

Licarin A is a candidate compound for the treatment of immediate hypersensitivity via inhibition of rat mast cell line RBL-2H3 cells

Takuya Matsui^{a,b}, Chihiro Ito^b, Satoru Masubuchi^a and Masataka Itoigawa^c

^aDepartment of Physiology, Aichi Medical University, ^bFaculty of Pharmacy, Meijo University and ^cSchool of Sport and Health Science, Tokai Gakuen University, Aichi, Japan

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Correspondence

Takuya Matsui, Department of Physiology, Aichi Medical University, 1-1 Yazako-karimata, Nagakute, Aichi 480-1195, Japan.
E-mail: tmatsui@aichi-med-u.ac.jp

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Abstract

Objectives We previously demonstrated that some phenylpropanoids are capable of inhibiting activated mast cells. This study evaluated the anti-allergic effects of licarin A, a neolignan isolated from various plants, on antigen-stimulated rat mast cell line.

Methods The inhibitory effects of licarin A on histamine release, tumour necrosis factor- α (TNF- α) and prostaglandin D2 (PGD2) production, and cyclooxygenase-2 (COX-2) expression in dinitrophenyl-human serum albumin (DNP-HSA) rat basophilic leukemia cells (DNP-HSA-stimulated RBL-2H3 cells), were investigated by spectrofluorometry, ELISA and immunoblotting.

Key findings Licarin A significantly and dose-dependently reduced TNF- α production (IC_{50} $12.6 \pm 0.3 \mu M$) in DNP-HSA-stimulated RBL-2H3 cells. Furthermore, the levels of PGD2 secretion in DNP-HSA-stimulated cells pretreated with licarin A were lower than those stimulated with DNP-HSA alone (positive control). Treatment with licarin A at $20 \mu M$ produced slight suppression of DNP-HSA-induced increases in COX-2 mRNA and protein levels. We identified several signalling pathways that mediated these pharmacological effects. Licarin A treatment tended to reduce phosphorylated protein kinase C α/β II (PKC α/β II) and p38 mitogen-activated protein kinase (MAPK) protein levels.

Conclusions Our results demonstrate that licarin A reduces TNF- α and PGD2 secretion via the inhibition of PKC α/β II and p38 MAPK pathways; this compound may be useful for attenuating immediate hypersensitivity.

Introduction

Licarin A (PubChem CID: 5281836) is found in plants such as *Aristolochia taliscana* (Aristolochiaceae),^[1,2] *Machilus thunbergii* (Lauraceae)^[3] and *Myristica fragrans* (Myristicaceae),^[4] which are used as spices and in folk medicines for nervous and digestive disorders.^[5,6] Licarin A is a neolignan that can be synthesized by oxidative coupling of the phenylpropanoid isoeugenol, which exhibits anti-inflammatory effects.^[7] As an isoeugenol analogue, licarin A is reported to also have anti-inflammatory effects in lipopolysaccharide-stimulated RAW264.7 cells.^[8] Licarin A reportedly elicits a wide range of pharmacological effects, including antitrypanosomal activity,^[9] cytotoxicity against tumour cells,^[10–12] free-radical scavenging^[13] and

neuroprotective activity.^[14] These reports suggest that licarin A induces different effects in different cell types. Previous reports have described an additional anti-inflammatory effect of licarin A in macrophages. We aimed to determine whether licarin A induces similar pharmacological effects in other inflammatory cells and to define the signalling pathways by which licarin A induces anti-inflammation.

To date, our work has largely focused on mast cells because they act as effector cells in acute and chronic inflammation. Recently, mast cells have been found to be involved in immediate hypersensitivity as well as innate immunity, such as providing protection against parasites.^[15]

In allergic inflammation, including allergic rhinitis and asthma, the activation of mast cells is initiated by the cross-linking of immunoglobulin E (IgE)-bound high-affinity IgE receptor with a cognate antigen.^[16,17] Activated mast cells synthesize or release various chemical mediators, such as histamine, prostaglandins and inflammatory cytokines. These mediators give rise to vascular leakage, bronchoconstriction and leucocyte migration, leading to exacerbation of inflammation.^[18,19] Hence, it is understood that techniques that inhibit the production or secretion of chemical mediators represent important therapeutic strategies for the control of allergic inflammatory diseases.

