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Sauchinone suppresses FcεRI-mediated mast cell signaling and anaphylaxis through regulation of LKB1/AMPK axis and SHP-1-Syk signaling module

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ABSTRACT

Sauchinone, the biologically active lignan of *Saururus chinensis*, has been reported to have anti-inflammatory, antitumor, antioxidant, and hepatoprotective properties. However, little is known about the effect of sauchinone on FcεRI-mediated mast cell activation. The aim of this study was to evaluate the anti-allergic activity of sauchinone and the underlying mechanism using mouse bone marrow-derived mast cells (BMMCs) and the mast cell-mediated passive cutaneous anaphylaxis (PCA) model. Sauchinone markedly suppressed FcεRI-mediated activation of positive signaling mediators, including Syk, linker for activation of T cells (LAT), phospholipase C (PLC)γ, mitogen-activated protein (MAP) kinases, Akt, IκB kinase (IKK), and intracellular Ca^{2+}, and increased the activation of negative signaling mediators, including liver kinase B (LKB)1/AMP-activated protein kinase (AMPK) and Src homology 2 domain-containing protein tyrosine phosphatase (SHP)-1. Interestingly, sauchinone increased the interaction between SHP-1 and Syk. Consequently, sauchinone significantly suppressed FcεRI-mediated BMMC degranulation and synthesis of eicosanoids and cytokines. These inhibitory effects of sauchinone were diminished in BMMCs treated with siRNAs targeting LKB1, AMPKα2, or SHP-1, and in BMMCs isolated from AMPKα2-deficient mice. In addition, administration of sauchinone markedly suppressed the IgE-mediated PCA reaction in wild-type mice, and this inhibitory effect was significantly reduced in *AMPKα2*$^{-/-}$ mice. Taken together, these data suggest that sauchinone suppresses FcεRI-mediated mast cell activation and anaphylaxis through modulation of the LKB1/AMPK and SHP-1/Syk pathways. Therefore, sauchinone might be a potential therapeutic agent for the treatment of allergic inflammatory diseases.

1. Introduction

Mast cells play a central role in allergic diseases such as rhinitis, asthma, and atopic dermatitis. Aggregation of IgE-bound FcεRI (the high-affinity IgE receptor) on mast cells triggers the release of preformed mediators, such as histamine and proteases, and newly synthesized lipid mediators, such as prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4), and various cytokines [1,2], which are responsible for the development of allergic inflammation and anaphylactic responses. The signaling pathways regulating FcεRI-mediated mast cell activation have been extensively studied. Antigen-induced cross-linking of FcεRI induces the activation of proximal FcεRI-associated Src kinases such as Fyn and Lyn, resulting in the phosphorylation of Syk, a central tyrosine kinase for mast cell activation. In turn, Syk phosphorylates linker for activation of T cells (LAT) and phospholipase C (PLC)γ, and activates several downstream signaling molecules including mitogen-activated protein kinases (MAPKs), protein kinase B (Akt), and IkB kinase (IKK)/nuclear factor κ-B (NF-κB), which are essential for allergic responses in mast cells [3–6]. In addition to these positive signaling pathways, IgE-mediated mast cell activation is negatively regulated by molecules such as the SH2 domain-containing protein tyrosine phosphatases (SHPs) and SH2 domain-containing inositol-polyphosphate 5-phosphatases (SHIPs) [1,7–9].

AMP-activated protein kinase (AMPK) is composed of one catalytic α subunit (α1 or α2) and two regulatory subunits, β (β1 or β2) and γ (γ1, γ2, or γ3). It is an essential signaling molecule for the maintenance of cellular and whole body energy homeostasis [10,11]. AMPK is activated during energy deficiency to restore the cellular energy state. In an energy-deficient state, liver kinase B1 (LKB1) plays an essential role as the principal AMPK upstream kinase. Recently, we reported that the

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LKB1-AMPK axis represents a novel negative regulatory module for FcεRI signaling, which is counter-regulated by Fyn and extracellular-related kinase (ERK) in mouse mast cells [12,13]. Therefore, AMPK activators might be promising therapeutic candidates for allergic diseases as well as metabolic syndromes.

Sauchinone is the biologically active lignin of *Saururus chinensis* (Saururaceae) and has been used in China and Korea as a traditional medicine for the treatment of edema, jaundice, gonorrhea, and several inflammatory diseases [14]. Sauchinone has diverse pharmacological activities, including hepatoprotective, antioxidant, and anti-allergic properties [15,16]. In addition, sauchinone has shown anti-inflammatory effects in various cell types, including vascular endothelial cells [17], neutrophils [18], microglial cells [19], and chondrocytes [20], *via* suppression of NF-κB signaling. It has been reported that sauchinone prevents iron-induced liver injury and increases macrophage phagocytosis through the activation of AMPK [15,21]. However, the anti-allergic activity of sauchinone in IgE/antigen (Ag)-stimulated mast cell activation and anaphylaxis and the underlying mechanisms have not been well established.

In the present study, sauchinone suppressed IgE/Ag-stimulated mast cell activation and anaphylaxis by regulating the LKB1-AMPK axis. In addition, the inhibitory effects of sauchinone on FcεRI signaling were also dependent on an LKB1/AMPK-independent pathway involving SHP-1 and Syk. These results elucidate the mechanisms underlying the anti-allergic activity of sauchinone.

2. Materials and methods

2.1. Materials

Antibodies against phosphorylated forms of LKB1 (Ser428), AMPKα2 (Thr172), ACC (Ser79), PLCγ1 (Tyr783), Akt (Ser473), p38 MAPK, ERK1/2, JNK, IKKα/β and those against LKB1, AMPKα, ACC, Akt, p38 MAPK, ERK1/2 and JNK were from Cell Signaling Technology (Danvers, MA). Antibodies against IKKα/β, PLCγ1, LAT, Syk, Fyn, Lyn and SHP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (pY) antibody was from Millipore and anti-AMPKα2 antibody was from Abcam (Cambridge, MA). Mouse anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA) and sauchinone were purchased from Sigma Chemical Co (St. Louis, MO). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) was from Calbiochem (San Diego, CA). Sauchinone was prepared by dissolving in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was adjusted to 0.1% (*v*/v) in culture medium. DMSO was used as a vehicle control in all cases.

