Suppressive effect of tamarixetin, isolated from *Inula japonica*, on degranulation and eicosanoid production in bone marrow-derived mast cells

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**Abstract**

**Background:** The aim of this study was to evaluate the inhibitory effect of tamarixetin on the production of inflammatory mediators in IgE/antigen-induced mouse bone marrow-derived mast cells (BMMCs).

**Materials and methods:** The effects of tamarixetin on mast cell activation were investigated with regard to degranulation, eicosanoid generation, Ca²⁺ influx, and immunoblotting of various signaling molecules.

**Results:** Tamarixetin effectively decreased degranulation and the eicosanoid generation such as leukotriene C₄ and prostaglandin D₂ in BMMCs. To elucidate the mechanism involved, we investigated the effect of tamarixetin on the phosphorylation of signal molecules. Tamarixetin inhibited the phosphorylation of Akt and its downstream signal molecules including IKK and nuclear factor κB. In addition, tamarixetin downregulated the phosphorylation of cytosolic phospholipase A₂ (cPLA₂) and p38 mitogen-activated protein kinase.

**Conclusions:** Taken together, this study suggests that tamarixetin inhibits degranulation and eicosanoid generation through the PLCγ as well as Akt pathways in BMMCs, which would be potential for the prevention of allergic inflammatory diseases.

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**KEYWORDS**

degranulation; leukotriene C₄ (LTC₄); prostaglandin D₂ (PGD₂); mast cells

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Suppressive effect of tamarixetin on degranulation and eicosanoid production in BMMCs

Introduction

Host response against inflammation is a conflict to infection resulting in the release of various inflammatory mediators produced by innate immune cells. However, if left uncontrolled, the inflammatory mediators become involved in the pathogenesis of many inflammatory disorders. Among immune cells, mast cells play a vital role in allergic diseases. Binding of antigen (Ag) to the high-affinity receptor for IgE (FcεRI) followed by crosslinking of IgE releases preformed inflammatory mediators (histamine, serotonin, and serine proteases) from their granules, generates eicosanoids [prostaglandin D\(_2\) (PGD\(_2\)) and leukotriene C\(_4\) (LTC\(_4\))] from arachidonic acid (AA), and synthesizes chemokines as well as cytokines.\(^5\)

Activated mast cells initiate the activation of Syk tyrosine kinase followed by phosphorylation of phospholipase C\(_{1}\gamma\) (PLC\(_{1}\gamma\)), which triggers calcium (Ca\(^{2+}\)) release from internal stores.\(^4\) FC\(_{ε}\)RI signaling also triggers the activation of three families of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB signaling pathways, which induce the expression of cyclooxygenase (COX)-2 and proinflammatory cytokines. Activated MAPKs also contribute to the phosphorylation of cytosolic phospholipase A\(_2\), and thus eventually lead to the AA release, a common precursor of eicosanoids.\(^3\)

Previously, we have shown anti-inflammatory and anti-allergic responses of britanin and tomentson.\(^6\) As a continuing study, tamarixetin was also isolated from Inulae flos. It is a natural flavonoid derivative of quercetin and has anti-inflammatory and anti-allergic responses of britanin and tomentson.\(^6\) The plant material

Tamarixetin (Figure 1) was isolated from the ethyl acetate extracts of the flowers of *Inula japonica* as previously described.\(^7\) Tamarixetin dissolved in dimethyl sulphoxide (DMSO) was 0.1% and DMSO was alone run in all cases.

Reagents

RPMI-1640, fetal bovine serum (FBS), and penicillin/streptomycin were acquired from Hyclone (Logan, UT, USA). Pokeweed mitogen, mouse anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), and specific inhibitors for MAPKs (SB203580, PD98059, and SP600125) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The enzyme immunoassay kits (EIA, LTC\(_4\) and PGD\(_2\)) and COX-2 antibody were obtained from Cayman Chemicals (Ann Arbor, MI, USA). The primary rabbit polyclonal antibodies specific for phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-PLC\(_{1}\gamma\), phospho-Akt, Akt, phospho-IKK\(\alpha/\beta\), phospho-IkB\(\alpha\), IkB\(\alpha\), and β-actin were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibody against phospho-cPLA\(_2\), lamI B and Bay 61-3606 (a Syk inhibitor) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The goat anti-rabbit secondary antibody was purchased from Cell Signaling.