We are interested in licarin A for two reasons. First, given that phenylpropanoids were found to inhibit mast cell degranulation in our previous study,^[20] we predicted that licarin A – which is synthesized from isoeugenol, itself a phenylpropanoid – may be capable of downregulating mast cell degranulation. Second, to our knowledge, there are no reports of investigations into whether licarin A might be useful for inhibiting activated mast cells. In this study, we aimed to elucidate the pharmacological effects of licarin A against antigen-stimulated mast cells. We selected and measured three well-known representative chemical mediators of inflammation – histamine, prostaglandin D2 (PGD2) and tumour necrosis factor- α (TNF- α) – and hypothesized that licarin A might be capable of attenuating mast cell activation during immediate hypersensitivity and be useful in the development of novel anti-inflammatory drugs.

Materials and Methods

Reagents

Licarin A (Figure 1) was synthesized by oxidative coupling of isoeugenol, as described previously.^[21] Minimum Eagle's medium, CellLytic M Cell Lysis Reagent, monoclonal anti-dinitrophenyl Clone SPE-7, dinitrophenyl-human serum albumin (DNP-HSA) and anti- β -actin antibody were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), and macrophage serum-free medium was purchased from Life Technologies Co. (Carlsbad, CA, USA). Mouse cyclooxygenase-2 (COX-2) antibody was purchased from

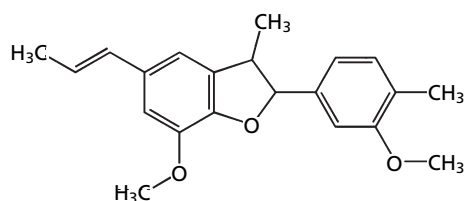


Figure 1 Chemical structure of licarin A.

Cayman Chemical Co. (Ann Arbor, MI, USA), and antibodies to p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK, p65 nuclear factor kappa-light chain enhancer of activated B cell (NF κ B), phospho-p65 NF κ B, I κ B α , phospho-I κ B α , phospho-protein kinase C (PKCs), phospho-c-Jun N-terminal kinases (JNK) and JNK from Cell Signaling Technology (Beverly, MA, USA). Western blotting detection reagents ImmunoStar LD were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture and cell viability

Rat basophilic leukaemia cells (RBL-2H3) were obtained from Tohoku University and grown in minimum Eagle's medium with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere containing 5% CO₂. To evaluate cytotoxic effects of licarin A on RBL-2H3 cells, cell viability was measured by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay (CellTiter 96 Aqueous One Solution Cell Proliferation assay, Promega, Madison, WI, USA).

Mast cell activation

RBL-2H3 cells were seeded at 5×10^4 cells/well in 96-well culture plates and incubated overnight. After removal of the medium, cells were sensitized with fresh medium containing 0.5 μ g/ml anti-DNP immunoglobulin (Ig) E (SPE-7 clone) for 24 h at 37°C. IgE-sensitized cells were washed with HEPES-Tyrodé's buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM phosphate buffer, 5.6 mM glucose, 25 mM HEPES and 0.1% bovine serum albumin (BSA), pH 7.4), or macrophage serum-free medium containing antibiotics, and pre-treated with licarin A at defined concentrations in the same buffers 1 h before antigen-stimulation. IgE-sensitized cells pre-treated with licarin A were stimulated with 0.2 μ g/ml DNP-HSA in HEPES-Tyrodé's buffer or macrophage serum-free medium at 37°C.

Histamine release

IgE-sensitized RBL-2H3 cells treated with licarin A were stimulated with DNP-HSA dissolved in HEPES-Tyrodé's buffer for 30 min. Conditioned medium was collected, and cells washed with HEPES-Tyrodé's buffer before 1% perchloric acid solution was added. Cells were centrifuged at $3000 \times g$ for 10 min at 4°C and the supernatant collected and stored. Histamine content was determined by HPLC coupled with post-column derivatization fluorometry, as described.^[20] The percentage of histamine released was calculated:

$$\begin{aligned} & \% \text{ Histamine release} \\ &= \frac{[\text{cultured medium}]}{[\text{cultured medium} + \text{cell(supernatant)}]} \times 100 \quad (1) \end{aligned}$$

Prostaglandin D₂ and tumour necrosis factor- α release assay

IgE-sensitized RBL-2H3 cells treated with licarin A were stimulated with DNP-HSA dissolved in macrophage serum-free medium, for 6 h (PGD₂) or 12 h (TNF- α). PGD₂ and TNF- α levels in conditioned medium or cells were determined using PGD₂ (Prostaglandin D₂ EIA kit and Prostaglandin D₂-MOX EIA kit, Cayman Chemical Co.) and Rat TNF- α ELISA kits (Quantikine Rat TNF- α , R&D Systems, Minneapolis, MN, USA), according to manufacturers' protocols.

