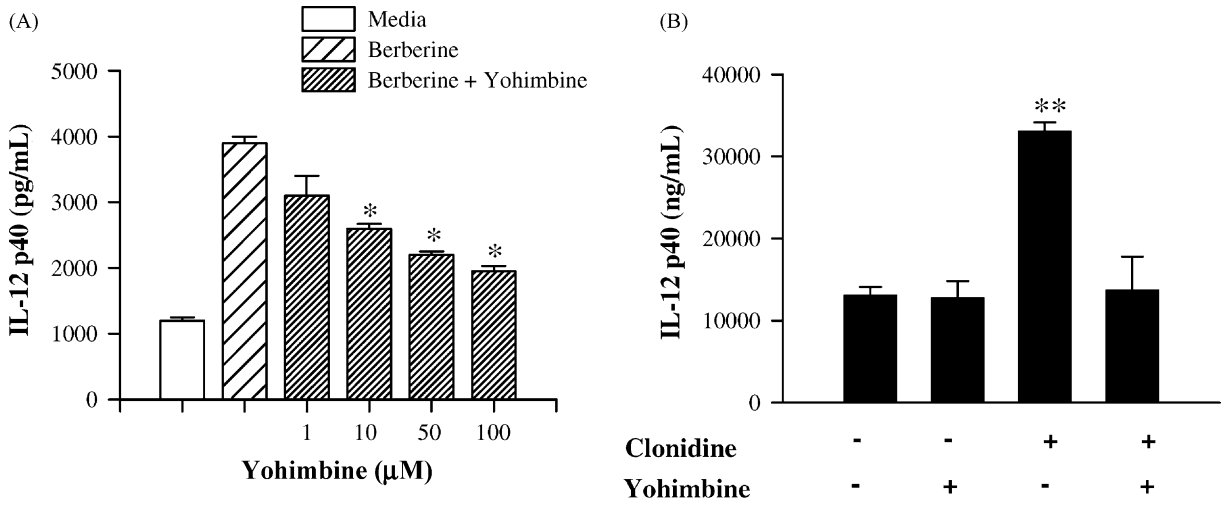

Fig. 6. Effect of yohimbine, an α_2-adrenergic receptor antagonist, on IL-12 p40 induction by berberine and clonidine in mouse macrophages. (A) Splenic macrophages were pretreated with yohimbine for 1 hr and then treated with 1 μg/mL berberine (A) or 20 μM clonidine (B). After 48 hr, the levels of IL-12 p40 protein in the culture supernatants were determined by a sandwich ELISA. The results are presented as the mean ± SEM ($n = 3$). (*) $P < 0.01$ vs. berberine-treated group in the absence of yohimbine. (**) $P < 0.01$ vs. clonidine-treated group in the absence of yohimbine.

by SB202190, an inhibitor of p38 MAPK, but not by SB202474 used as a negative control chemical of p38 MAPK inhibitor (Fig. 5B). These results (Figs. 4 and 5) demonstrated that IL-12 p40 induction by berberine might be mediated through p38 MAPK signaling pathway.

3.5. IL-12 p40 production by berberine is, at least in part, mediated via α_2-adrenergic receptor

To further determine any involvement of α_2-adrenergic receptor in the berberine-induced IL-12 p40 production, macrophages were treated with berberine in the absence or presence of yohimbine, an α_2-adrenergic receptor antagonist. As shown in Fig. 6A, treatment with yohimbine significantly suppressed the berberine-induced IL-12 p40 production in a dose-dependent manner. Furthermore, clonidine, a well-known α_2-adrenergic receptor agonist, also induced IL-12 p40 production in macrophages, which was significantly inhibited by yohimbine (Fig. 6B). These results indicate that the induction of IL-12 p40 production in macrophages by berberine may be, at least in part, mediated *via* α_2-adrenergic receptor.

3.6. Berberine enhances IL-12 p40 production when combined with LPS

LPS, a major component of the outer membrane of the cell wall in gram-negative bacteria, is a potent activator of