Cell culture and viability

Mouse bone marrow-derived mast cells (BMMCs) from Balb/c mice (Koatek, Seoul, Korea) were isolated and cultured for up to 10 weeks in RPMI 1640 containing 10% FBS, penicillin/streptomycin (100 U/mL/0.1%), 20% pokeweed mitogen-spleen cell-conditioned medium as a source of IL-3 as described previously.\(^13\) Mice care and experimental procedures were performed under the approval of the animal care committee of the National Research Institute of Korean Medicine. A colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA) was performed to assess the cytotoxicity of tamarixetin on BMMCs as described previously.\(^14\)

Quantification of β-hexosaminidase (hex) and eicosanoid generation

After pretreatment with or without tamarixetin for 1 h and stimulation with DNP-HSA (Ag, 100 ng/mL) for 15 min, the release of β-hex was quantified as described previously.\(^13\) To measure LTC\(_4\) generation, BMMCs (1 x 10\(^6\) cells/mL) sensitized with anti-DNP IgE (500 ng/mL) were pretreated with different concentrations of tamarixetin for 1 h and stimulated with DNP-HSA for 15 min followed by centrifugation at 120 g for 5 min at 4°C. The culture supernatants were collected and the LTC\(_4\) concentration was determined using an EIA kit (Cayman Chemical) in accordance with the manufacturer’s protocols. To quantify COX-2-dependent PGD\(_2\) production, BMMCs (1 x 10\(^6\) cells/mL) were preincubated with aspirin (10 µg/mL) for 2 h to inactivate preexisting COX-1 and washed. BMMCs were pretreated with tamarixetin for 1 h, incubated with Ag for 7 h at 37°C, and then the supernatants were quantified using PGD\(_2\) EIA kit (Cayman Chemical).
Measurement of intracellular Ca^{2+} level

BMMCs sensitized with anti-DNP IgE were preincubated with FluoForte™ dye-loading solution for 1 h at room temperature. After washing the dye with HBSS, the cells (5 × 10^4 cells) were seeded and pretreated with tamarixetin for 1 h before stimulation with DNP-HSA for 5 min. The supernatant was subjected to Calcium assay according to the manufacturer’s instructions (FluoForte Calcium Assay Kit, Enzo Life Sciences, Ann Arbor, MI, USA).

Western blot analysis

To obtain whole-cell protein lysates, cells were lysed using RIPA buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktail. The nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear Protein Extraction kit (Pierce) according to the manufacturer's instructions. Equal amounts of proteins (20-30 μg) were run on 10% SDS-PAGE and transferred to nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine (Schleicher and Schull, Dassel, Germany). The membranes were blocked via incubation in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% non-fat milk for 1 h and incubated with a variety of first antibodies overnight. After washing with TTBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase for 1 h and specific protein bands were visualized using an ECL system (Pierce). The densities of bands were measured with the ImageQuant LAS 4000 luminescent image analyzer and quantified using the ImageQuant software system (GE Healthcare, Little Chalfont, UK).

Statistical analysis

All data are presented as the mean ± S.E.M. One-way ANOVA by Duncan’s multiple range test was utilized to determine statistical differences and a p-value < 0.05 was considered statistically significant.

Results

Tamarixetin decreases degranulation and intracellular Ca^{2+} through inhibition of PLC\(\gamma_1\) phosphorylation

We performed an MTS assay to determine the cytotoxicity of tamarixetin in BMMCs and found that tamarixetin was not toxic at a concentration up to 6.3 μM (Figure 2A). Thus, concentrations of equal to or lower than 6.3 μM were used for subsequent experiments. To examine the inhibitory effect of tamarixetin on degranulation, IgE-sensitized BMMCs were pretreated with tamarixetin for 1 h and stimulated with DNP-HSA (Ag) for 15 min. The amount of released β-hex into the supernatant was quantified (B) and cell lysates were used to assess for PLC\(\gamma_1\) phosphorylation (D). IgE-sensitized BMMCs were preincubated with FluFore™ Dye-Loading for 1 h and washed with HBSS. BMMCs were pretreated with tamarixetin for 1 h before stimulation with DNP-HSA. Intracellular Ca^{2+} levels were measured with a multilabel plate reader at an excitation of 485 nm and emission of 535 nm (B). Data are expressed as the means ± S.E.M. * p < 0.05, ** p < 0.01, and *** p < 0.001 were compared to DNP-HSA stimulated-BMMCs.
BMMCs were pretreated with various concentrations of tamarixetin for 1 h followed by 15 min of Ag stimulation and the supernatants were quantified to determine β-hex release. The result showed that β-hex release was increased in Ag treatment on IgE-sensitized BMMCs, but pretreatment with tamarixetin suppressed β-hex release in a dose-dependent manner (Figure 2B).