Cyclooxygenase-2 protein expression

IgE-sensitized RBL-2H3 cells treated with licarin A were incubated with DNP-HSA dissolved in macrophage serum-free medium for 6 h. Cells were washed twice with phosphate-buffered saline (–) and lysed in CelLytic M Cell Lysis Reagent, according to manufacturer's protocol. Cell lysates were centrifuged at 10000 $\times g$ for 15 min at 4°C and supernatants collected. After quantification, supernatants (10 μ g) were boiled together with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Samples underwent SDS-PAGE in reducing conditions and were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Co., Billerica, MA, USA). Membranes were incubated with 5% skim milk for 1 h at room temperature, incubated with anti-COX-2 antibody at 4°C overnight, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies (Cell Signaling Technology) for 1 h at room temperature. Blots were probed with ImmunoStar LD, according to manufacturer's instructions. Band densities were measured using Image J and corrected to the relative density of non-stimulated cells.

Quantitation of tumour necrosis factor- α and cyclooxygenase-2 mRNAs

IgE-sensitized RBL-2H3 cells treated with licarin A were stimulated with DNP-HSA dissolved in macrophage serum-free medium. TNF- α and COX-2 mRNA levels were determined at 1, 3 and 5 h using a reported method.^[22]

Measurement of calcium kinetics

Laser scanning confocal microscopy (LSM 710, Carl Zeiss Microscopy Co., Oberkochen, Germany) was used to

measure Ca²⁺ kinetics in RBL-2H3 cells loaded with Ca²⁺-reactive fluorescent probe (Fluo-4). RBL-2H3 cells in a confocal imaging dish (μ Clear black plate; Greiner Bio One Co., Hannover, Germany) were sensitized with 0.5 μ g/ml anti-DNP IgE for 24 h. After washing twice with HEPES-Tyrod's buffer, cells were incubated with Fluo-4 solution (Fluo-4 NW Calcium assay kit, Life Technologies Co.) in HEPES-Tyrod's buffer for 45 min at 37°C. Cells were washed twice and incubated with 20 μ M licarin A solution for 30 min. The fluorescence of the solution was monitored before and after DNP-HSA stimulation (0.2 μ g/ml). Fluo-4-loaded RBL-2H3 cells were excited at 494 nm, and fluorescence was observed at 516 nm. Images were collected for 5 min, and changes in Ca²⁺ levels were analysed using Zeiss Efficient Navigation 2011 (ZEN 2011).

Signalling molecule expression

Licarin A (20 μ M) pre-treated RBL-2H3 cells sensitized with IgE were stimulated with DNP-HSA for 0, 5, 15 and 30 min. Cell lysates (10 μ g) were electrophoresed on 5–20% SDS-PAGE gels and electrotransferred to polyvinylidene difluoride membrane. Membranes were incubated with 5% skim milk or BSA for 1 h at room temperature, followed by antibodies against phosphorylated or non-phosphorylated forms of signalling molecules (p65 NF κ B, p38 MAPK, PKC α / β II, JNK) separately overnight at 4°C. Bands were detected by incubation with HRP-conjugated anti-mouse IgG or anti-rabbit IgG, and bands were visualized using ImmunoStar LD. Band densities were measured using Image J and corrected to the relative density of non-stimulated cells.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Tukey's test to compare the responses of RBL-2H3 cells pre-treated with licarin A before DNP-HSA stimulation with cells under DNP-HSA stimulation alone (positive control) using Statistical Package for the Social Sciences (version 16.00; SPSS Inc., Chicago, IL, USA). Results were expressed as means $\pm$ SD. *P*-values < 0.05 were considered statistically significant.

Results

Histamine release

Histamine is one of several preformed mediators present in mast cell granules and released immediately after antigen stimulation; thus, we investigated whether licarin A treatment affected histamine release from DNP-HSA-stimulated RBL-2H3 cells. Cells treated with licarin A at a concentration of 20 μ M showed a significant reduction in histamine release (28.2%) compared with DNP-HSA-only stimulated

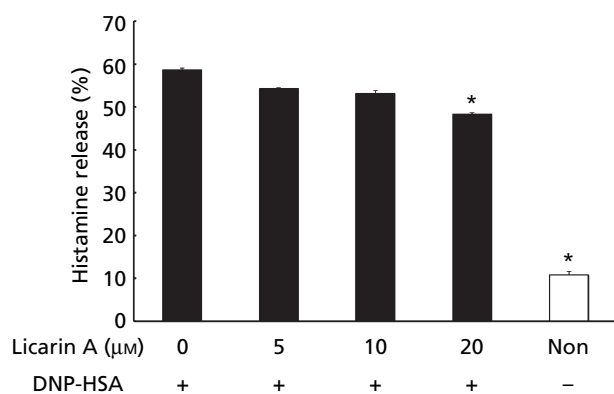


Figure 2 The inhibitory effect of licarin A on histamine release from dinitrophenyl-human serum albumin (DNP-HSA)-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were pretreated with licarin A 1 h before antigen stimulation. Cells were stimulated with DNP-HSA and cultured for 30 min. Licarin A dose-dependently decreased histamine release from DNP-HSA-stimulated RBL-2H3 cells. Values represent means $\pm$ SD of at least three experiments. * $P < 0.05$ vs DNP-HSA stimulation alone.