2.2. Mice

Balb/cJ and C57BL/6J mice were obtained from Samtako, INC (Seoul, Korea). *AMPKα2*$^{-/-}$ mice on the C57BL/6J background were reported previously [22]. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Yeungnam University [12].

2.3. Preparation and activation of mouse bone marrow-derived mast cells (BMMCs)

The preparation and activation of BMMCs were done as the same protocol as described previously [12]. Briefly, BMMCs isolated from male Balb/cJ or C57BL/6J mice were cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin (Thermo Fisher Scientific), 10 mM HEPES (Sigma-Aldrich), 100 μM MEM non-essential amino acid solution (Invitrogen, Grand Island, NY) and 20% PWM-SCM (pokeweed mitogen-spleen cell conditioned medium) as a source of IL-3. For cell stimulation, 1×10^6 cells/ml were sensitized with 500 ng/ml mouse anti-DNP IgE overnight and then stimulated with 100 ng/ml DNP-HSA typically for 15 min at 37 °C. When the effects of sauchinone and AICAR were examined, they were added 1 h and 5 h, respectively, prior to the addition of DNP-HSA. Intracellular Ca^{2+} levels at 5 min, releases of β-hexosaminidase (a marker of mast cell degranulation) and eicosanoids (LTC_4 and PGD_2) at 15 min, and production of cytokines (IL-6 and TNF-α) at 6 h were evaluated as described previously [12]. PGD_2, LTC_4, IL-6 and TNF-α were quantified using respective immunoassay kits for eicosanoids (Cayman Chemicals) and cytokines (R&D Systems).

2.4. Measurement of intracellular Ca^{2+} level

Intracellular Ca^{2+} level was determined with the FluoForte Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, MI) as described previously [12]. Briefly, IgE-sensitized BMMCs were preincubated with FluoForte Dye-Loading Solution for 1 h at room temperature. After washing with HBSS, the cells (5×10^4) were seeded into 96-well microplates. Then, cells were pretreated with indicated concentration of sauchinone for 1 h before adding DNP-HSA. The fluorescence was measured using a flurometric imaging plated reader at an excitation of 485 nm and an emission of 520 nm on a BMG Labtechnologies FLUOStar OPITIMA platereader (Offenburg, Germany).

2.5. Immunoblotting and immunoprecipitation

Immunoprecipitation (IP) was performed by the same method as our previous report [12]. Cell lysates were collected using modified lysis buffer (0.1% Nonidet P-40, 50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 0.5 mM dithiothreitol). Total cell lysate (1 mg protein equivalent) was incubated with various antibodies for 2 h at 4 °C and then the immunocomplexes were precipitated with 20 μl of protein A-Sepharose. These precipitates were extensively washed 3 times with ice-cold lysis buffer. These precipitates or cell lysates were subjected to SDS-PAGE and immunoblotted with corresponding antibodies.

2.6. Transfection of siRNA

Gene silencing with specific siRNA was carried out as described previously [12]. Mouse LKB1, SHP-1 and non-specific siRNA was obtained from Santa Cruz Biotechnology and AMPKα2 siRNA was from Dharmacon (Lafayette, CO). BMMCs were cultured in 12-well plates for 16 h in serum-free medium and transfected with a siRNA transfection reagent (Santa Cruz Biotechnology) containing siRNA (100 nM per well). After 24 h, BMMCs were sensitized with IgE in the presence or absence of sauchinone or AICAR and then stimulated with DNP-HSA as described in our previous report [12].

2.7. IgE-mediated passive cutaneous anaphylaxis (PCA) in mice

PCA was carried out by the same protocol as described previously [12]. Briefly, 80 ng of mouse anti-DNP IgE was intradermally injected into one ear of 7-wk-old mice, followed 24 h later by oral administration of indicated dose of sauchinone or 50 mg/kg fexofenadine-HCl (Korea Pharma, Seoul). After 1 h, mice were challenged intravenously with 60 μg of DNP-HSA containing Evans blue ($n = 6$ per group). Evans blue from ear tissue was extracted with formamide at 63 °C overnight and quantified by absorbance at 630 nm. Blood was collected by cardiac puncture at 1 h after Ag challenge to determine serum LTC_4 and PGD_2 levels as described above.

2.8. Statistical analysis

Data calculation and statistical analysis were performed using GraphPad Prism 3.0 software. The statistical significance of differences between two groups was determined with unpaired Student's *t*-test and multiple comparisons were analyzed using one-way ANOVA. All data

