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RESEARCH ARTICLE

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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 C_4 and prostaglandin D_2 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 A_2 ($cPLA_2$) and p38 mitogen-activated protein kinase.
Conclusions: Taken together, this study suggests that tamarixetin inhibits degranulation and eicosanoid generation through the PLCγ1 as well as Akt pathways in BMMCs, which would be potential for the prevention of allergic inflammatory diseases.

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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.[1,2]

Activated mast cells initiate the activation of Syk tyrosine kinase followed by phosphorylation of phospholipase Cγ1 (PLCγ1), which triggers calcium (Ca^{2+}) release from internal stores.[3,4] FcεRI 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.[5]

Previously, we have shown anti-inflammatory and anti-allergic responses of britanin and tomentson.[6-9] As a continuing study, tamarixetin was also isolated from Inulae flos. It is a natural flavonoid derivative of quercetin and has the inhibitory effects of acetylcholinesterase and xanthine oxidase *in vitro* assays.[10,11] Recently, one study showed that tamarixetin reduced the secretion of inflammatory cytokines by dendritic cells.[12] However, the effect of tamarixetin on degranulation and AA generation in activated mast cells has not been studied to date.

In this study, we investigated the anti-inflammatory effect of tamarixetin and found that it suppresses degranulation through the attenuation of PLCγ1 phosphorylation in IgE/Ag-induced mast cells. Tamarixetin also inhibits effectively the generation of 5-lipoxygenase (LO)-dependent LTC_4 and COX-2-dependent PGD_2 via the regulation of p38 as well as NF-κB pathways.

Materials and methods

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 sulfoxide (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-PLCγ1, phospho-Akt, Akt, phospho-IKKα/β, phospho-IκBα, IκBα, and β-actin were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibody against phospho-$cPLA_2$, lamin 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.

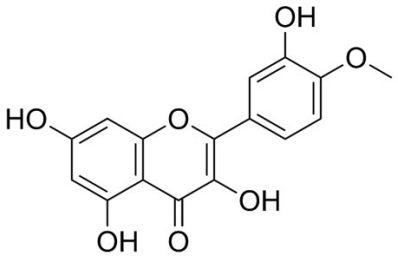

Figure 1 Chemical structure of tamarixetin.

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 Development Institute of Korean Medicine. A colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-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 (1×10^6 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 (1×10^6 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 PLCγ1 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