Table 1 Licarin A showed no cytotoxicity towards RBL-2H3 cells, licarin A (5–20 μM) did not affect cell viability at 1 or 12 h after DNP-HSA stimulation, as measured by MTT assay

Licarin A did not have cytotoxicity on RBL-2H3 cells		
Licarin A (μM)	1 h (%)	12 h (%)
5	99 $\pm$ 4.0	100 $\pm$ 3.5
10	97 $\pm$ 1.9	100 $\pm$ 1.1
20	96 $\pm$ 2.4	100 $\pm$ 2.5
Licarin A (μM) + DNP-HSA	1 h (%)	12 h (%)
0	103 $\pm$ 4.0	100 $\pm$ 4.4
5	100 $\pm$ 3.8	99 $\pm$ 7.1
10	100 $\pm$ 3.3	97 $\pm$ 6.0
20	100 $\pm$ 1.5	90 $\pm$ 5.8
Non-treated	100 $\pm$ 0.4	100 $\pm$ 2.8

DNP-HSA, dinitrophenyl-human serum albumin. Cells stimulated with licarin A alone also showed no cytotoxicity.

cells (positive control) (Figure 2). However, at lower concentrations of licarin A (5 μM), histamine release was not reduced significantly. No concentrations of licarin A tested (5–20 μM; Table 1) had an effect on cell viability. Unlike other tumour cells, RBL-2H3 cells appear to have low sensitivity to the cytotoxic effect of licarin A at the tested concentrations. These results suggest that licarin A has slight inhibitory activity against histamine release in DNP-HSA-stimulated RBL-2H3 cells.

Licarin A inhibited tumour necrosis factor- α and prostaglandin D2 release

We next sought to determine whether licarin A might also block the release of PGD2 and TNF- α from DNP-HSA-

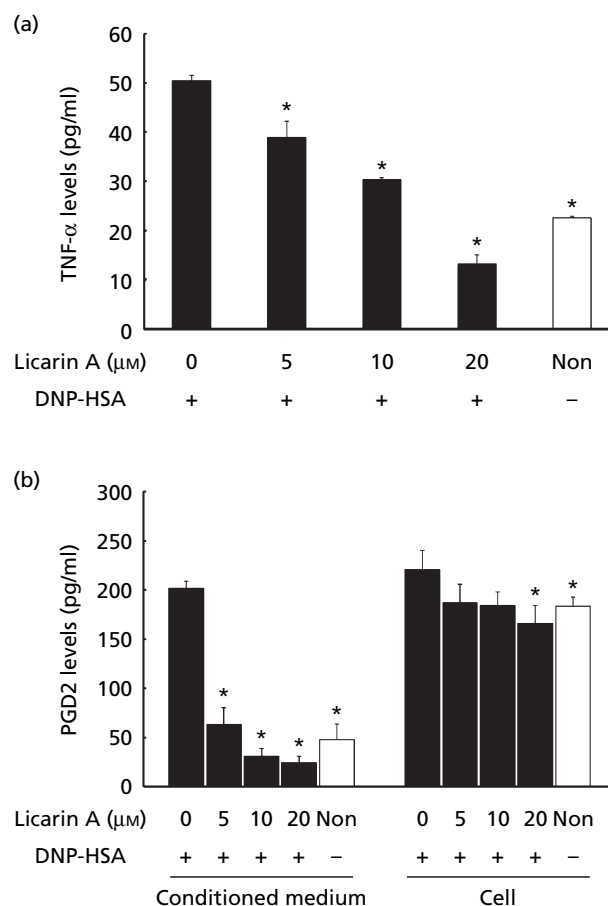


Figure 3 Effects of licarin A on tumour necrosis factor- α and prostaglandin D2 protein levels. (a) Licarin A dose-dependently decreased tumor necrosis factor- α (TNF- α) in conditioned medium from dinitrophenyl-human serum albumin (DNP-HSA)-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were pretreated with licarin A at each concentration. Cells were stimulated with DNP-HSA and cultured for 12 h. (b) Licarin A at 5 and 20 μM significantly reduced prostaglandin D2 (PGD2) levels in conditioned medium. Licarin A-treated cells were stimulated with DNP-HSA and cultured for 6 h. Values represent means $\pm$ SD of at least three experiments. * $P < 0.05$ vs DNP-HSA stimulation alone.