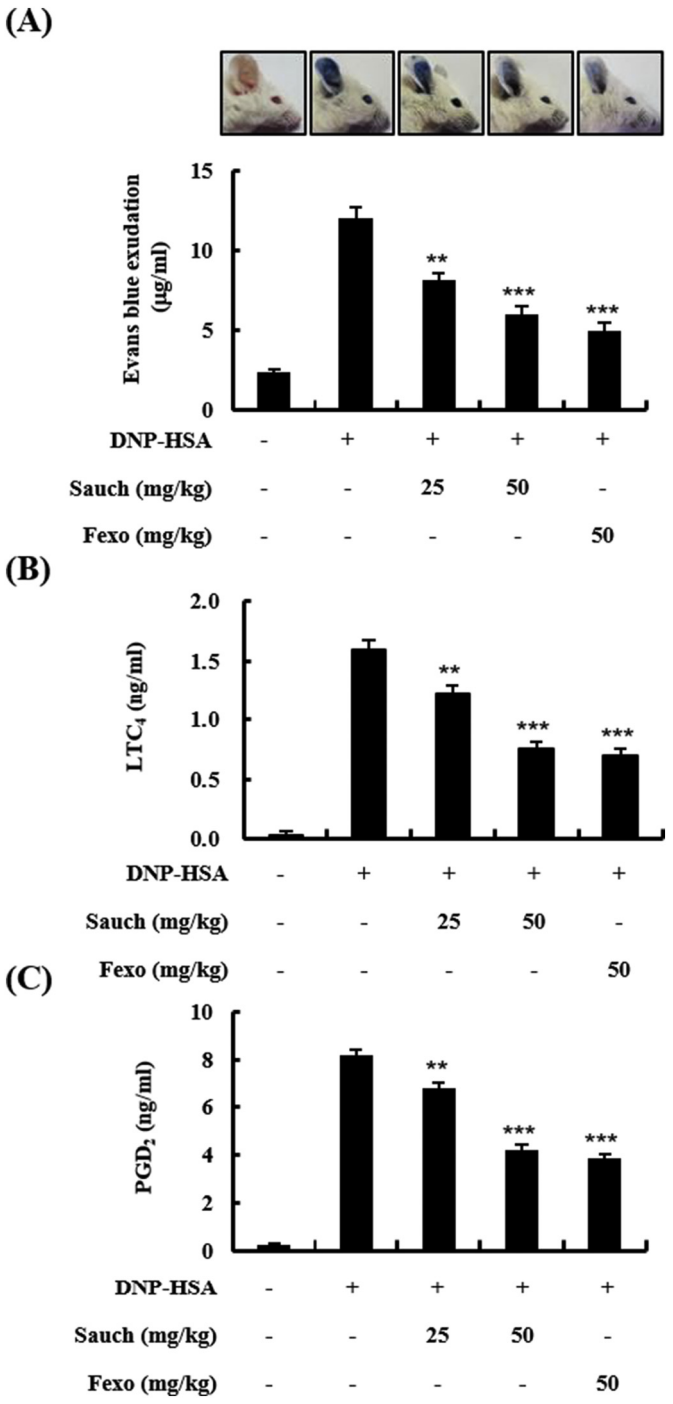

Fig. 1. Sauchinone inhibits IgE/Ag induced PCA reaction.
Balb/cJ mice were injected intradermally with anti-DNP IgE into one ear, followed by intravenous challenge with DNP-HSA or saline together with Evans blue. Sauchinone (Sauch) and fexofenadine-HCl (Fexo) were orally administered 1 h before DNP-HSA treatment. The amounts of Evans blue dye exudation are presented. Top panels show representative photos of ears with dye extravasation at 1 h (A). The levels of serum LTC_4 (B) and PGD_2 (C) were evaluated. Data represents mean ± S.E.M ($n = 6$ mice per group; $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* DNP-HSA alone). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

are presented as means ± S.E.M. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Sauchinone inhibits anaphylaxis in mice

Anaphylaxis is a profound allergic reaction initiated by allergen-induced cross-linking of specific IgE-bound FcεRI. Since previous reports have shown that sauchinone suppresses allergen-induced airway inflammation [16,23], we evaluated the anti-allergic activity of sauchinone in the PCA mouse model. Mice sensitized with anti-DNP-IgE were challenged with DNP-HSA after oral administration of sauchinone or fexofenadine-HCl, a selective histamine H1 blocker [24]. As shown in Fig. 1A, sauchinone reduced the amount of dye extravasation by 30.6% ($p < 0.01$) and 48.4% ($p < 0.001$) at 25 and 50 mg/kg, respectively. In addition, sauchinone significantly reduced the serum levels of LTC_4 by 23.4% ($p < 0.01$) and 50% ($p < 0.001$) (Fig. 1B), and the levels of PGD_2 by 18.8% ($p < 0.01$) and 46.9% ($p < 0.001$) (Fig. 1C), at 25 and 50 mg/kg, respectively. The inhibitory potency of 50 mg/kg sauchinone on the PCA reaction was comparable to that of 50 mg/kg fexofenadine-HCl. These results demonstrate that sauchinone suppresses FcεRI-mediated anaphylaxis *in vivo*.

3.2. Sauchinone dampens IgE/Ag-stimulated mast cell activation

As sauchinone effectively suppressed IgE/Ag-induced allergic reactions *in vivo*, the effect of sauchinone on FcεRI signaling in BMMCs was investigated. In a previous study, we reported that the LKB1/AMPK axis suppresses FcεRI signaling, including FcεRI-mediated activation of PLCγ1, ERK1/2, c-Jun N-terminal kinase (JNK), and NF-κB, without affecting Akt or p38 MAPK, thereby limiting mast cell activation [12,13]. We therefore investigated the effect of sauchinone on IgE/Ag-stimulated BMMC activation in the context of the LKB1-AMPK pathway. First, to determine a concentration of sauchinone that induces AMPK activation, BMMCs were treated with sauchinone (10, 20, or 30 μM) for 1 h. Sauchinone increased the phosphorylation of LKB1, AMPK, and acetyl-CoA carboxylase (ACC), a downstream target of AMPK, in a dose-dependent manner. Since 20 μM sauchinone showed maximal effects (data not shown), this concentration was used in subsequent experiments. As mentioned, sauchinone (20 μM) alone markedly increased the phosphorylation of LKB1, AMPK, and ACC which were slightly decreased in FcεRI-activated mast cells (Fig. 2A). The increase in the phosphorylation of LKB1/AMPK/ACC by sauchinone (20 μM) was time-dependent and reached the maximal increase at 120 min (Fig. 2B). Concurrently, sauchinone impaired the IgE/Ag-induced activation of FcεRI downstream signaling molecules, including PLCγ1, ERK1/2, JNK, and IKK. In addition, sauchinone inhibited the phosphorylation of Akt and p38 MAPK (Fig. 2C), which are positively regulated by Syk but not by the LKB1/AMPK axis [12,13]. Consistent with the inhibitory effects of sauchinone on FcεRI signaling, IgE/Ag-induced degranulation, as assessed by exocytosis of β-hexosaminidase (Fig. 2D); production of LTC_4 (Fig. 2E), PGD_2 (Fig. 2F), IL-6 (Fig. 2G), and TNF-α (Fig. 2H); and intracellular Ca^{2+} mobilization (Fig. 2I) were significantly reduced by sauchinone. These results suggest that sauchinone dampens FcεRI-mediated mast cell signaling through both LKB1/AMPK-dependent and -independent pathways.

