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European Journal of Pharmacology 467 (2003) 223–231
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Stimulation of interleukin-12 production in mouse macrophages via activation of p38 mitogen-activated protein kinase by α_2-adrenoceptor agonists

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Received 19 September 2002; received in revised form 12 March 2003; accepted 14 March 2003

Abstract

Interleukin-12 is a cytokine primarily produced by monocytes and macrophages. It plays an essential role in the development of cell-mediated immunity and stimulates T helper type 1 (Th1) immune responses. This study was designed to determine if α_2-adrenoceptor agonists are involved in the induction of interleukin-12 production by macrophages. α_2-adrenoceptor agonists such as clonidine, guanfacine, and oxymetazoline significantly induced interleukin-12 secretion and interleukin-12 mRNA expression by macrophages in a concentration-dependent manner. Moreover, stimulation of α_2-adrenoceptor by their agonists triggered the activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Inhibitors of p38 MAPK prevented the stimulatory effects of α_2-adrenoceptor agonists on IL-12 production. Yohimbine and 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)4,5-dihydro-1*H*-imidazole (RX821002), α_2-adrenoceptor antagonists, significantly blocked agonist-induced interleukin-12 production and p38 MAPK activation, indicating that the effects of the agonists were mediated through α_2-adrenoceptor. In addition, protein kinase C (PKC) inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and chelerythrine, significantly inhibited guanfacine-induced interleukin-12 production and p38 MAPK in a concentration-dependent manner. These findings show that α_2-adrenoceptor agonists induce interleukin-12 production in mouse macrophages via a PKC/p38 MAPK signaling pathway and suggest that the effect of α_2-adrenoceptor agonists on interleukin-12 secretion may be a new and novel means of augmenting cell-mediated immune responses.

Keywords: α_2-Adrenoceptor agonist; Interleukin-12; p38 Mitogen-activated protein kinase; Macrophage

1. Introduction

Interleukin-12 is a heterodimeric cytokine comprised of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by separate genes. Secretion of p40 or p70 is limited to cells of the macrophage/monocyte lineage and occurs only after activation of these cells. In contrast, the interleukin-12 p35 chain is produced by a number of cell types and is constitutively expressed (Trinchieri, 1998). Thus, p40 expression governs the production of the bioactive p70, which induces the production of interferon-γ and, in turn, drives the production of a number of inflammatory cytokines. Additionally, interleukin-12-induced interferon-γ can direct activated $CD4^+$ T lymphocytes to differentiate into T helper type 1 (Th1) cells. Adequate production of interleukin-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 interleukin-12 biosynthesis (Gately et al., 1998).

Catecholamines are a class of endogenous mediators that may potentially direct the responsiveness of macrophages through α- or β-adrenoceptors. Expression of α_2- and β-adrenoceptors was reported on macrophages, which can be activated by the endogenous ligand norepinephrine and by adrenergic drugs frequently used in clinical practice (Liggett, 1989; Spengler et al., 1990). Through stimulation of these receptors, norepinephrine and epinephrine affect lymphocyte trafficking (Benschop et al., 1996), migration (Schedlowski et al., 1996) and proliferation (Chambers et

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doi:10.1016/S0014-2999(03)01628-5

al., 1993). They also modulate cytokine production and the functional activity of different lymphoid cells (Chou et al., 1996). Recent evidence shows that agents such as catecholamines that stimulate the β_2-adrendoreceptor-cAMP-protein A pathway inhibit the production of type 1/proinflammatory cytokines such as interleukin-12, tumor necrosis factor-α (TNF-α) and interferon-γ by antigen-presenting cells or Th1 cells (Severn et al., 1992; Panina-Bordignon et al., 1997; Borger et al., 1998). In contrast, the same compounds stimulate the production of type 2/anti-inflammatory cytokines such as interleukin-10 and transforming growth factor-β (Elenkov et al., 1996). Stimulation of cells via the α_2-adrenoceptor resulted in an augmentation of TNF-α release by murine peritoneal macrophages stimulated with lipopolysaccharide as well as their phagocytic and tumoricidal activity (Spengler et al., 1990). Although the α_2-adrenoceptor has been reported to have a potential immunomodulatory role, little is known regarding the effect α_2-adrenoceptor agonists on the regulation of cytokine secretion.