stimulated RBL-2H3 cells for the following reasons: (1) PGD2 is synthesized as a COX-2 metabolite in activated mast cells and released in the subacute phase of immediate hypersensitivity; and (2) TNF- α accelerates leucocyte migration to inflammatory lesions. TNF- α is both stored in granules and synthesized *de novo* after antigen stimulation. TNF- α is closely involved in triggering late-phase reactions. We found that the protein levels of TNF- α in conditioned medium were reduced dose-dependently by licarin A, with a 50% inhibitory concentration (IC₅₀) of 12.6 ± 0.3 μM (Figure 3a). Next, we measured PGD2, which is induced in the subacute phase of immediate allergy, in DNP-HSA-

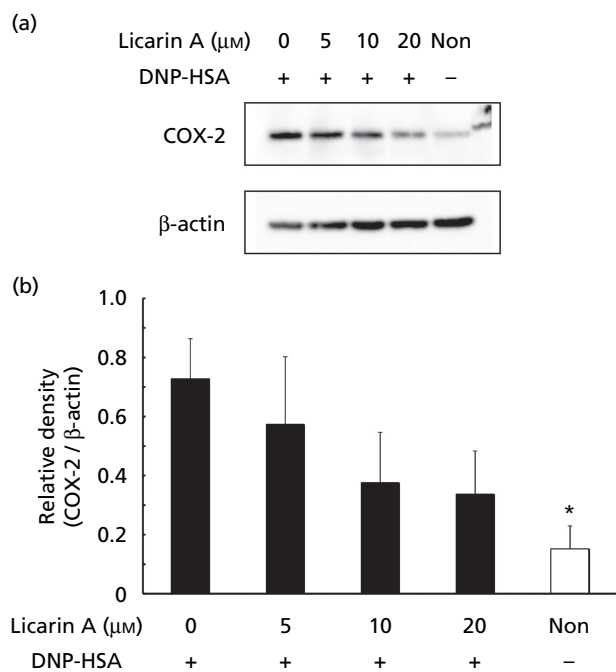


Figure 4 Licarin A downregulated cyclooxygenase-2 expression. Licarin A-treated cells were stimulated with dinitrophenyl-human serum albumin (DNP-HSA) and cultured for 6 h. (a) Cyclooxygenase-2 (COX-2) protein expression of treated cell lysates were determined by immunoblotting. (b) COX-2 protein levels were revealed as relative density of the bands. Values represent means $\pm$ SD of at least three experiments.

stimulated RBL-2H3 cells treated with or without licarin A, and in the conditioned medium. The levels of PGD2 in conditioned medium from cells treated with licarin A at all defined concentrations were markedly decreased and the reduction rate of 20 μ M licarin A-treated cells was 84.7% (vs positive control 201.7 $\pm$ 7.0 pg/ml) (Figure 3b). In contrast, intracellular PGD2 levels in the same cells were reduced subtly compared with positive controls.

Cyclooxygenase-2 protein expression

Since licarin A inhibited PGD2 secretion from DNP-HSA-stimulated RBL-2H3 cells, we sought to investigate whether altered COX-2 protein expression contributed to the underlying decrease in PGD2 production. Despite the dramatic decrease in PGD2, licarin A did not significantly suppress COX-2 protein expression, which was only slightly decreased (Figure 4).

Licarin A effect on tumour necrosis factor- α and cyclooxygenase-2 mRNA expression

Because licarin A greatly reduced TNF- α and PGD2 levels in the conditioned medium, and because this might be con-