3.3. LKB1 and AMPK knockdown decrease the inhibitory effect of sauchinone on IgE/Ag-induced mast cell activation

To investigate the involvement of LKB1/AMPK in the effects of sauchinone on FcεRI-induced mast cell signaling, LKB1 and AMPK were knocked down with LKB1- and AMPKα2-specific siRNAs. As expected, LKB1 siRNA prevented the phosphorylation of LKB1, AMPK, and ACC (Fig. 3A), whereas AMPKα2 siRNA inhibited the phosphorylation of

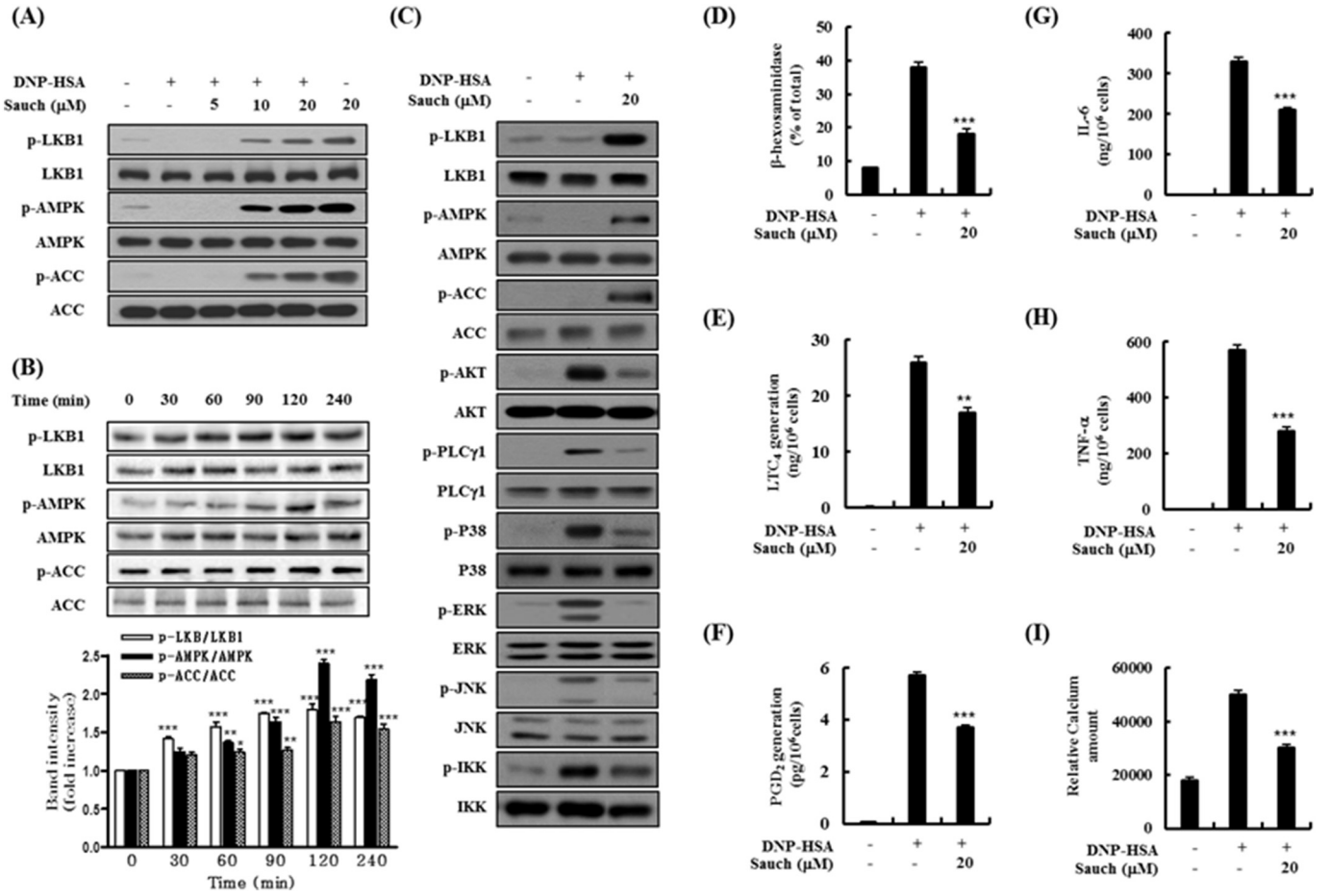

Fig. 2. Sauchinone inhibits IgE/Ag-stimulated mast cell activation.
BMMCs were treated with 20 μM of sauchinone (Sauch) alone for 1 h or Ig*E*-sensitized BMMCs were pretreated with different concentrations (5, 10, 20 μM) of Sauch for 1 h and then, stimulated with Ag (A, C) or BMMCs were treated with 20 μM of Sauch for different times (0–240 min) (B). Effects of Sauch on phosphorylation of signaling molecules were evaluated by immunoblotting (A–C). The relative ratios of band intensity of phosphorylated signaling molecules to their total proteins (panel Fig. 2B) were determined by scanning densitometry. The data from three independent experiments with different BMMCs are expressed as fold increase in bar graphs ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. time 0). Releases of β-hexosaminidase (D), LTC_4 (E) and PGD_2 (F), secretion of IL-6 (G) and TNF-α (H) and influx of Ca^{2+} (I) were evaluated. The immunoblot data (A, B) is a representative of three independent experiments, and the values (B–G) indicate the means ± S.E.M. from three independent experiments with different BMMCs. ($^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. DNP-HSA alone).

AMPK and ACC but not LKB1, as it is an upstream kinase of AMPK (Fig. 3E). In agreement with our previous studies [12,13], treatment of BMMCs with AICAR, an AMPK activator, resulted in increased phosphorylation of LKB1, AMPK, and ACC, and decreased phosphorylation of PLCγ1, ERK, JNK, and IKK, but not Akt or p38 MAPK (Fig. 3A, E). The inhibitory effect of AICAR was almost completely abolished in LKB1 or AMPKα2 siRNA-treated cells. Importantly, in LKB1- or AMPKα2-silenced cells, the inhibitory effect of sauchinone on the phosphorylation of PLCγ1, ERK, JNK, and IKK was largely, if not completely, abolished, whereas the phosphorylation of Akt and p38 MAPK was only modestly affected (Fig. 3A, E). Consistently, the inhibitory effects of sauchinone and AICAR on IgE/Ag-dependent degranulation (Fig. 3B, F) and generation of LTC_4 (Fig. 3C, G) and PGD_2 (Fig. 3D, H) were reversed by silencing of LKB1 or AMPKα2. These results suggest that the inhibitory effects of sauchinone on mast cell activation are mediated in part by the LKB1/AMPK axis.

