Manassantin B Isolated from Saururus chinensis Inhibits Cyclooxygenase-2-Dependent Prostaglandin D₂ Generation by Blocking Fyn-Mediated Nuclear Factor-kappaB and Mitogen Activated Protein Kinase Pathways in Bone Marrow Derived-Mast Cells

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The authors investigated the effect of manassantin B (Man B) isolated from Saururus chinensis (S. chinensis) on cyclooxygenase-2 (COX-2)-dependent prostaglandin D₂ (PGD₂) generation in mouse bone marrow derived-mast cells (BMMCs). Man B inhibited the generation of PGD₂ dose-dependently by inhibiting COX-2 expression in immunoglobulin E (IgE)/Ag-stimulated BMMCs. To elucidate the mechanism responsible for the inhibition of COX-2 expression by Man B, the effects of Man B on the activation of nuclear factor-kappaB (NF-κB), a transcription factor essential and mitogen-activated protein kinases (MAPKs) for COX-2 induction, were examined. Man B attenuated the nuclear translocation of NF-κB p65 and its DNA-binding activity by inhibiting inhibitors of kappa B (IκB) phosphorylation. In addition, Man B suppressed phosphorylation of MAPKs including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH₂-terminal kinase (JNK) and p38. It was also found that Man B suppressed Fyn kinase activation and consequent downstream signaling processes, including those involving Syk, Gab2, and Akt. Taken together, the present results suggest that Man B suppresses COX-2 dependent PGD₂ generation by primarily inhibiting Fyn kinase in FcεRI-mediated mast cells.

Key words Saururus chinensis; manassantin B; cyclooxygenase-2; nuclear factor-kappaB; Fyn kinase; bone marrow-derived mast cell

Mast cells are involved in various allergy and inflammatory responses due to the aggregation of high-affinity immunoglobulin E (IgE) receptor (FcεRI) on their surfaces. The aggregation of FcεRI results in the release of preformed mediators (e.g., histamine and proteases), newly synthesized lipid mediators (e.g., prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄)) and various cytokines. IgE/Ag stimulation of mast cells activates a variety of Src family kinases such as Lyn and Fyn. Subsequently, immunoreceptor tyrosine-based activation motifs (ITAM) of the β and γ subunits of FcεRI become phosphorylated by the Lyn and associated with tyrosine kinase Syk. Syk then phosphorylates linker for activation of T cells (LAT), phospholipase Cγ (PLCγ), and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76). These reactions then stimulate mitogen-activated protein kinases (MAPKs) pathways as well as transcription factors such as nuclear factor-kappaB (NF-κB) and AP-1 which mainly contribute to induce the pro-inflammatory genes including cyclooxygenase (COX)-2 and pro-inflammatory cytokines, for propagation of inflammation.

Manassantin B (Man B) is a neolignan isolated from the roots of Saururus chinensis (S. chinensis) and has been reported to exert various biological activities, including anti-inflammatory, anti-septic, anti-human immunodeficiency virus (HIV), and anti-tumor activities. In addition, Man B has been shown to inhibit hyperpigmentation and the expressions of adhesion molecules. However, the effects of Man B on PGD₂ generation in mast cells have not been previously reported. The purpose of this study was to investigate the effects of Man B on COX-2 dependent PGD₂ generation, which is known to be associated with the anti-inflammatory activity of S. chinensis in bone marrow-derived mast cells (BMMCs).

MATERIALS AND METHODS

Reagents The rabbit polyclonal antibodies specific for phospho-Akt, Gab2, inhibitors of kappa B (IκB) kinase (IκK) α/β, extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), p38 and IκBα as well as those against total forms of Akt, ERK, JNK, p38, Gab2, IκBα and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Antibody against COX-2, PGD₂ assay kit and NF-κB (p65) transcription factor assay kit were purchased from Cayman (Ann Arbor, MI, U.S.A.). Polyclonal antibodies against NF-κB p65, Syk Fyn, Gab2, IKKα/β and lamin B, rabbit anti-goat IgG-horse-radish peroxidase (HRP) and goat anti-rabbit IgG-HRP were obtained from Santa Cruz Biotechnological (Santa Cruz, CA, U.S.A.). Antibody for phosphotyrosine was from Millipore (Billericia, MA, U.S.A.). PP2, mouse anti-dinitrophenyl (DNP) IgE and DNP-human serum albumin (HSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

Plant Materials Manassantin B (Fig. 1A) was isolated from the methanol extract of the dried roots of S. chinensis and structurally identified as described previously. ManB was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was adjusted to 0.1% (v/v) in culture media. Control experiments indicated that DMSO at this concentration had no effect on PGD₂ generation.

Preparation and Activation of BMMCs Bone marrow cells from male Balb/cJ mice (purchased from Sam Taco, Seoul) were cultured for up to 10 weeks in 50% enriched

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medium (RPMI 1640 containing 2 mM l-glutamine, 0.1 mM nonessential amino acids, antibiotics and 10% fetal calf serum) and 20% pokeweed mitogen-stimulated spleen condition medium (PWM-SCM) as a source of interleukin-3 (IL-3). After 3 weeks, >98% of cells was found to be BMMCs using a previously described procedure.