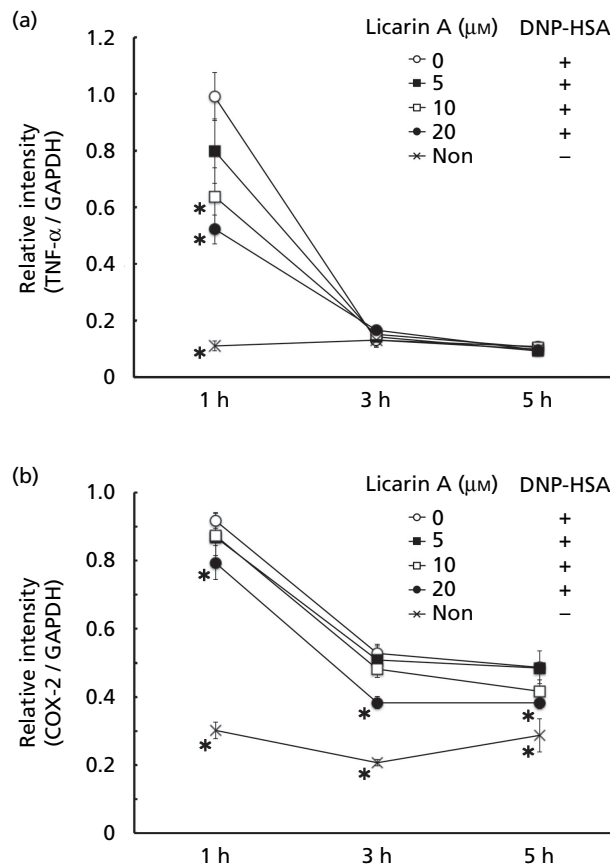


Figure 5 Effects of licarin A on the expression of tumour necrosis factor- α and cyclooxygenase-2 mRNA. IgE-sensitized RBL-2H3 cells were pretreated with licarin A at defined concentrations. Cells were stimulated with dinitrophenyl-human serum albumin (DNP-HSA) and cultured for 1, 3 and 5 h. (a) Tumour necrosis factor- α (TNF- α) and (b) Cyclooxygenase-2 (COX-2). Open circles: DNP-HSA stimulation alone (positive control). Closed squares, open squares, closed circles: licarin A at 5, 10 and 20 μ M, respectively. Crosses: untreated cells (negative control). Values represent means $\pm$ SD of at least three experiments. * P < 0.05 vs DNP-HSA stimulation alone.

nected to COX-2 expression, we next evaluated the temporal changes in TNF- α and COX-2 mRNA expression in DNP-HSA-stimulated RBL-2H3 cells treated with or without licarin A at concentrations of 5, 10 and 20 μ M. At 1 h after DNP-HSA stimulation, RBL-2H3 cells showed high levels of TNF- α mRNA expression. However, levels at 3 and 5 h after stimulation were the same as in the negative control. We found that licarin A significantly and dose-dependently decreased the mRNA levels of TNF- α at 1 h. TNF- α mRNA levels were reduced by 35.6% and 47.3% in cells treated with licarin A at 10 and 20 μ M, respectively (Figure 5a). Licarin A therefore appears to affect the production of pro-inflammatory cytokines.

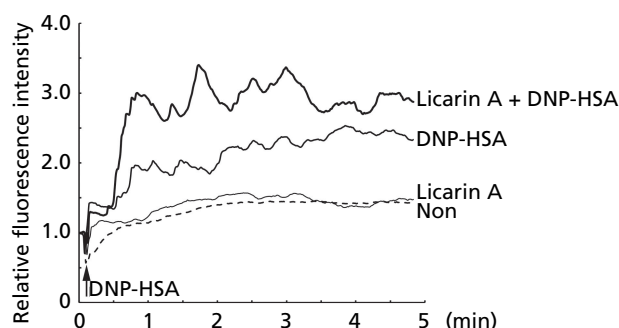


Figure 6 Effect of licarin A on intracellular Ca^{2+} kinetics. IgE-sensitized RBL-2H3 cells were pretreated with licarin A at a concentration of $20\text{ }\mu\text{M}$. Cells were stimulated with dinitrophenyl-human serum albumin (DNP-HSA) and intracellular Ca^{2+} levels measured immediately after stimulation (solid line). The intracellular Ca^{2+} alterations in untreated cells (dotted line), cells treated with licarin A alone (thin solid line), and cells treated with licarin A + DNP-HSA (bold solid line) were monitored for 5 min after antigen stimulation by confocal microscopy.

The expression of COX-2 mRNA was also upregulated 1 h after DNP-HSA stimulation, similar to the expression of TNF- α mRNA in the positive control. Unlike TNF- α mRNA, levels at 3 and 5 h after stimulation were higher than in the negative control. At $20\text{ }\mu\text{M}$, licarin A moderately inhibited COX-2 mRNA levels. Indeed, we found reductions in COX-2 mRNA levels of 13.4%, 27.6% and 21.3% at all times in cells treated with licarin A at $20\text{ }\mu\text{M}$ (Figure 5b). Thus, these findings raise the possibility that the inhibitory effects of licarin A on COX-2 expression partially underpin the reduction of PGD2 levels.