In this study, we investigated the effects of α_2-adrenoceptor agonists on the production of interleukin-12 as well as the mechanism underlying these effects. We found that α_2-adrenoceptor agonists enhanced both interleukin-12 mRNA expression and protein secretion in mouse macrophages, via the receptor. Furthermore, our results demonstrated that p38 mitogen-activated protein kinase (MAPK) pathway was involved in the α_2-adrenoceptor-mediated induction of interleukin-12 production in macrophages. These results suggest a possible mechanism for α_2-adrenoceptor agonist-mediated modulation of cellular immune responses and α_2-adrenergic compounds may have important immunological effects that may be used in the treatment of impaired cell-mediated immunity.

2. Materials and methods

2.1. Materials

Anti-IL-12 p40 monoclonal antibodies C17.8 and C15.6 were purified from ascitic fluid by ammonium sulfate precipitation followed by DEAE-Sephagel chromatography (Sigma, St. Louis, MO). Anti-interleukin-12 p35 monoclonal antibody Red-T/G297-289 was obtained from PharMingen (San Diego, CA). Anti-p38 MAPK monoclonal antibody and anti-phosphotyrosine monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Yohimbine, wortmannin and protein A were purchased from the Sigma. Recombinant murine interleukin-12 was generously provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). Clonidine and oxymetazoline were purchased from Calbiochem-Novabiochem (La Jolla, CA) and guanfacine was purchased from BIOMOL Research Laboratory (Plymouth Meeting, PA). 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole (SB203580), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole (SB202190), 4-ethyl-2-(4-acetylphenyl)-5-(4-pyridyl)-imidazole (SB202474), 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), and foskolin were purchased from Calbiochem-Novabiochem. Chelerythrine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059), and 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)4,5-dihydro-1*H*-imidazole (RX821002) were purchased from the Tocris Cookson (UK). Female DBA/2 mice were obtained from Japan SLC (Tokyo, Japan) and used at 6–10 weeks of age. The mice were maintained and treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Preparation of splenic macrophages

Splenic macrophages were prepared as previously described (Chung et al., 2000). In brief, spleen cells were cultured at 10^6 cells per milliliter for approximately 3 h at 37 °C. The nonadherent cells were removed by washing with warm Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (Life Technologies) 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 α_2-adrenoceptor agonists, α_2-adrenoceptor antagonist, or selective inhibitors of various signaling components.

2.3. Cytokine assay

The quantities of interleukin-12 p40 and interleukin-12 p70 in culture supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies specific for each cytokine, as previously described (Kang et al., 1999). The monoclonal antibody for coating the plates and the biotinylated second monoclonal antibody were as follows: C17.8 and C15.6 for interleukin-12 p40; Red-T/G297-289 and C17.8 for interleukin-12 p70. Standard curves were generated using recombinant cytokine. The lower limits of detection were 30 pg/ml for interleukin-12 p40 and 50 pg/ml for interleukin-12 p70, respectively.

2.4. Preparation of cell lysates

Cells stimulated with α_2-adrenoceptor agonists or medium alone were washed twice with ice-cold PBS 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 phenylmethylsulfonyl fluoride) by incubation on ice for 30 min. Lysates were then centrifuged for 10 min at

4 °C (13,000 × *g*), and the supernatants were transferred to fresh tubes and stored at − 70 °C until required. Protein concentrations of the lysates were determined using the Coomassie protein assay reagent (Bio-Rad, Richmond, CA).

2.5. Immunoprecipitation and Western blot analysis

The cell lysates were incubated overnight with protein A-Sepharose beads (Pharmacia, Piscataway, NJ) coupled with anti-phosphotyrosine monoclonal antibody and the beads were washed and samples were eluted under nonreducing conditions as previously described (Smith et al., 1993). Immunoprecipitates were analyzed by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gels before transfer to poly(vinylidene fluoride) membranes using a Semi-Phor (Hoefer Scientific Instrument, San Francisco, CA). The membranes were then incubated with washing buffer (PBS containing 0.1% Tween 20) containing 2% bovine serum albumin for at least 1 h to block nonspecific protein binding. Primary monoclonal antibody was diluted up to 1:1000 in washing buffer and applied to the membrane for 1 h at room temperature. Following washing, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary monoclonal antibody (diluted up to 1:3000 in washing buffer) for 1 h at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