3.4. AMPKα2 deficiency dampens the inhibitory effect of sauchinone on IgE-dependent mast cell activation and anaphylaxis

To obtain additional evidence for the requirement of AMPK for the inhibitory effect of sauchinone on FcεRI-mediated mast cell activation, BMMCs from AMPKα2-deficient mice were used. Consistent with the experiments using AMPKα2 siRNA, the suppressive effect of AICAR on IgE/Ag-induced phosphorylation of PLCγ1, ERK, JNK, and IKK in wild-type (WT) BMMCs was largely abolished in *AMPKα2*$^{-/-}$ BMMCs (Fig. 4A). As expected, in *AMPKα2*$^{-/-}$ BMMCs, the inhibitory effect of sauchinone on the phosphorylation of PLCγ1, ERK, JNK, and IKK was largely abolished, whereas the phosphorylation of Akt and p38 MAPK was only marginally affected (Fig. 4A). These results further suggest that the inhibitory effects of sauchinone on mast cell activation are mediated by both LKB1/AMPK-dependent and -independent (Syk-dependent) mechanisms. Consistent with the reduced effects on cell signaling, IgE/Ag-induced degranulation (Fig. 4B) and generation of LTC_4 (Fig. 4C) and PGD_2 (Fig. 4D) were significantly increased in *AMPKα2*$^{-/-}$ BMMCs compared with WT BMMCs.

Next, we evaluated the effect of sauchinone on the PCA reaction using *AMPKα2*$^{-/-}$ mice to confirm the AMPK-dependent anti-allergic activity of sauchinone *in vivo*. Consistent with results observed in *AMPKα2*$^{-/-}$ BMMCs (Fig. 4B-D), *AMPKα2*$^{-/-}$ mice showed greater vascular permeability, as measured by IgE/Ag-induced dye extravasation, than WT mice (Fig. 4E). Moreover, IgE/Ag-induced increases in serum levels of LTC_4 (Fig. 4F) and PGD_2 (Fig. 4G) were significantly enhanced in *AMPKα2*$^{-/-}$ mice compared with WT mice, and the inhibitory effects of sauchinone were partially diminished in *AMPKα2*$^{-/-}$ mice. These results strongly suggest that the anti-allergic effects of

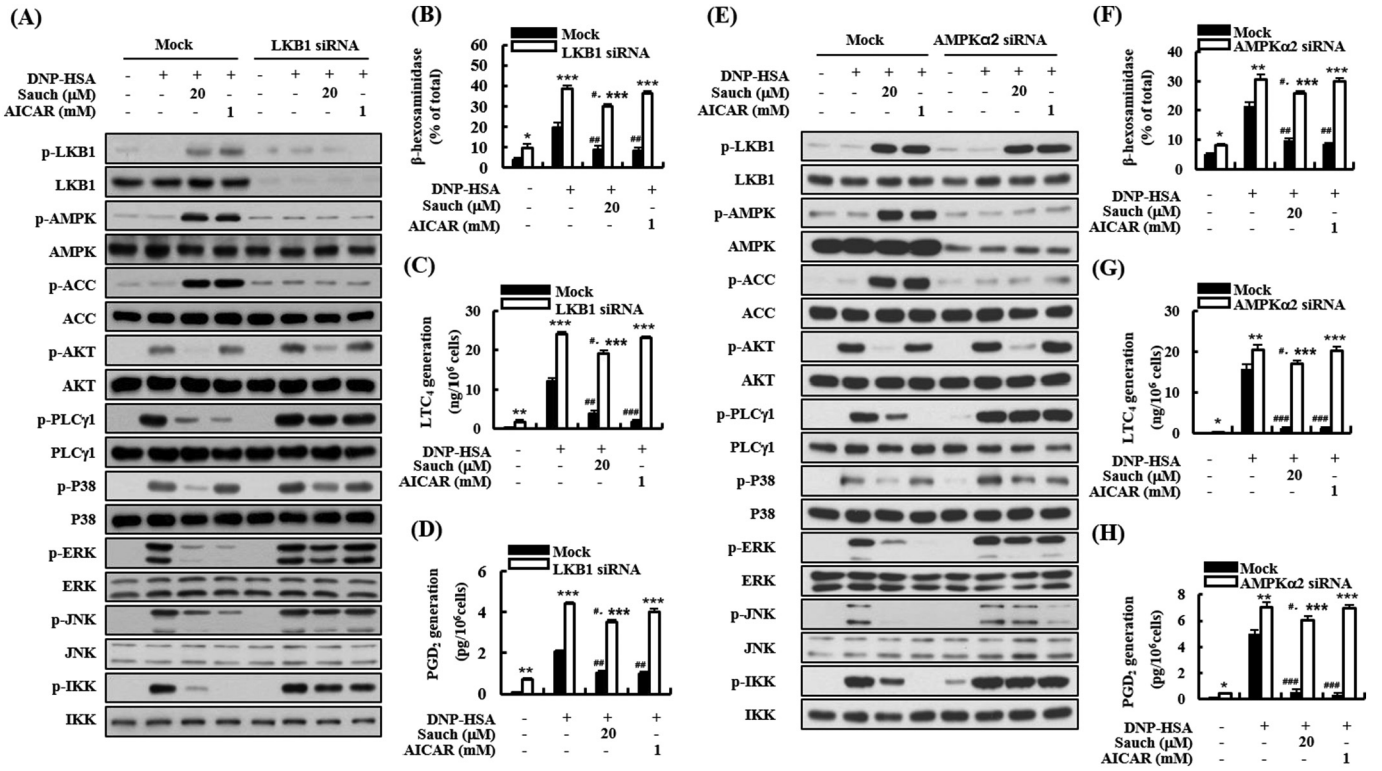

Fig. 3. LKB1 or AMPKα2 knockdown decreases the inhibitory activity of sauchinone on IgE/Ag-stimulated BMMCs.
BMMCs were treated with siRNA specific for LKB1 (A-D), AMPKα2 (E-H) or control siRNA (Mock) for 48 h. BMMCs were sensitized with anti-DNP IgE in the presence or absence of sauchinone (Sauch) or AICAR and then, stimulated with DNP-HSA. The immunoblot data (A, E) is a representative of three independent experiments. Ag-induced release of β-hexosaminidase (B, F), LTC_4 (C, G) and PGD_2 (D, H) were evaluated. The values indicate the mean ± S.E.M. from three independent experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. mock in each treatment; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs*. DNP-HSA alone in each group).

sauchinone rely on the activation of AMPK both *in vitro* and *in vivo*.