Kinetics of intracellular Ca^{2+}

The above results provide evidence that licarin A inhibited histamine, TNF- α and PGD2 production, or release from DNP-HSA-stimulated RBL-2H3 cells. We next focused on the mechanisms by which licarin A might inhibit the release of chemical mediators from activated mast cells. We investigated whether licarin A affects the kinetics of intracellular Ca^{2+} levels, which are closely involved with histamine release. Immediately after DNP-HSA stimulation, intracellular Ca^{2+} levels rose sharply and reached a plateau both in licarin A pre-treated cells and positive control cells. However, the steady-state Ca^{2+} levels in licarin A-treated cells were elevated compared with the positive control cells (Figure 6). In Ca^{2+} ionophore (A23187)-stimulated cells, licarin A also increased steady-state intracellular Ca^{2+} levels (data not shown). Thus, these results revealed that licarin A had no effects on the rise time of intracellular Ca^{2+} levels mediated by antigen and A23187 stimulation.

Signalling molecules

The discrepancy between the reduction in chemical mediator release and the kinetics of intracellular Ca^{2+} levels in licarin A-treated cells led us to explore the downstream signalling molecules activated by intracellular Ca^{2+} . The expression of phosphorylated PKC α/β II, a Ca^{2+} -dependent protein kinase C isomer that induces exocytosis in antigen-stimulated RBL-2H3 cells, was measured by immunoblotting lysates of cells treated with $20\text{-}\mu\text{M}$ licarin A. The blots showed that licarin A tended to decrease the expression of phosphorylated PKC α/β II compared with the positive control at 30 min (Figure 7a and 7b).

Our previous report indicated that RBL-2H3 cells treated with SB202190 or dexamethasone, which act as p38 MAPK and p65 NF κ B inhibitors, respectively, secreted lower levels of TNF- α into the conditioned medium and showed decreased COX-2 expression,^[22] suggesting that p38 MAPK and p65 NF κ B play important roles in the production of pro-inflammatory molecules. We therefore examined whether licarin A regulated the expression of p38 MAPK, JNK or p65 NF κ B proteins. For p38 MAPK, the relative band densities for licarin A-treated cells tended to be lower than those of the positive control at all times (Figure 7a and 7c). However, licarin A did not alter the levels of phosphorylated JNK isoforms at 45 or 56 kDa. Licarin A also decreased the expression of phosphorylated p65 NF κ B. Thus, licarin A showed inhibitory effects on key signalling molecules in response to the increase in intracellular Ca^{2+} levels.

Discussion

Licarin A belongs to the phenylpropanoid family and has various pharmacological effects, including anti-trypanocidal and anti-cancer activity.^[11,23] It attenuates glutamate-induced neurotoxicity in rat cortical cells^[14] and inhibits COX-2 and iNOS expression in lipopolysaccharide-stimulated RAW264.7 cells, suggesting anti-inflammatory activity.^[8,24,25] Thus, it has multiple pharmacological functions and induces different effects in different cells. We sought to examine the pharmacological effects of licarin A in allergic inflammation. To our knowledge, the regulation and mechanisms of the production or release of chemical mediators in antigen-stimulated mast cells by licarin A have not been determined.

Licarin A at $20\text{ }\mu\text{M}$ slightly inhibited histamine release from DNP-HSA-stimulated RBL-2H3 cells and significantly and dose-dependently reduced the mRNA levels of pro-inflammatory cytokine TNF- α . Licarin A also downregulated TNF- α protein levels in conditioned medium from DNP-HSA-stimulated RBL-2H3 cells. These results suggested that licarin A inhibits TNF- α production