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 performed. The sequences of PCR primers used in the experiments are as follows: mouse interleukin-12 p40 (sense, 5′CAG AAG CTA ACC ATC TCC TGG TTT G3′; antisense, 5′TCC GGA GTA ATT TGG TGC TTC ACA C3′), interleukin-12 p35 (sense, 5′TCA GCG TTC CAA CAG CCT C3′; antisense, 5′CGC AGA GTC TCG CCA TTA TG3′), TNF-α (sense, 5′GGC AGG TCT ACT TTG GAG TCA TTG C3′; antisense, 5′ACA TTC GAG GCT CCA GTG AAT TCG G3′), interleukin-18 (sense, 5′ACT GTA CAA CCG GAG TAA TAC GG3′; antisense, 5′AGT GAA CAT TAC AGA TTT ATC CC3′), and β-actin (sense, 5′TGG AAT CCT GTG GCA TCC ATG AAA C3′; antisense, 5′TAA AAC GCA GCT CAG TAA CAG TCC G3′). The PCR reactions were run for 35 cycles for 94 °C (30 s), 58 °C (45 s), 72 °C (30 s) using a MJ Thermal Cycler (Watertown, MA). After the amplification, the RT-PCR products were separated in 1.5% (w/v) agarose gels and stained with ethidium bromide.

2.7. Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical significance of differences between values for various experimental and control groups. A P value of <0.05 was considered as significant.

3. Results

3.1. α_2-Adrenoceptor agonists induce interleukin-12 production in mouse macrophages

We examined the effect of various α_2-adrenoceptor agonists on the production of interleukin-12 by primary mouse macrophages. Splenic macrophages were treated with clonidine, guanfacine, and oxymetazoline for 48 h and the levels of interleukin-12 p40 and interleukin-12 p70 in the culture supernatants were determined by sandwich ELISAs. As shown in Fig. 1, all three α_2-adrenoceptor agonists significantly stimulated production of interleukin-12 heterodimer as well as the p40 subunit in a concentration-dependent manner. Clonidine and guanfacine were more effective than oxymetazoline in stimulating interleukin-12 production at the protein level ($P<0.01$ at >20 μM).

To determine whether the induction of interleukin-12 by α_2-adrenoceptor agonists was the result of increased mRNA production, we analyzed the effect of α_2-adrenoceptor agonists on the expression of interleukin-12 p40 and interleukin-12 p35 mRNA. Total RNA was isolated from splenic macrophages treated with α_2-adrenoceptor agonists for 24 h, and the expression of interleukin-12 was evaluated using primer-specific RT-PCR. The expression of interleukin-12 p40 was increased in a concentration-dependent manner, consistent with the results observed from ELISA analysis of protein levels (Fig. 2A). In addition, the expression of interleukin-12 p35 was also upregulated in the presence of α_2-adrenoceptor agonists (Fig. 2B). In agreement with published data, splenic macrophages expressed large

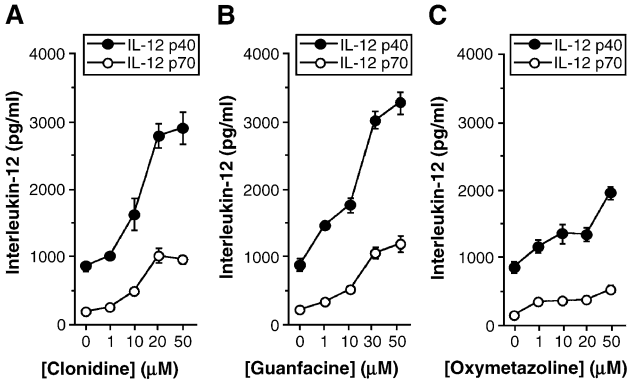

Fig. 1. Induction of interleukin-12 production in primary mouse macrophages by α_2-adrenoceptor agonists. Splenic macrophages were incubated in the presence or absence or of varying concentrations of clonidine (A), guanfacine (B), and oxymetazoline (C) for 48 h. The levels of interleukin-12 p40 and interleukin-12 p70 proteins in the culture supernatants were determined by sandwich ELISAs. The results are presented as the mean ± S.E.M. ($n=4$).