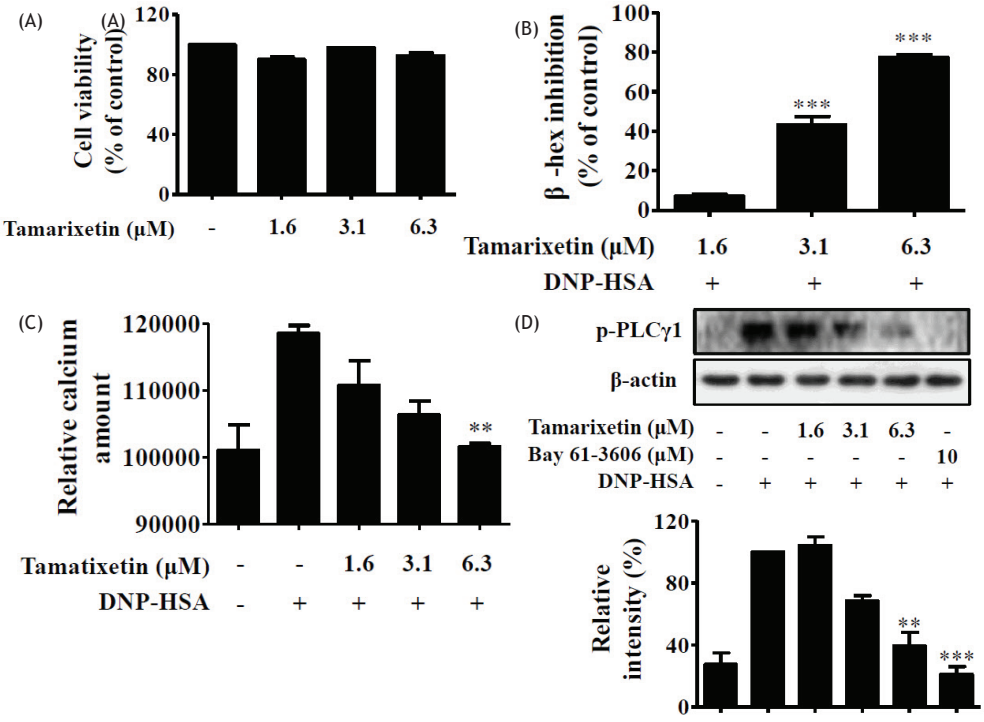

Figure 2 Effect of tamarixetin on degranulation, Ca^{2+} mobilization, and PLCγ1 phosphorylation. IgE-sensitized BMMCs were pretreated with tamarixetin for 1 h and stimulated with DNP-HSA (Ag) for 15 min. The amount of released β-hex into the supernatant was quantified (B) and cell lysates were used to assess for PLCγ1 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 PLCγ1 phosphorylation. Figure 2D showed that the phosphorylation of PLCγ1 was increased by Ag treatment on IgE-sensitized BMMCs, but tamarixetin treatment dose-dependently inhibited PLCγ1 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