Next, we determined the effect of tamarixetin on the intracellular Ca\(^{2+}\) levels. The activation of BMMCs induced an increase of intracellular Ca\(^{2+}\) levels and this increase was decreased by tamarixetin treatment (Figure 2C). We also examined the effect of tamarixetin on PLC\(\gamma\)1 phosphorylation. Figure 2D showed that the phosphorylation of PLC\(\gamma\)1 was increased by Ag treatment on IgE-sensitized BMMCs, but tamarixetin treatment dose-dependently inhibited PLC\(\gamma\)1 phosphorylation.

**Tamarixetin inhibits LTC\(_4\) generation by decreasing cPLA\(_2\) phosphorylation**

cPLA\(_2\) is a key enzyme for AA release and LT synthesis. To determine the effect of tamarixetin on the IgE-induced LTC\(_4\) generation, the amount of LTC\(_4\) was measured with or without tamarixetin pretreatment. The Ag stimulation significantly increased the LTC\(_4\) levels, but tamarixetin consistently reduced LTC\(_4\) generation in a dose-dependent manner (Figure 3A). Also, we examined the effect of tamarixetin on cPLA\(_2\) phosphorylation. The result showed that the phosphorylation of cPLA\(_2\) was increased after Ag stimulation, but suppressed by tamarixetin (Figure 3B), suggesting that tamarixetin decreased LTC\(_4\) generation by inhibiting cPLA\(_2\) phosphorylation.

To study the involvement of MAPKs in cPLA\(_2\) phosphorylation, we investigated the effect of tamarixetin on

![Figure 3](image-url)  
*Figure 3* Effect of tamarixetin on LTC\(_4\) and the phosphorylation of cPLA\(_2\) and MAPKs. IgE-sensitized BMMCs were pretreated with the tamarixetin for 1 h followed by stimulation with DNP-HSA for 15 min. LTC\(_4\) generation in the supernatant was determined using EIA kit (A). Cell lysates were used for Western blot analysis to evaluate the phosphorylation of cPLA\(_2\) (B). BMMCs were pretreated with tamarixetin for 1 h and stimulated with DNP-HSA for 30 min. Total lysates were subjected to Western blot analysis for phosphorylation of p38, JNK, and ERK MAPKs (C). Data are expressed as the means ± S.E.M. * p < 0.05, ** p < 0.01, and *** p < 0.001 were compared to DNP-HSA stimulated-BMMCs.
phosphorylation of MAPKs in IgE-sensitized BMMCs. Ag stimulation in IgE-sensitized BMMCs induced the phosphorylation of all three MAPKs without changing total protein and tamarixetin attenuated p38 phosphorylation (Figure 3C). However, it seems that tamarixetin did not affect the phosphorylation of JNK and ERK at all.

Tamarixetin suppresses COX-2-dependent PGD\(_2\) production by decreasing Akt phosphorylation or Akt-mediated signal pathway

PGD\(_2\) is a major prostanoid synthesized from AA via the catalytic activities of COX and PGD\(_2\) synthase in mast cells. To assess COX-2-dependent PGD\(_2\) generation, BMMCs were pretreated with aspirin to abolish preexisting COX-1 activity. Tamarixetin dose-dependently suppressed the PGD\(_2\) generation with a reduction of COX-2 expression in the concentration of tamarixetin on 6.3 \(\mu\text{M}\) (Figure 4A and B). Since NF-\(\kappa\)B, an essential transcription factor for COX-2 expression, lies downstream of the Akt pathway, we examined the effect of tamarixetin on the NF-\(\kappa\)B signal pathway in IgE/Ag-stimulated BMMCs. As shown in Figure 4C, tamarixetin inhibited the phosphorylation of Akt, IKK, and I\(\kappa\)B\(_{\alpha}\), the degradation of I\(\kappa\)B\(_{\alpha}\), and the nuclear translocation of NF-\(\kappa\)B, implying that the Akt-mediated signal pathway regulates the decreased generation of COX-2-dependent PGD\(_2\).