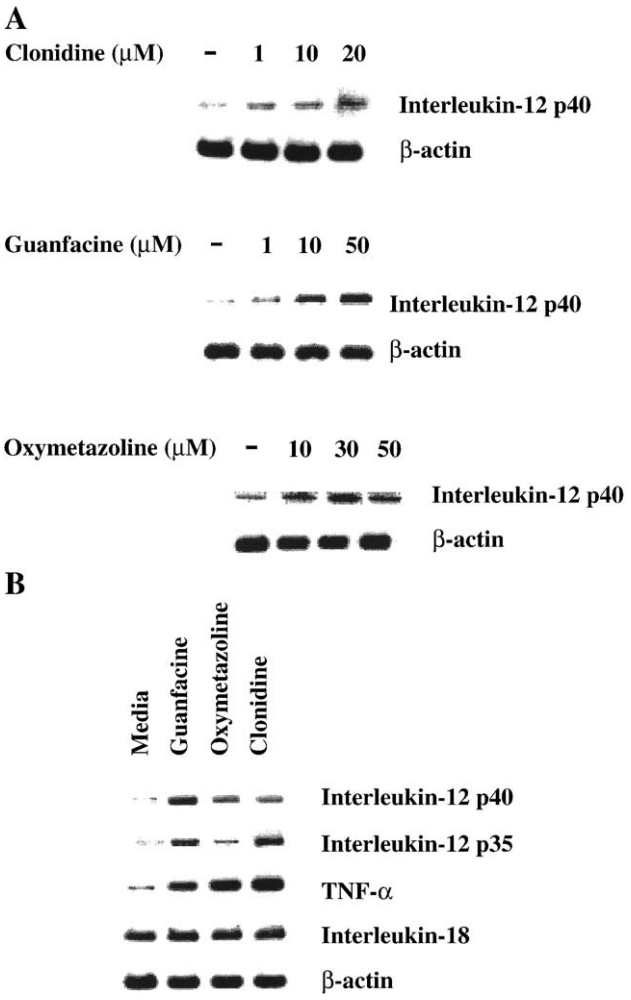

Fig. 2. Increased interleukin-12 mRNA expression by α_2-adrenoceptor agonists. (A) Splenic macrophages were incubated for 24 h in the presence or absence of varying concentrations of clonidine, guanfacine, and oxymetazoline, after which total RNA was prepared from the cells. (B) Splenic macrophages were incubated with media, clonidine (20 μM), guanfacine (50 μM), and oxymetazoline (50 μM) for 24 h and total RNA was prepared from the cells. RT-PCR was conducted with the respective primer pairs and the products were analyzed in 1.5% agarose gels.

amounts of TNF-α in the presence of α_2-adrenoceptor agonists (Fig. 2B). In contrast, treatment with α_2-adrenoceptor agonists did not increase the expression of interleukin-18 and β-actin mRNA, suggesting that the stimulatory effects of α_2-adrenoceptor agonists on interleukin-12 induction were not the result of a general activation of the cells.

3.2. Induced interleukin-12 p40 production by α_2-adrenoceptor agonists is mediated via their receptor

The α_2-adrenoceptor antagonists yohimbine and RX821002 were used to determine if the increases in interleukin-12 p40 production were mediated via the α_2-adrenoceptor. Macrophages were cultured with yohimbine or RX821002 before treatment of α_2-adrenoceptor agonists (20 μM clonidine, 50 μM guanficine, and 50 μM oxy-

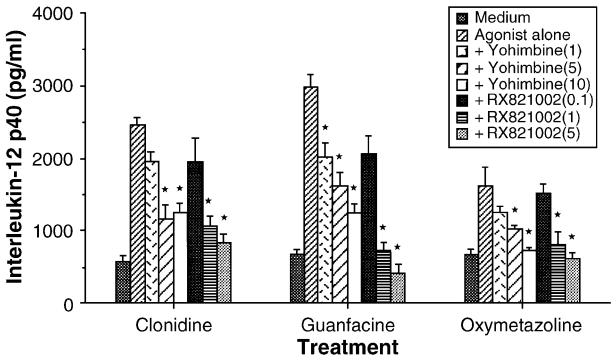

Fig. 3. Effect of yohimbine and RX 821002 on α_2-adrenoceptor agonist-induced interleukin-12-production by mouse macrophages. Splenic macrophages were preincubated with media or varying concentrations of yohimbine (1–10 μM) or RX821002 (0.5–5 μM) for 2 h before the addition of the α_2-adrenoceptor agonists. After 48-h incubation, the culture supernatants were harvested and the levels of interleukin-12 protein were determined by a sandwich ELISA. The results are presented as the mean ± S.E.M. ($n=3$). *$P<0.05$, relative to an α_2-adrenoceptor agonists-treated group in the absence of yohimbine.