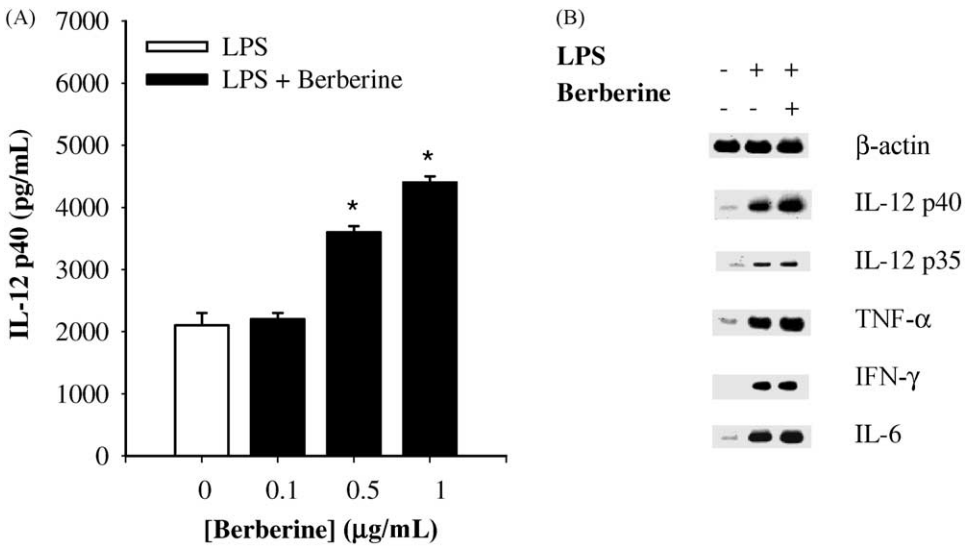

Fig. 7. Augmentation of LPS-induced IL-12 p40 expression in mouse macrophages by berberine. Splenic macrophages were incubated with LPS (5 μg/mL) in the absence or presence of varying concentrations of berberine for 48 hr (A) or 8 hr (B), followed by a sandwich ELISA for IL-12 p40 determination and RT-PCR for mRNA expression, respectively. The results for IL-12 p40 ELISA are presented as the mean ± SEM ($n = 3$). (*) $P < 0.01$ vs. a group treated with LPS alone.

the immune and inflammatory responses. Macrophages stimulated with LPS lead to the generation of IL-12 p40, which forms the biologically active heterodimeric form of IL-12 p70 with the constitutively expressed IL-12 p35 [2]. We investigated whether berberine could enhance IL-12 p40 production in mouse macrophages when costimulated with LPS. Mouse macrophages were stimulated with LPS in the absence or presence of varying concentrations of berberine. Berberine significantly enhanced IL-12 p40 production in a dose-dependent manner when combined with LPS (Fig. 7A). Furthermore, to examine whether the enhanced IL-12 p40 production by berberine is the results of enhanced IL-12 p40 mRNA expression, the effect of berberine on the expression of IL-12 p40 mRNA was analyzed in LPS-stimulated macrophages in the absence or presence of berberine. Berberine significantly enhanced mRNA level of IL-12 *p40* gene, indicating that the enhanced IL-12 p40 production with berberine occurred at the transcriptional level. In contrast, treatment with berberine did not enhance IL-12 p35, IFN-γ and IL-6 mRNA expression when combined with LPS (Fig. 7B).

4. Discussion

In this report we have demonstrated that berberine can significantly induce IL-12 p40 production through p38 MAPK activation and can synergize to induce IL-12 p40 production when combined with LPS. IL-12 plays an essential role in the optimal generation of IFN-γ-secreting Th1 cell under many experimental conditions. Through this activation, and through its ability to induce directly IFN-γ secretion from both T and NK cells, IL-12 plays a central role in both innate and adaptive immunity important to host defense against predominantly intracellular pathogens. Recombinant IL-12 has striking therapeutic effects in mouse models of tumor [35,36], infectious disease [37,38], and airway inflammation [39,40]. Based on these results, clinical trials investigating the potential therapeutic effects of IL-12 have been initiated in human cancer patients, HIV-infected patients, and patients with chronic viral hepatitis. IL-12 may also have utility as a vaccine adjuvant [41]. Clinical trials using direct administration and gene therapy approaches have yielded promising results. These findings have raised great interest in identifying enhancers of IL-12 production for the treatment of diseases associated with pathologic Th2 responses such as allergic disorders and asthma. Recently, paclitaxel was reported to enhance macrophage IL-12 production in tumor-bearing hosts through nitric oxide [42]. In addition, cyclosporin was shown to enhance IL-12 production by CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides [43]. Substance P was reported to stimulate IL-12 production through NK-1 receptor [44]. Chitin-particle initiated IL-12 production through mannose receptor-mediated phagocytosis [45]. IL-12 induction by hyaluronan through CD44 interaction was also reported [46].

The induced IL-12 p40 production by berberine might occur at the mRNA level, as demonstrated by IL-12 p40 RT-PCR experiments (Fig. 2). Treatment of macrophages with cycloheximide before berberine addition inhibited the IL-12 p40 mRNA expression (Fig. 2C), indicating that IL-12 p40 transcription by berberine might be regulated by a *de novo* synthesized protein factor(s). However, we can't exclude any possibility of the increased IL-12 mRNA stability by berberine, resulting in the induction of IL-12 p40 production in mouse macrophages. MAP kinases have been demonstrated to increase the stability of cytokine RNAs [47].