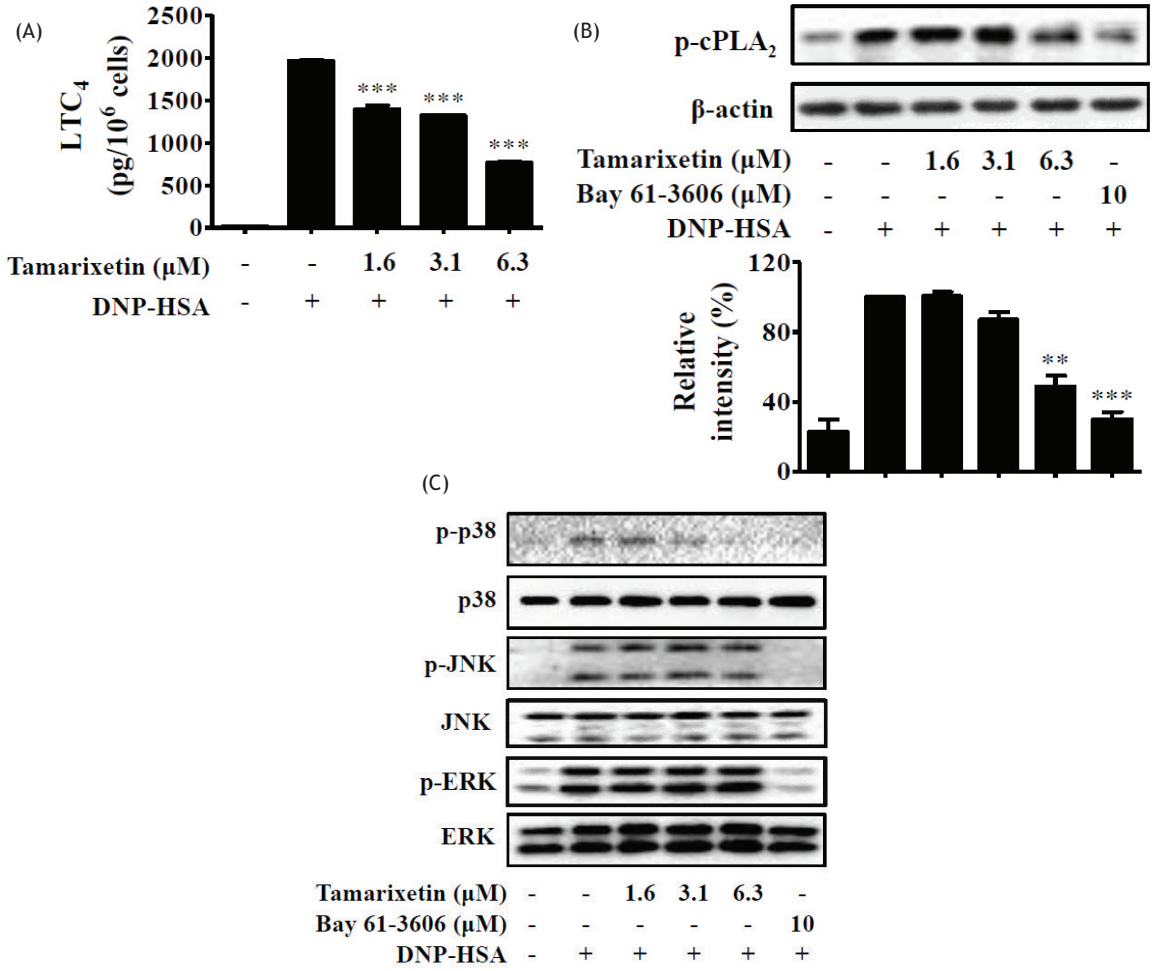

Figure 3 Effect of tamarixetin on LTC_4 and the phosphorylation of $cPLA_2$ and MAPKs. IgE-sensitized BMMCs were pretreated with the tamarixetin for 1 h followed by stimulation with DNP-HSA for 15 min. LTC_4 generation in the supernatant was determined using EIA kit (A). Cell lysates were used for Western 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.

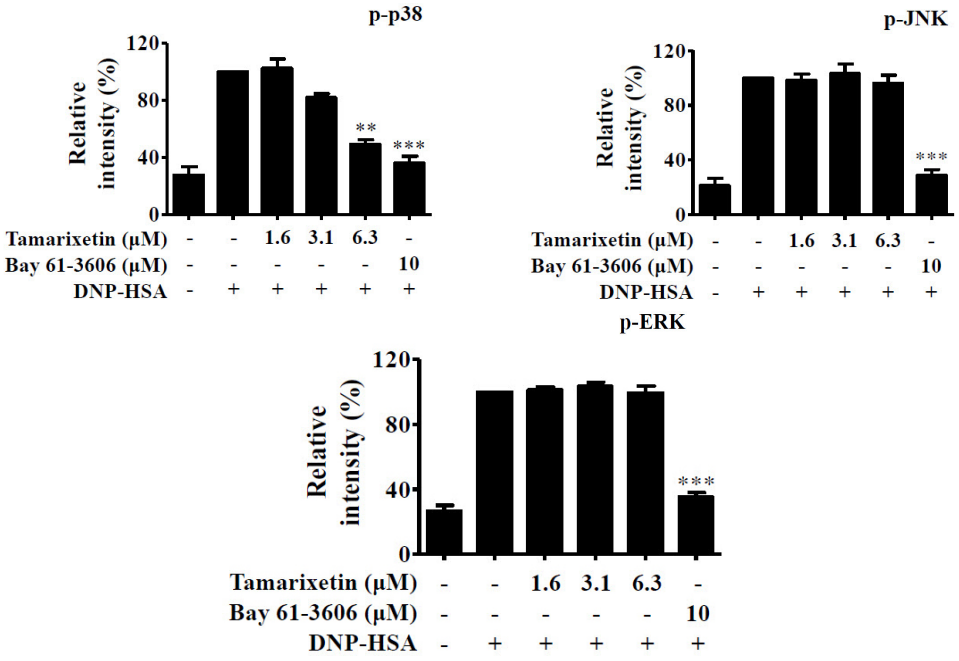

Figure 3 (Continued)

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 synthesis 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 μM (Figure 4A and B). Since NF-κB, an essential transcription factor for COX-2 expression, lies downstream of the Akt pathway, we examined the effect of tamarixetin on the NF-κB signal pathway in IgE/Ag-stimulated BMMCs. As shown in Figure 4C, tamarixetin inhibited the phosphorylation of Akt, IKK, and IκBα, the degradation of IκBα, and the nuclear translocation of NF-κB, 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 β-hex release dose-dependently in BMMCs (Figure 2B). Since PLCγ-mediated Ca^{2+} mobilization is a prerequisite for mast cell degranulation in activated mast cells,[15] we examined intracellular Ca^{2+} influx and PLCγ1 phosphorylation by tamarixetin treatment. The results showed tamarixetin decreased intracellular Ca^{2+} levels and PLCγ1 phosphorylation activation (Figure 2C and D), suggesting that the inhibitory effect of tamarixetin on mast cell degranulation resulted from the suppression of PLCγ1 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. LTC_4 is a potent lipid mediator involved in asthma and inflammation. LTC_4 synthesis is orchestrated by translocation to the nuclear envelope along with $cPLA_2$, 5-LO, and FLAP in response to the concentration of increased intracellular Ca^{2+}.[16,17] $cPLA_2$ plays a key role in mediating AA and eicosanoid production.[18] 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,[5] 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.[19,20] 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 IκBα, the degradation of IκBα, 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,[21-23] 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 PLCγ1 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