Determination of PGD$_2$ BMMCs were sensitized with 500 ng/mL of DNP-specific IgE overnight. On next days, BMMCs were pretreated with aspirin (1 µg/mL) for 2 h, to inactivate preexisting COX-1 irreversibly. After washing, BMMCs were pretreated with Man B or PP2 for 1 h and then activated with 100 ng/mL of DNP-HSA for 7 h. All reactions were stopped by centrifugation at 3000 rpm at 4°C for 5 min. Concentrations of PGD$_2$ in the supernatants were measured using PGD$_2$ assay kits according to the manufacturer’s instruction and cells were used for immunoblotting.

Immunoprecipitation (IP) Cell lysates were obtained using modified lysis buffer (0.1% Nonidet P-40, 50 mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.0), 250 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol (DTT)). Protein in total cell lysate (1 mg) was incubated with anti-Fyn or Syk antibodies for 2 h at 4°C and immunocomplexes were precipitated with 20 µL of protein A-Sepharose. Precipitates were extensively washed 3 times with ice-cold lysis buffer, and precipitates or total cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with indicated antibodies.

Preparation of Nuclear and Cytosolic Extracts Cells were suspended in wash buffer containing 10 mM HEPES buffer (pH 8.0), 1.5 mM MgCl$_2$, 10 mM KCl, 1 mM PMSF and 1 mM protease inhibitor cocktail (Merck Biosciences, Darmstadt, Germany) and then lysed in wash buffer containing 0.1% (v/v) NP40 by incubating on ice for 10 min. After centrifugation at 10000×g for 4 min, supernatants were used as a cytosolic fraction. Nuclear pellets were washed and resuspended in a buffer containing 20 mM HEPES (pH 8.0), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, and protease inhibitor cocktail. This suspension was incubated for 30 min at 4°C followed by centrifugation at 10000×g, and resultant supernatants were used as nuclear fraction.

Reverse Transcriptase-Polymerase Chain Reaction Analysis (RT-PCR) BMMCs were sensitized with 500 ng/mL of DNP-specific IgE overnight. Aspirin (1 µg/mL) pretreated BMMCs were incubated with Man B or PP2 for 1 h and stimulated with 100 ng/mL of DNP-HSA for 4 h. Extraction of total RNA and RT-PCR was performed as described previously.

Western Blot Analysis IgE-sensitized mast cells were stimulated with Ag for the indicated times with or without Man B or other inhibitors. BMMCs (2×10$^6$) were then subjected to 8% (w/v) SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and immunoblotted. The membranes were then washed once with 10 mM Tris-buffered saline (TBS, pH 7.2) containing 0.1% Tween-20
(TBS-T) and then blocked for 1 h in TBS-T containing 3% skim milk. After washing the membranes with TBS-T, antibodies against phosphorylated forms of IKKα/β and IκBα those against total IKKα/β and IκBα, or those against NF-κB, β-actin and lamin B were added at a dilution of 1:1000–3000 in TBS-T. The immunoreactive proteins were incubated with the use of HRP-coupled secondary antibodies diluted 1:3000 for 1 h at room temperature. The antibody-reactive bands were visualized with an ECL system (Pierce Biotechnology, Rockford, IL, U.S.A.).

NF-κB Activation Assay Nuclear fractions were prepared as described previously.17) NF-κB transactivation capacity was measured by using a NF-κB (p65) transcription factor assay kit (Cayman Chemicals, Ann Arbor, MI, U.S.A.) according to the manufacturer’s instructions. The results from the NF-κB (p65) transcription factor assay kit are presented as percent activation with the stimulated control set to 100%.

Statistical Analysis All values are presented as the arithmetic means±S.D. from three separate experiments. One-way ANOVA was utilized to determine the statistical significance.

RESULTS AND DISCUSSION

Initially, we examined the cytotoxicity of Man B (Fig. 1A) on BMMCs using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with different doses (1 to 20 µM) of Man B for 8h. Man B did not affect cell viability at concentrations up to 20 µM (data not shown). Therefore, we decide to use Man B at a concentration of less than 0.5 µM in subsequent experiments. It was found that Man B inhibited PGD2 generation and COX-2 expression in IgE/Ag-induced BMMCs. To understand how Man B inhibited COX-2 expression, we first investigated the AKT-mediated IKK/IκBα/NF-κB and upstream Src kinase pathways.