The transcription factors NF-κB and Ets are proposed to play a key role in the regulation of *IL-12* gene. Others and we have reported that some compounds including retinoids regulated IL-12 production in macrophages, monocytes and dendritic cells by controlling NF-κB activity [48–51]. Little is known regarding the molecular mechanisms underlying the induction of biologically active IL-12 apart from the fact that the NF-κB appears to be important in the LPS-mediated stimulation of the IL-12 *p40* gene transcription. LPS-induced NF-κB binding activity in murine IL-12 p40 promoter was independent of protein kinase C, protein kinase A and extracellular signal-regulated kinase [32]. Recent reports have shown that SB203580, a p38 MAPK inhibitor, inhibited in part the LPS-induced IL-12 production in macrophages/monocytes [24,25] and also inhibited CD40-induced IL-12 p40 production in DCs [52], suggesting that the p38 MAPK, activated through MKK3, may be a factor to regulate the production of IL-12. However, the target of p38 MAPK that mediates IL-12 p40 expression has not been identified yet. In this study we have shown that berberine significantly induced IL-12 p40 production in mouse macrophages in a dose-dependent manner and also stimulated p38 MAPK activation. In addition, we showed that the inducing effect of berberine on IL-12 production closely associated with p38 MAPK activation. These results are consistent with other's report that p38 MAPK promoted the up-regulation of IL-12 and NF-κB complexes did not transduce the p38 MAPK in IL-12 induction [24,25].

The involvement of p38 MAPK was reported for IFN-γ and IL-6 production in various immune and non-immune cells [15,53]. However, there was little effect on IFN-γ and IL-6 mRNA expression by berberine in mouse splenic macrophages (Fig. 7B). One possibility may be the differential expression of p38 MAPK family members in various cell lineages and tissues. In addition, the involvement of MAPK in the regulation of cytokine gene expression may be different depending on the type of cytokines. LPS-stimulated macrophages isolated form MKK-3 deficient mice made normal levels of IL-6, IL-1, TNF-α and IL-12, although IL-12 production was significantly reduced in $MKK3^{-/-}$ macrophages [24].

Furthermore, berberine could increase IL-12 p40 mRNA expression and p40 production when combined with LPS, a well-known inducer of IL-12. However, IL-12 p40 induction by LPS was reduced in the presence of nitric oxide donor but IL-12 p40 induction by berberine was not reduced under the same condition (unpublished observation). Nitric oxide was suggested to prevent the excessive amplification of Th1 cells inhibiting IL-12 production [54]. In addition, berberine did not activate IL-12 p40 promoter containing the NF-κB site and also did not stimulate NF-κB binding to the κB site while LPS strongly did activation of IL-12 p40 promoter and stimulation of NF-κB binding. These findings suggest that berberine induces IL-12 p40 production in mouse macrophages, at least in part, through a distinct pathway with that of LPS.

Berberine was reported to have similar pharmacological effects to clonidine, an α_2-adrenegic agonist, and to interact with α_2-adrenergic receptor [34]. In this study we have demonstrated that yohimbine, known as an α_2-adrenergic antagonist, significantly inhibited the berberine-induced IL-12 p40 production in a dose-dependent manner, indicating that α_2-adrenergic receptor on macrophages is involved in the regulation of IL-12 p40 production by berberine. Furthermore, treatment of macrophages with clonidine, an α_2-adrenegic agonist, significantly induced IL-12 p40 production in mouse macrophages and also enhanced LPS-induced IL-12 p40 production (Fig. 6B). These results suggest that berberine-induced IL-12 p40 production in mouse macrophages, at least in part, through an α_2-adrenergic receptor. Since cells such as macrophages that lack specific receptors for antigens are the predominate secretors of IL-12 [55], the identification of receptor-mediated signals that augment IL-12 production is critical for an understanding of the events that regulates the secretion of this cytokine. Since the berberine-mediated IL-12 p40 production in mouse macrophages was partially inhibited by yohimbine, an α_2-adrenergic receptor antagonist, berberine might induce IL-12 p40 production by another pathway such as modulation of Ca^{2+} movement, not through α_2-adrenergic receptor. Berberine possess an inhibitory effect on the influx of extracellular Ca^{2+} and Ca^{2+}-release from intracellular stores in the smooth muscle cells of colon [56], and IL-12 production is subject to modulation by changes in Ca^{2+} movement [57,58]. The inhibitors of MEK, protein kinase C and casein kinase II were little effective on IL-12 p40 induction by berberine (unpublished observation).

In conclusion, we have shown that berberine induces IL-12 p40 production through p38 MAPK activation not modulating NF-κB activity. Furthermore, combined with a costimulus (i.e. bacterial LPS), berberine enhances IL-12 p40 production in macrophages. Because of IL-12's pivotal role in directing immunity toward cell-mediated or Th1-mediated responses, these findings suggest a promising role of berberine as a potential immunomodulator to generate the required cell-mediated immunity by induction of IL-12 production.

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