Discussion

Mast cells are effector cells of innate immunity and can serve to amplify adaptive immunity. Activated mast cells degranulate and release preformed mediators such as histamine and AA metabolites that are responsible for various inflammatory diseases.\(^1\) In this study, we demonstrated that tamarixetin decreased \(\beta\)-hex release dose-dependently in BMMCs (Figure 2B). Since PLC\(_\gamma\)-mediated Ca\(^{2+}\) mobilization is a prerequisite for mast cell degranulation in activated mast cells,\(^{15}\) we examined intracellular Ca\(^{2+}\) influx and PLC\(_\gamma\)1 phosphorylation by tamarixetin treatment. The results showed tamarixetin decreased intracellular Ca\(^{2+}\) levels and PLC\(_\gamma\)1 phosphorylation activation (Figure 2C and D), suggesting that the inhibitory effect of tamarixetin on mast cell degranulation resulted from the suppression of PLC\(_\gamma\)1 phosphorylation and intracellular Ca\(^{2+}\) levels.

cPLA\(_2\) is a key enzyme for AA release and LT synthesis. To determine the effect of tamarixetin on the IgE-induced LTC\(_4\) generation, the amount of LTC\(_4\) was measured with or without tamarixetin pretreatment. The Ag stimulation significantly increased the LTC\(_4\) levels, but tamarixetin consistently reduced LTC\(_4\) generation in a dose-dependent manner (Figure 3A). In addition, we examined the effect of tamarixetin on cPLA\(_2\) phosphorylation. The result showed that the phosphorylation of cPLA\(_2\) was increased after Ag stimulation, but suppressed by tamarixetin (Figure 3B), suggesting that tamarixetin decreased LTC\(_4\) generation by inhibiting cPLA\(_2\), phosphorylation. The present study showed that tamarixetin suppressed LTC\(_4\) generation by inhibiting cPLA\(_2\) phosphorylation (Figure 3A and B). Moreover, since MAPK can synthesize LTC\(_4\) through cPLA\(_2\) phosphorylation,\(^3\) we examined the involvement of MAPK and demonstrated that tamarixetin suppressed the increased phosphorylation of p38 (Figure 3C). Thus, these data indicate that tamarixetin inhibits LTC\(_4\) synthesis by suppressing cPLA\(_2\) and p38 activation as well as intracellular Ca\(^{2+}\) release in BMMCs.
In mast cells, AA can be metabolized to PGD$_2$ through the COX pathway that occurs in a biphasic manner. COX-1 dependent PGD$_2$ production occurs within a few minutes followed by the delayed phase of PGD$_2$ production that is dependent on de novo-induced COX-2. NF-κB, a transcription factor, regulates a number of genes, including COX-2 and Akt activates the NF-κB pathway through direct activation of IKK. Our study showed that tamarixetin inhibited the PGD$_2$ generation and COX-2 expression along with the reduced phosphorylation of Akt, IKK, and IκB$_{α}$, the degradation of IκB$_{α}$, and the nuclear translocation of NF-κB (Figure 4), implying that Akt-mediated signal pathway regulates the decreased generation of COX-2-dependent PGD$_2$. Moreover, since several reports demonstrated that the MAPKs as well as NF-κB/Akt pathways are critical for COX-2-dependent PGD$_2$ synthesis, tamarixetin may suppress COX-2-dependent PGD$_2$ production by p38 inactivation.

In summary, this study demonstrated that tamarixetin reduced degranulation and LTC$_4$ generation by suppressing the phosphorylation of PLC$_{γ}$1 with subsequent Ca$^{2+}$ and cPLA$_{2}$ inhibition (Figure 5). It also inhibited PGD$_2$ production and COX-2 expression through the Akt signal pathway.

Figure 4  Effect of tamarixetin on COX-2-dependent PGD$_2$ production and Akt/NF-κB pathway. IgE-sensitized BMMCs were pretreated with aspirin for 2 h to inactivate preexisting COX-1 followed by washing and pretreated with tamarixetin for 1 h followed by DNP-HSA stimulation for 7 h. PGD$_2$ released into the supernatant was quantified using EIA kit (A) and cell lysates were used for COX-2 expression (B). BMMCs were pretreated with tamarixetin for 1 h and stimulated with DNP-HSA for 30 min. Total cell lysates and cytosolic/nuclear fractions were subjected to Western blot analysis with antibodies for Akt, IKK$α$/β, IκB$_{α}$, cytosolic, and nuclear NF-κB (C). Data are expressed as the means±S.E.M. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ were compared to DNP-HSA stimulated-BMMCs.
with the inactivation of p38 in IgE/Ag-stimulated BMMCs (Figure 5).

Conflicts of interest
The authors declare that they have no conflicts of interest.

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