3.5. Sauchinone inhibits Syk phosphorylation by promoting the interaction between SHP-1 and Syk

We have previously reported that the activation of LKB1 and AMPK in mast cells is negatively regulated by Fyn [12]. Moreover, sauchinone exerts its anti-allergic function *via* AMPK-dependent and -independent pathways, both of which are downstream of Syk signaling. We therefore investigated the effect of sauchinone on the FcεRI-proximal tyrosine kinases Lyn, Fyn, and Syk. Sauchinone inhibited FcεRI-mediated tyrosine phosphorylation of Syk and its target adaptor LAT, but not Fyn and Lyn, indicating that the Syk pathway is an inhibitory target of sauchinone (Fig. 5A).

The tyrosine phosphorylation status of proteins is determined by the balance between the activity of protein tyrosine kinases and protein tyrosine phosphatases. Previous studies have demonstrated that SHP-1, a cytosolic tyrosine phosphatase, is an important negative regulator of allergic inflammation and anaphylaxis [25–28]. Importantly, it has been reported that SHP-1 reduces Syk phosphorylation [29,30] and negatively regulates FcεRI-mediated mast cell activation [31]. Recently, we reported that Bay 61-3606, a selective Syk inhibitor, increased SHP-1 phosphorylation and promoted the interaction between SHP-1 and Syk, thereby reducing Syk phosphorylation [32]. Based on these findings, we hypothesized that sauchinone would increase the phosphorylation of SHP-1 and the interaction between SHP-1 and Syk in BMMCs. As shown in Fig. 5B, SHP-1 was not phosphorylated and did not interact with Syk in resting or IgE/Ag-stimulated BMMCs in the absence of sauchinone. However, sauchinone markedly increased SHP-1 phosphorylation and its interaction with Syk in FcεRI-stimulated cells. To further verify the involvement of SHP-1 in the inhibitory effects of sauchinone on the Syk pathway, BMMCs were pretreated with a SHP-1-specific siRNA. The ability of sauchinone to reduce Syk phosphorylation and promote the interaction between SHP-1 and Syk was markedly reduced in SHP-1 siRNA-treated BMMCs compared with control siRNA (Mock)-treated cells (Fig. 5C). Consistently, the effects of sauchinone on the release of β-hexosaminidase and the generation of LTC_4 and PGD_2 were significantly reduced in SHP-1 knockdown cells (Fig. 5D–F), indicating that SHP-1 is another target of sauchinone in FcεRI-stimulated mast cells.

4. Discussion

Mast cells play a crucial role in the progression of allergic disorders. IgE/Ag-stimulated mast cell activation *via* cross-linking of FcεRI activates proximal FcεRI-associated tyrosine kinases including Fyn, Lyn, and Syk, eventually leading to the release of various preformed mediators and the secretion of newly synthesized lipid mediators, cytokines, and chemokines [1–4,6]. FcεRI activation is also regulated by negative signaling molecules whose interaction with positive signaling mediators determines the extent of mast cell activation [33–35]. Therefore, pharmacologic agents that regulate these signaling pathways involved in mast cell activation could be potential candidates for anti-allergic agents.

We recently reported that IgE/Ag-mediated signaling in mast cells is negatively regulated by the LKB1/AMPK axis, suggesting that AMPK activators could be useful for the treatment of allergic diseases [12,13]. Since it has been reported that sauchinone exerts hepatoprotective effects through the activation of AMPK [15,36], here, we investigated the effect of sauchinone on IgE/Ag-stimulated mast cell activation. Our results using siRNA knockdown and genetic deletion of LKB1 or AMPK clearly demonstrate that sauchinone suppressed FcεRI-mediated mast cell activation and mast cell-mediated anaphylaxis by regulating the LKB1/AMPK pathway. Similar to the effects of AICAR, sauchinone