Several lines of evidence show that PGD2 is the major prostanooid produced by activated mast cells. Furthermore, PGD2 has long been implicated in the manifestations of various inflammatory diseases.18) Therefore, we investigated the
effects of Man B on the generation of COX-2 dependent PGD2 in BMMCs. Cells showed biphasic PGD2 generation over time, in addition to COX-1-dependent immediate and COX-2-dependent delayed responses. Immediate PGD2 generation occurs within 2h due to preexisting COX-1 activation, whereas delayed PGD2 generation, which occurs after several hours of culture (2–10h), is associated with the induction of COX-2.\textsuperscript{19,20} To assess COX-2-mediated PGD2 generation, IgE-sensitized BMMCs were pre-treated with aspirin to abolish preexisting COX-1 activity, then briefly washed and stimulated with Ag for 7h with or without Man B. We found that Man B inhibited PGD2 generation and concomitant reduced COX-2 protein levels (Figs. 1B and C). Next, we examined whether Man B inhibits COX-2 mRNA expression in IgE/Ag-induced BMMCs. Pretreatment of BMMCs with Man B inhibited expression of COX-2 mRNAs in a dose dependent manner (Fig. 1C). Furthermore, PP2 (a general Src-family kinase inhibitor) almost completely inhibited induction of both COX-2 protein and mRNA, suggesting that Man B might be affected phosphorylation of Src family kinase(s).

Since it is well known that NF-κB is a major transcription factor that regulates the expressions of COX-2 and proinflammatory cytokines,\textsuperscript{20} we investigated whether Man B inhibits the NF-κB pathway. NF-κB (a heterodimer) is located in the cytoplasm as an inactive complex associated with IκBα, which is phosphorylated and subsequently degraded by IKK complex. After IgE/Ag activation, phosphorylations of IKK complex (p-IKKα/β) and IκBα (p-IκBα) were clearly increased, total IκBα protein was concomitantly decreased and the nuclear translocation of NF-κB (N-NF-κB) were increased. As shown in Fig. 2A, Man B suppressed increases in p-IKKα/β and p-IκBα, IκBα reduction, and the appearance of N-NF-κB. Additional experiments were carried out to determine whether Man B affected the nuclear binding activity of NF-κB in response to IgE/Ag stimulation using an NF-κB activation assay kit. Nuclear extracts were obtained from BMMCs that had been pretreated with Man B or PP2 for 1h and then stimulated with DNP-HSA for 30min. As shown by Fig. 2B, both Man B and PP2 markedly reduced IgE/Ag-induced NF-κB-DNA binding activity, indicating that Man B inhibits the NF-κB pathway by inhibiting the IKK-IκBα-NF-κB signaling pathway. PP2 also inhibited these responses, suggesting that the IKK-IκBα-NF-κB pathway was under the control of certain Src-family kinase(s).

Our previous study showed that MAP kinases (MAPKs) were phosphorylated within 10–15min and transcriptional induction of COX-2 in IgE/Ag-stimulated BMMCs.\textsuperscript{22} Therefore, we investigate the effect of Man B or PP2 on MAPKs phosphorylation, which lie downstream of Syk. As shown in Fig. 2C, when BMMCs were pretreated with Man B or PP2, both compounds inhibited IgE/Ag-induced phosphorylation of three MAPKs including ERK1/2, JNK and p38. These results suggest that the inhibition of COX-2 gene expression and PGD2 generation by Man B may be mediated through the inhibition of phosphorylation of MAPKs.

Fyn is one of the Src family tyrosine kinases, which required for critical downstream signaling pathways in mast cell. Fyn is required for the activation of Syk, which also plays an essential role in the IgE-dependent activation of mast cells. Activation of MAPKs downstream of Syk is believed to play an important role in COX-2 expression. Another critical downstream signaling pathway which mediated by Fyn is Gab2/phosphatidylinositol-3-kinase (PI3K)/IKK pathway. Studies using Fyn-deficient BMMCs have suggested that decreased PI3K activation, as reflected by AKT phosphorylation, is accompanied by reductions in the activations of the IKK/IκBα/NF-κB and MAPK/AP-1 pathways.\textsuperscript{23} In this study, both Man B and PP2 inhibited Fyn phosphorylation, therefore suppressed other downstream signal events including IKK-IκBα-NF-κB and MAPKs pathways.

To understand how Man B suppressed COX-2 dependent PGD2 generation, we examined effects of Man B on the FcεRI-mediated activation of Fyn and its downstream signaling events, including those involving Syk, Gab2, and Akt. As shown in Fig. 3, the phosphorylations of Fyn, Syk, Gab2, and Akt were also suppressed by Man B or PP2, which suggests that Man B inhibits the IgE/Ag-induced phosphorylation of Fyn, and that this in turn regulates the activations of transcription factors leading to COX-2 expression.

In conclusion, the present study shows that Man B isolated from \textit{S. chinensis} strongly inhibits the expression of COX-2 in IgE/Ag induced mast cells. Furthermore, this inhibitory activity of Man B was closely associated with NF-κB inactivation via both blocking of IKK dependent IκBα phosphorylation, degradation and MAPKs phosphorylation. Furthermore, Man B suppressed Fyn kinase activation and downstream signal molecules in FcεRI-mediated mast cells. Since NF-κB is an essential transcription factor that regulates the transcription of COX-2, inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines, we believe the inhibition of these transcription factors by Man B provides a possible therapeutic approach to the treatment of inflammatory disorders.

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