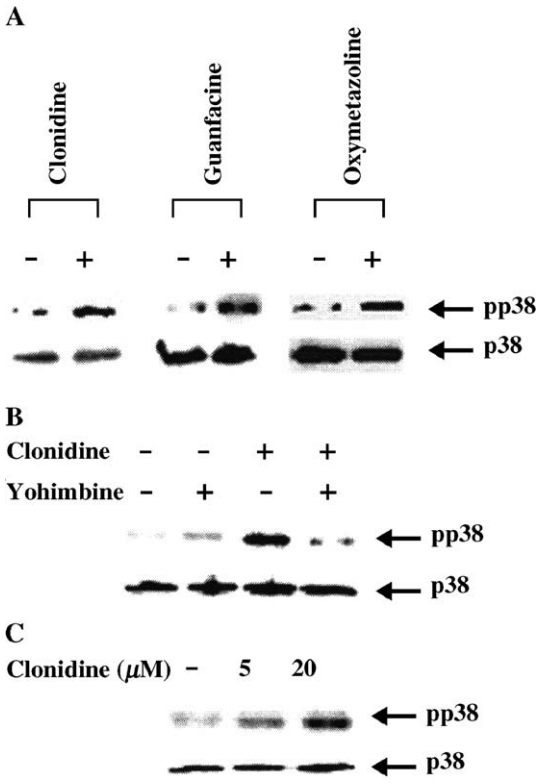

Fig. 4. α_2-Adrenoceptor agonist-mediated phosphorylation of p38 MAPK in mouse macrophages. (A) Splenic macrophages were cultured for 45 min in media containing clonidine (20 μM), guanfacine (50 μM), or oxymetazoline (50 μM). (B) Splenic macrophages were preincubated for 2 h with media or yohimbine (5 μM) before clonidine (20 μM) was added. After 45 min, macrophages were harvested. (C) Splenic macrophages were cultured in the absence or presence of clonidine (5 and 20 μM) for 45 min. The cell lysates were immunoprecipitated using anti-phosphorylated tyrosine monoclonal antibody and blotted with anti-p38 MAPK monoclonal antibody (pp38), or the cell lysates were directly probed with anti-p38 MAPK monoclonal antibody (p38).

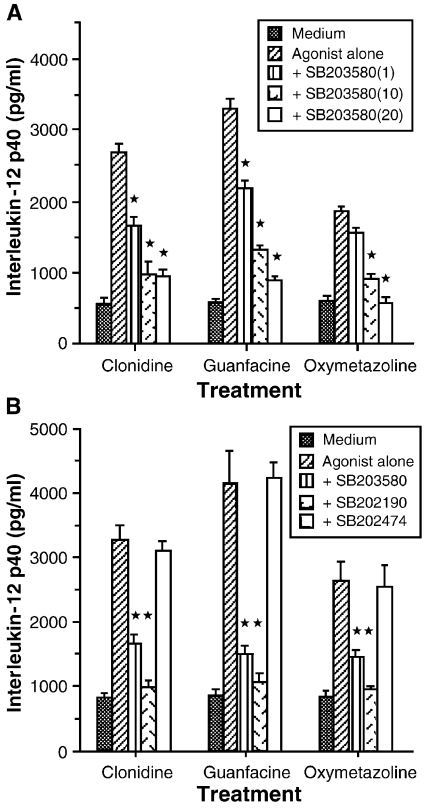

Fig. 5. Suppression of α_2-adrenoceptor agonist-induced interleukin-12-production by p38 MAPK inhibitors. (A) Splenic macrophages were cultured in the absence or presence of varying concentrations of SB203580 (1–20 μM) for 2 h, followed by treatment with the α_2-agonists. *$P<0.01$, relative to an α_2-adrenoceptor agonist-treated group in the absence of SB203580. (B) Splenic macrophages were cultured in the absence or presence of SB203580, SB202190, or SB202474 (10 μM each) for 2 h, followed by treatment with α_2-adrenoceptor agonists. After 48-h culture, the levels of interleukin-12 protein in the cell supernatants were evaluated by a sandwich ELISA. The results are presented as the mean ± S.E.M. ($n=3$). *$P<0.01$, relative to an α_2-adrenoceptor agonist-treated group in the presence of SB202474.

metazoline) for 48 h. As shown in Fig. 3, treatment with yohimbine or RX821002 significantly suppressed the α_2-adrenoceptor agonists-induced interleukin-12 p40 production. These results suggested that the stimulation of interleukin-12 p40 production by α_2-adrenoceptor agonists was mediated via their receptor.

3.3. α_2-Agonists activate p38 MAPK in mouse macrophages

We next examined the molecular requirements for the induction of interleukin-12 production in the macrophages by α_2-adrenoceptor agonists. To assess the involvement of p38 MAPK in the induction of interleukin-12 p40 production in mouse macrophages exposed to α_2-adrenergic agonists, we examined the activation of p38 MAPK. Splenic macrophages were incubated for 45 min with α_2-adrenoceptor agonists and the activation of p38 MAPK was determined by measuring the phosphorylated form of p38 MAPK. We found that the phosphorylation of p38 MAPK was increased by all the α_2-agonists tested (Fig. 4A). To determine if the increase in p38 MAPK phosphorylation was mediated via the α_2-adrenoceptor, splenic macrophages were cultured in medium containing yohimbine before exposure to clonidine. As shown in Fig. 4B, the presence of yohimbine significantly blocked the phosphorylation of p38 MAPK by clonidine. Clonidine alone (5 and 20 μM) increased the phosphorylation of p38 MAPK in a concentration-dependent manner (Fig. 4C).