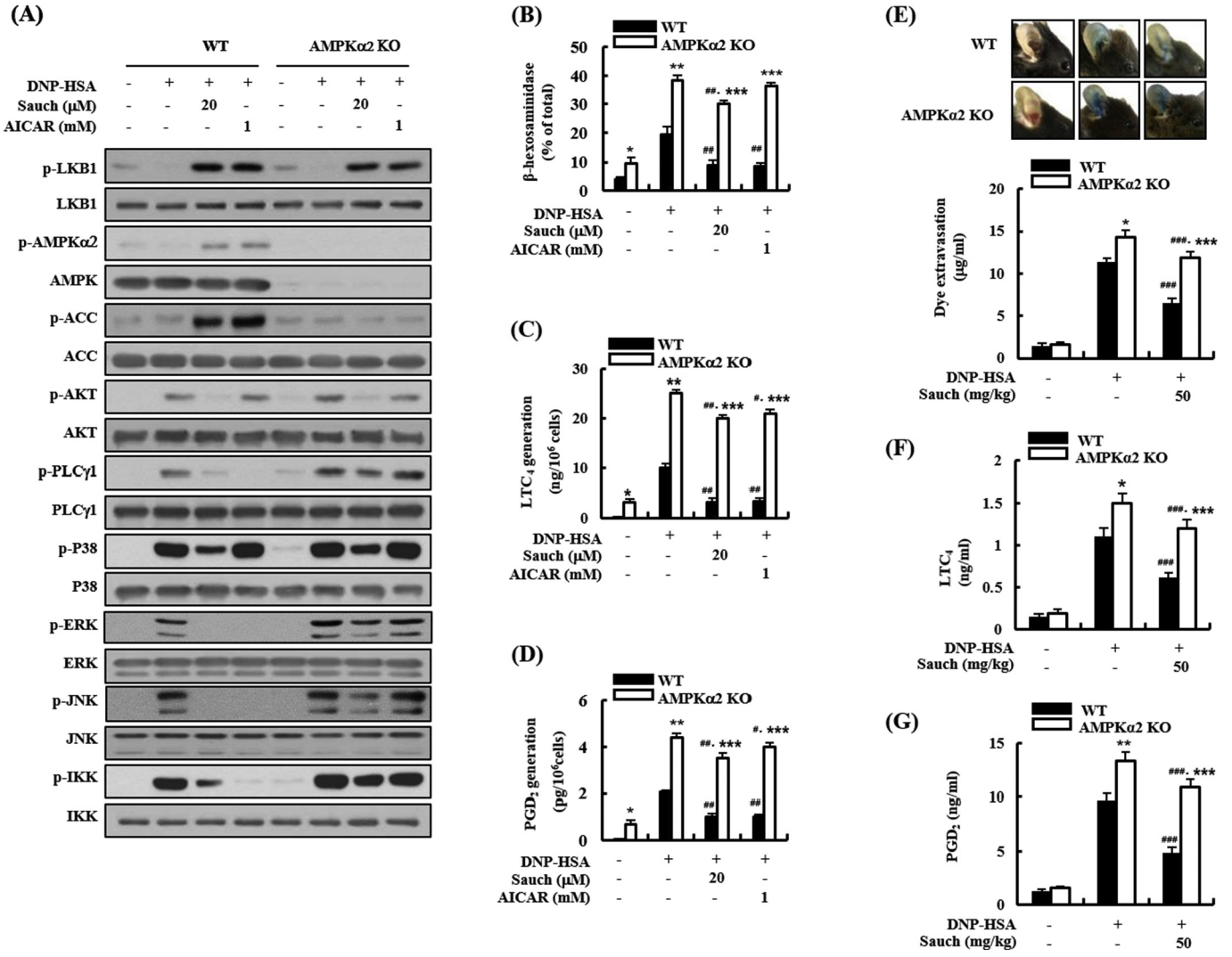

Fig. 4. Inhibition of mast cell activation by sauchinone was significantly decreased by gene ablation of AMPKα2.
BMMCs from *AMPKα2*$^{-/-}$ (KO) or WT mice were sensitized with IgE in the presence or absence of sauchinone (Sauch) or AICAR and then stimulated with DNP-HSA (A–D). Cell lysates were analyzed by immunoblotting. A representative blot of three independent experiments is shown (A) and releases of β-hexosaminidase (B), LTC_4 (C) and PGD_2 (D) were evaluated. The values indicate the means ± S.E.M. from three independent experiments with different BMMCs. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. WT in each treatment; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs*. DNP-HSA alone in each group). (E–G) IgE/Ag-induced PCA in *AMPKα2*$^{-/-}$ or WT mice. The amounts of Evans blue dye exudation are presented (E). Top panels show representative photos of ears with dye extravasation at 1 h. The levels of serum LTC_4 (F) and PGD_2 (G) were evaluated (n = 6 mice per group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. WT in each treatment; $^{\#\#\#}P < 0.001$ *vs*. DNP-HSA alone in each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased the phosphorylation of LKB1 and AMPK, and decreased the phosphorylation of PLCγ1, ERK1/2, JNK, and IKK and the concomitant cellular functions in IgE/Ag-stimulated mast cells. These effects of sauchinone were abolished by knockdown of LKB1 and AMPK with specific siRNAs. The LKB1/AMPK-dependent inhibitory effects of sauchinone on mast cell activation were further confirmed using *AMPKα2*$^{-/-}$ mice. The anti-allergic effect of sauchinone was abrogated by AMPKα2 gene deletion, lending strong support to our conclusions. Although the underlying mechanism for the upregulation of LKB1/AMPK signaling by sauchinone is not completely understood, we previously reported that LKB1/AMPK signaling is negatively regulated by Fyn [12] but positively regulated by Sirt1 [37]. However, sauchinone had no effect on Fyn activation. Therefore, it will be interesting to evaluate the involvement of Sirt1 in the effect of sauchinone on LKB1/AMPK.

In addition to effects similar to AICAR, sauchinone markedly inhibited the phosphorylation of Akt and p38 MAPK, which are not regulated by the LKB1/AMPK pathway but lie downstream of the Syk pathway [12,13]. This result suggests that the inhibitory effect of sauchinone is also mediated by AMPK-independent pathways. Aggregation of FcεRI in mast cells results in the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the β and γ subunits of FcεRI by Lyn and Fyn, which are constitutively associated with FcεRI. The phosphorylated ITAMs then serve as docking sites for Syk and other downstream signaling molecules [5,6]. Notably, pharmacologic or genetic inhibition of Syk blunts the intracellular Ca^{2+} mobilization, degranulation, and activation of MAP kinases and NF-κB, indicating that phosphorylation of Syk is an essential step for all known IgE/Ag-stimulated allergic responses in mast cells [1–4,6]. Syk signaling is regulated by tyrosine phosphatases such as SHP-1 as well as by upstream tyrosine kinases [29,30]. SHP-1 is recruited to the phosphorylated ITAMs of FcεRIγ and dephosphorylates the FcεRI β- and γ-chains and Syk [27,28,32]. In the present study, we have shown that sauchinone selectively reduced the phosphorylation of Syk without affecting Lyn or Fyn. This suggests SHP-1 as a potential target of sauchinone in mast cells. Indeed, sauchinone increased the phosphorylation of SHP-1