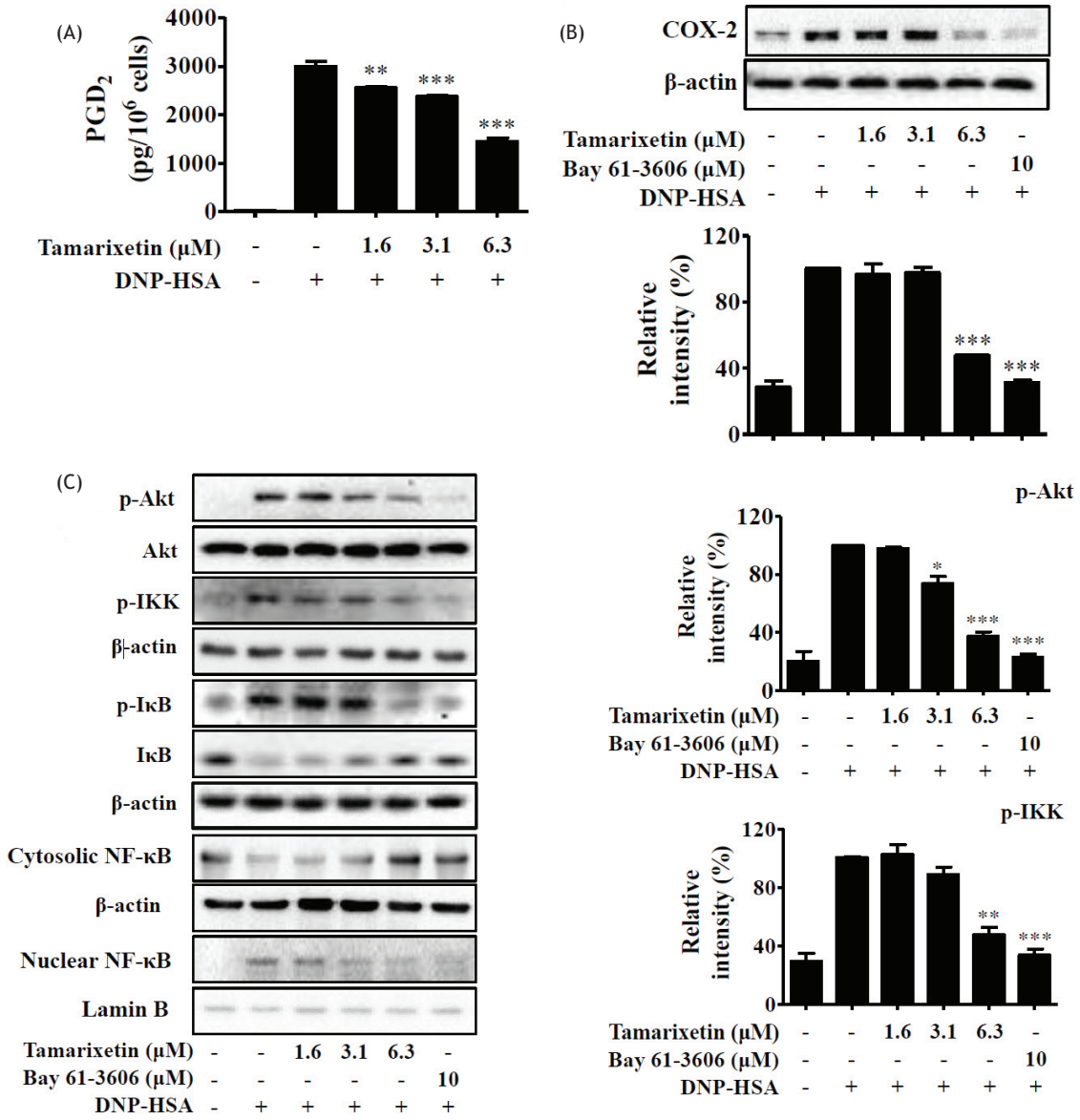

Figure 4 Effect of tamarixetin on COX-2-dependent PGD_2 production and Akt/NF–κB pathway. IgE-sensitized BMMCs were pretreated with aspirin for 2 h to inactivate preexisting COX-1 followed by washing and pretreated with tamarixetin for 1 h followed by DNP-HSA stimulation for 7 h. PGD_2 released into 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, IKKα/β, IκBα, 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.