3.4. Selective p38 MAPK inhibitors suppress the α_2-adrenoceptor agonist-induced production of interleukin-12 p40

To further determine whether p38 MAPK activation was required for the induction of interleukin-12 p40 by α_2-adrenoceptor agonists, we investigated the effect of p38 MAPK inhibitors on α_2-adrenoceptor agonist-induced interleukin-12 p40 production in mouse macrophages. SB203580, a selective inhibitor of p38 MAPK, inhibited the α_2-agonist-induced interleukin-12 p40 production in a concentration-dependent manner (Fig. 5A). Moreover, the α_2-adrenoceptor agonist-induced interleukin-12 production was also inhibited by SB202190, a selective inhibitor of p38 MAPK, but not by SB202474, a chemical used as a negative control (Fig. 5B). SB202474 is structurally related to two well-known pyridinyl inhibitors of p38 MAPK inhibitors SB203580 and BS202190 but does not inhibit p38 MAPK (Lahti et al., 2002). These

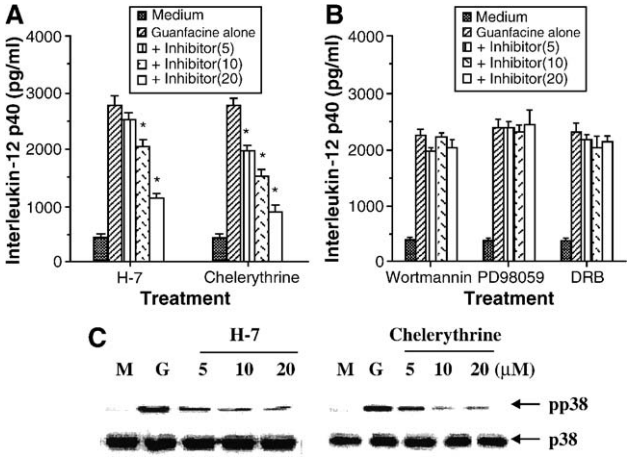

Fig. 6. Effects of inhibitors for PKC, PI3K, MEK, and CK II on interleukin-12 production and p38 phosphorylation stimulated with guanfacine. Splenic macrophages were cultured in the absence or presence of PKC inhibitors (A) or inhibitors for PI3K, or MEK or CK II (B) (5, 10, 20 μM) for 2 h, followed by treatment with 50 μM guanfacine. After 48-h culture, the levels of interleukin-12 protein in the cell supernatants were evaluated by a sandwich ELISA. The results are presented as the mean ± S.E.M. ($n=3$). *$P<0.01$, relative to a group treated with guanfacine alone. The levels of p38 MAPK were determined by Western blot analysis at 45 min after treatment with guanfacine (C). The cell lysates were immunoprecipitated using anti-phophorylated tyrosine monoclonal antibody and blotted with anti-p38 MAPK monoclonal antibody (pp38), or the cell lysates were directly probed with anti-p38 MAPK monoclonal antibody (p38). Lane M, medium alone; lane G, guanfacine alone.

results (Figs. 4 and 5) demonstrated that interleukin-12 p40 induction by α_2-adrenoceptor agonists was mediated through the p38 MAPK signaling pathway.

3.5. Involvement of protein kinase C (PKC) and adenylate cyclase in the α_2-adrenoceptor agonist-induced production of interleukin-12 p40

To further characterize the involvement of other signaling components in the induction of interleukin-12 production by the α_2-adrenoceptor agonists, macrophages were first treated with selective inhibitors of various signaling components such as PKC, PI3-K, and MEK, followed by incubation in medium containing guanfacine. Afterward, the levels of interleukin-12 in the cell culture supernatants were determined. As shown in Fig. 6, both PKC inhibitors, H-7 and chelerythrine, significantly inhibited guanfacine-induced interleukin-12 production in a concentration-dependent manner. In contrast, there was little effect on guanfacine-induced interleukin-12 production by the PI3-K inhibitor (wortmannin), a casein kinase inhibitor (DRB) or a MEK inhibitor (PD98059). Furthermore, PKC inhibitors inhibited guanfacine-induced activation of p38 MAPK in a concentration-dependent manner. The involvement of adenylate cyclase was also studied. As shown in Fig. 7, an activator of adenylate cyclase (foskolin) inhibited interleukin-12 production and p38 MAPK activation stimulated by guanfacine, indicating that adenylate cyclase might be involved in the induction of interleukin-12 production by α_2-adrenoceptor agonists.