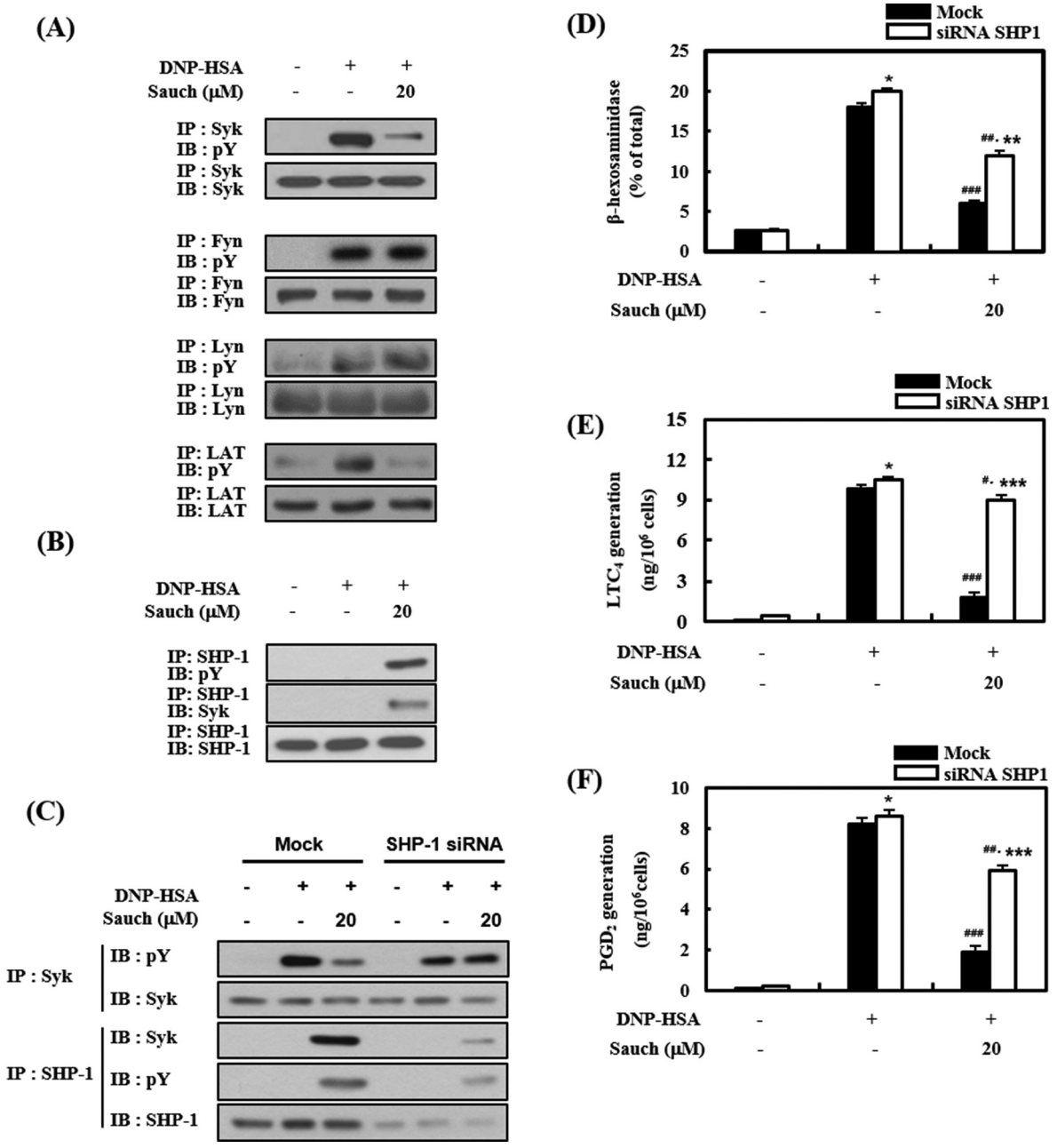

Fig. 5. Sauchinone decreases the phosphorylation of Syk and increases the phosphorylation of SHP-1 in IgE/Ag-stimulated BMMCs.
IgE-sensitized BMMCs were preincubated with sauchinone (Sauch) for 1 h, and then stimulated with DNP-HSA (A, B). BMMCs were treated with control (Mock) or SHP-1 siRNA for 48 h and then sensitized with anti-DNP IgE in the presence or absence of sauchinone (Sauch) followed by stimulation with DNP-HSA (C–F). Cell lysates were subjected to immunoprecipitation and immunoblot analysis for the phosphorylation forms of Lyn, Fyn, Syk, LAT and SHP-1 and for protein interaction between SHP-1 and Syk (A–C). The releases of β-hexosaminidase (D), LTC_4 (E) and PGD_2 (F) were evaluated. The values indicate the means ± S.E.M. from three independent experiments with different BMMCs. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* mock in each treatment; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs.* DNP-HSA alone in each group).

and the physical interaction between SHP-1 and Syk, leading to decreased Syk phosphorylation. This result shows that the SHP-1/Syk module is an additional target of sauchinone in mast cells.

An overall model for the mechanism of the inhibition of mast cell activation by sauchinone is summarized in Fig. 6. Sauchinone attenuates FcεRI-mediated phosphorylation of PLCγ1, ERK1/2, JNK, IKK, Akt, and p38 MAPK, and also attenuates Ca^{2+} influx. Consequently, sauchinone inhibits mast cell degranulation, generation of eicosanoids (LTC_4 and PGD_2), and secretion of pro-inflammatory cytokines (TNF-α and IL-6) by activating LKB1/AMPK pathway and SHP-1/Syk signaling. Taken together, these data are the first to show that sauchinone inhibits FcεRI-mediated mast cell activation by regulating both the LKB1/AMPK axis and SHP-1/Syk signaling module, demonstrating that sauchinone could be a promising therapeutic agent for the treatment of allergic inflammatory diseases.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

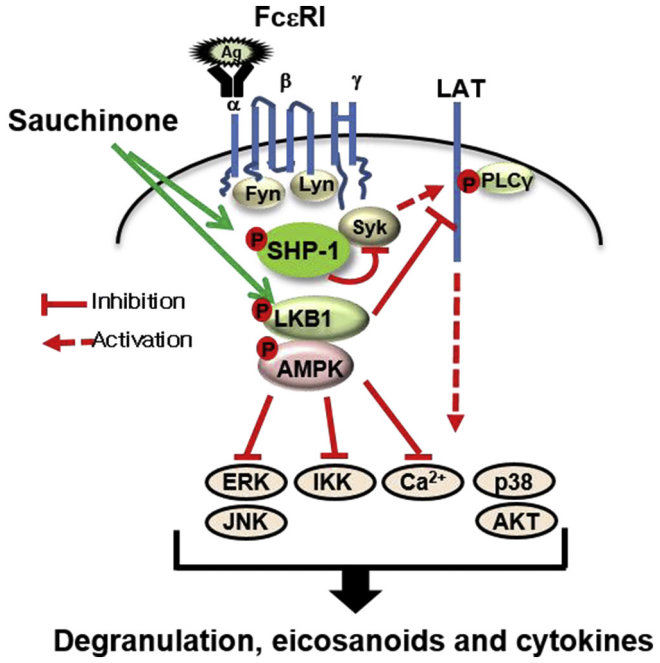

Fig. 6. Possible inhibitory mechanism of sauchinone on IgE/Ag-stimulated mast cell activation.
For details, please see text.

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