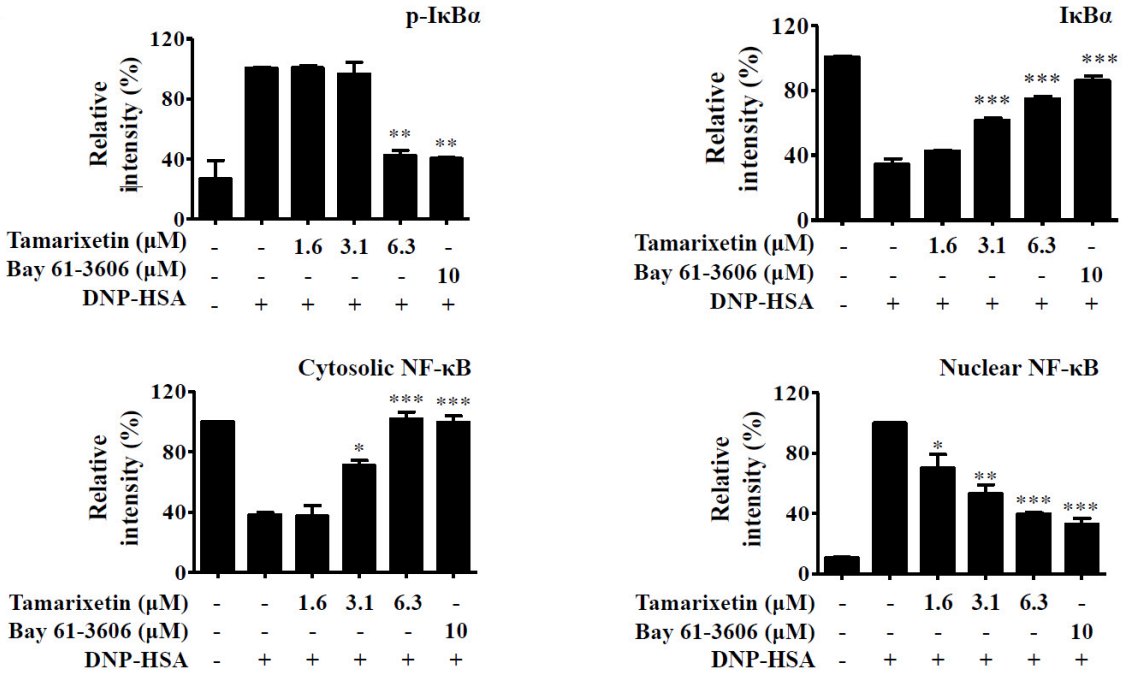

Figure 4 (Continued)

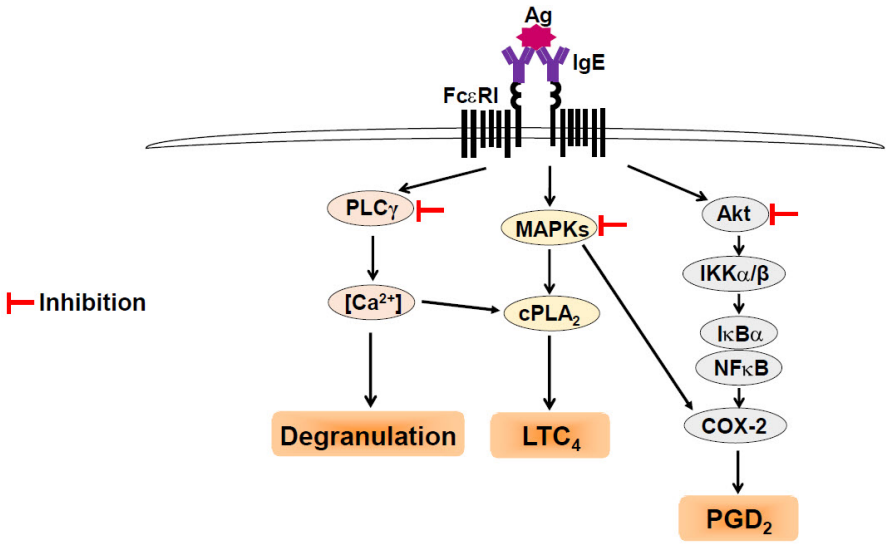

Figure 5 The schematic diagram for tamarixetin to suppress the IgE/Ag-mediated activation of mast cells.

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.

Funding

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