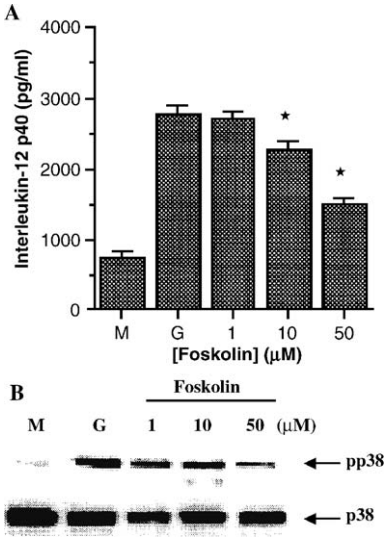

Fig. 7. Effect of foskolin on interleukin-12 production and p38 phosphorylation stimulated with guanfacine. Splenic macrophages were cultured in the absence or presence of foskolin for 2 h, followed by treatment with 50 μM guanfacine. After 48-h culture, the levels of interleukin-12 protein in the cell supernatants were evaluated by a sandwich ELISA (A). The results are presented as the mean ± S.E.M. ($n = 3$). *$P < 0.05$, relative to a group treated with guanfacine alone. The levels of p38 MAPK were determined by Western blot analysis at 45 min after treatment with guanfacine (B). Lane M, medium alone; lane G, guanfacine alone.

4. Discussion

In this report, we have demonstrated that α_2-adrenoceptor agonists such as clonidine, guanfacine, and oxymetazoline, significantly enhanced interleukin-12 production by mouse macrophages. Furthermore, we found that the enhancement of interleukin-12 production by α_2-adrenoceptor agonists was mediated by a PKC/p38 MAPK signaling pathway. Interleukin-12 plays a central role in both innate and adaptive immunity through its ability to induce interferon-γ secretion directly from both T and natural killer cells. It is critically important in host defense, predominantly against intracellular pathogens. Recombinant interleukin-12 has striking therapeutic effects in mouse models of tumor immunotherapy, prevention of infectious disease, and inflammation of airways. Clinical trials designed to determine the potential therapeutic effects of interleukin-12 have been initiated in human cancer patients, HIV-infected patients, and patients with chronic viral hepatitis (Gollob et al., 2001). Direct administration of interleukin-12 and modification of cells to form interleukin-12 by gene therapy have yielded promising results in some cancers, allergic asthmas, and mycobacterial infections (Bryan et al., 2000; Gajewski et al., 2001; Holland, 2001). These findings have generated great interest in identifying enhancers of interleukin-12 production, especially in the treatment of diseases such as allergic disorders and asthma associated with pathologic Th2 responses. Recently, paclitaxel (Taxol), an anti-cancer agent, was reported to enhance interleukin-12 production by macrophages in tumor-bearing hosts through the production of nitric oxide (Mullins et al., 1999). Substance P was reported to stimulate interleukin-12 production through the natural killer-1 receptor (Kincy-Cain and Bost, 1997). Chitin particle initiated interleukin-12 production through mannose receptor-mediated phagocytosis (Shibata et al., 1997). The induction of interleukin-12 synthesis by hyaluronan through its interaction with CD44 has also been reported (Hodge-Dufour et al., 1997).

The results of the study reported here indicate that α_2-adrenoceptor agonists such as clonidine, guanfacine, and oxymetazoline significantly augment interleukin-12 production by mouse macrophages in a concentration-dependent manner via the receptor. The adrenergic receptors are part of the superfamily of G-protein-coupled receptors that bind the endogenous agonists norepinephrine and epinephrine. These seven transmembrane spanning receptors coupled to multiple effectors via Gi/Go proteins, decrease adenyl cyclase activity (Simonds et al., 1989), activate receptor-mediated K^+ channels (Bunemann et al., 2001), and inhibit voltage-gated Ca^{2+} channels (Tateyama et al., 2001), among other effects. The endogenous agonists, norepinephrine and epinephrine, were reported to stimulate resident peritoneal

macrophages from BALB/c mice to suppress the growth of *Mycobacterium avium*, an effect mediated by α_2-adrenoceptors (Miles et al., 1996). This was in accord with the study of Spengler et al. (1990), who found an α_2-adrenoceptor-mediated stimulatory effect of norephinephrine on the production of TNF-α by mouse peritoneal macrophages. Furthermore, experimental hemorrhage in mice, a condition associated with elevations of systemic catecholamine concentration, increased the expression of TNF-α and interleukin-1 by lung mononuclear cells through stimulation of the α_2-adrenoceptor (Le Tulzo et al., 1997).

Ligands of α_2-adrenoceptors are therapeutically administrated in the management of severe sepsis (Pastores et al., 1996). They maintain perfusion pressure for cardiovascular support. However, this study shows that these agents can affect the production of inflammatory mediators, which are responsible for the deleterious effects of shock-inducing responses. In this respect, our findings may provide a rationale for the observations that treatment with α_2-adrenergic antagonists has a beneficial effect. They shift the inflammatory process in an anti-inflammatory direction by inhibition of the release of pro-inflammatory mediators in septic shock. Administration of α_2-adreneric antagonists has been reported to exert protective effects in animal models of endotoxic shock (Maitra and Sayeed, 1992).

Little is known about the coupling of α_2-adrenoceptors to MAPK signaling pathways. The present study shows the possibility that stimulation of the α_2-adrenoceptor expressed by splenic macrophages activated p38 MAPK and mediated the induction of interleukin-12. The p38 MAPK pathway is activated by environmental perturbation (e.g., osmotic changes, heat shock) and by inflammatory cytokines including TNF-α and interleukin-1. This pathway has been proposed to function in the regulation of cytokine production, B cell and T cell proliferation and differentiation, the innate immune response, cell cycle control, and apoptosis (Amrani et al., 2001; Rincon et al., 1998; Han et al., 1998). Recent studies have shown that the p38 MAPK, activated through MAPK kinase 3 (MKK3), might be involved in the production of proinflammatory cytokines by both antigen-presenting cells and $CD4^+$ T cells (Feng et al., 1999). It has been shown that SB203580, an inhibitor of p38 MAPK, inhibited lipopolysaccharide-induced interleukin-12 production in macrophages/monocytes and reduced CD40-induced interleukin-12 p40 production in dendritic cells (Aicher et al., 1999; Zhang and Kaplan, 2000), suggesting that the p38 MAPK might be a factor in regulating the production of interleukin-12. As yet, the target of p38 MAPK that controls interleukin-12 p40 expression has not been identified. The transcription factors nuclear factor-κB (NF-κB) and Ets are proposed to play a key role in the regulation of interleukin-12 gene (Gri et al., 1998). However, α_2-adrenoceptor agonists did not activate the interleukin-12 p40 promoter containing NF-κB sites and also did not increase NF-κB-DNA binding activity (unpublished observations), suggesting that the induced interleukin-12 p40 production by α_2-adrenoceptor agonists is not mediated through NF-κB. Recently, a novel c-Rel (a member of the NF-κB family)-independent pathway was reported to be involved in interleukin-12 p40 production during toxoplasmosis (Mason et al., 2002).

Inhibitor studies on several components of signal transduction pathways showed that PKC was involved in the induction of interleukin-12 production by α_2-adrenoceptor agonists (Fig. 6A). Furthermore, PKC inhibitors suppressed p38 MAPK activation (Fig. 6C), suggesting the involvement of PKC as an upstream component of p38 MAPK activation. In addition, adenylate cyclase and cAMP may also be involved in the induction of interleukin-12 production by α_2-adrenoceptor agonists, as demonstrated by inhibitory effects of foskolin (an activator of adenylate cyclase) (Fig. 7). Previous reports showed that cyclic AMP-elevating molecules such as prostaglandin E_2, pentoxifylline, and isoproterenol could suppress interleukin-12 production, leading to possible therapeutic effects on Th1-dominated diseases (van der Pouw Kraan et al., 1995; Elenkov et al., 1996). The administration of pentoxifylline inhibited interleukin-12 production in peripheral blood mononuclear cells of multiple sclerosis patients (Rieckmann et al., 1996). Treatment of endotoxemic mice with isoproterenol or rolipram suppressed interleukin-12 production and consequently decreased mortality (Hasko et al., 1998). The α_2-adrenoceptor couples to inhibit adenylate cyclase and, subsequently, the formation of cAMP (Keularts et al., 2000).

In summary, we have shown that α_2-adrenoceptor agonists induced the production of interleukin-12 in splenic macrophages by stimulation of α_2-adrenoceptors and subsequent activation of p38 MAPK. Because of interleukin-12's pivotal role in directing immunity toward cell-mediated or Th1-mediated responses, these results suggest that α_2-adrenoceptor-mediated interleukin-12 regulation could be a potent target of immunomodulation.

Acknowledgements

The authors thank Drs. S. Wolf, Y.K. Choe, and J. Cheong for their generous gift of valuable reagents, and Drs. E.P. Cohen, J.W. Lee, and Y.C. Lee for helpful discussions and critical reading. This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ1-PG3-21200-0021).

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