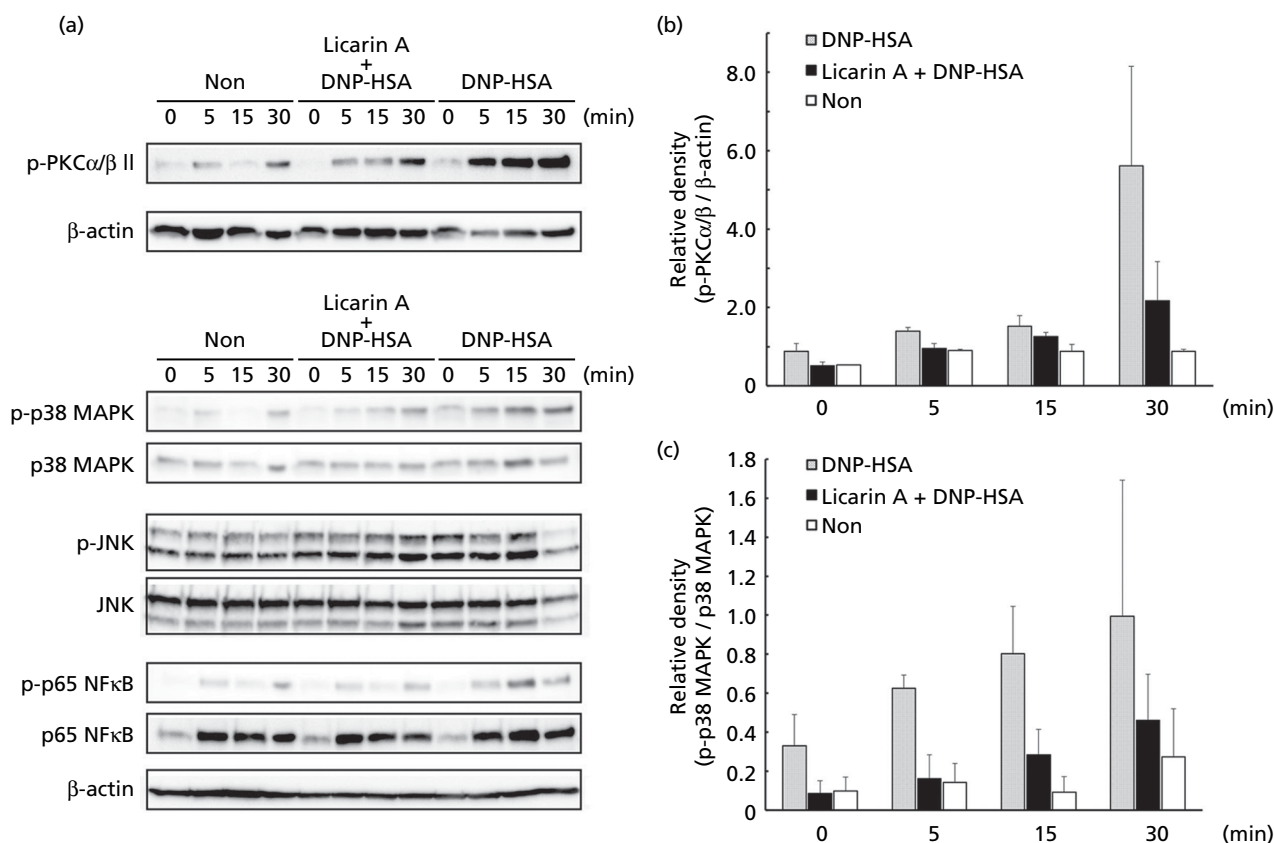


Figure 7 Effects of licarin A on the expression of signalling molecules. (a) RBL-2H3 cells were pretreated with licarin A at 20 μ M. Cells were stimulated with dinitrophenyl-human serum albumin (DNP-HSA) and cultured for 0, 5, 15 and 30 min. Cells were then subjected to immunoblotting using antibodies against phosphorylated PKC α/β II, and phosphorylated (p-) and non-phosphorylated p38 MAPK, JNK and p65 NF κ B. (b, c) The relative densities of phosphorylated PKC α/β II and p38 MAPK were corrected using β -actin and non-phosphorylated forms. Values represent means $\pm$ SD of at least three experiments.

or secretion. Licarin A also blocked PGD2 synthesis and secretion in the subacute phase of immediate hypersensitivity. PGD2 levels significantly decreased in conditioned medium, demonstrating the inhibitory effect of licarin A on its secretion. Meanwhile, intracellular PGD2 levels in licarin A-treated cells reduced to a lesser degree. We investigated whether the reduction of PGD2 by licarin A was mediated through COX-2. Licarin A at 20 μ M decreased the expression of COX-2 mRNA, and maintained it for 5 h, although the reduction in COX-2 protein expression was slight. This downregulation of COX-2 by licarin A corresponds with a report by Coy *et al.*^[26] Thus, we have shown that licarin A, at least partly, inhibited PGD2 through the suppression of COX-2 production. These results suggest that licarin A is efficacious in reducing the activation of RBL-2H3 cells in response to DNP-HSA stimulation. Our observations support previous reports showing the anti-inflammatory effects of licarin A on lipopolysaccharide-stimulated RAW264.7 cells.^[8,24,25]

Next, we focused on the signalling molecules that regulate the production and secretion of chemical mediators. These experiments measured licarin A-induced changes in selected representative signalling molecules in DNP-HSA-stimulated RBL-2H3 cells. Histamine release from antigen-stimulated mast cells occurs after rapid upregulation of intracellular Ca^{2+} levels; thus, we examined whether licarin A treatment altered intracellular Ca^{2+} levels. The kinetics of intracellular Ca^{2+} levels in licarin A-treated cells were the same as positive controls. Ma *et al.* reported that licarin A suppressed intracellular Ca^{2+} in glutamate-injured rat cortical cells.^[14] This may result from fundamental differences between the mast cells (immune cells) and cortical cells (neuronal cells). While licarin A stimulation alone did not alter intracellular Ca^{2+} levels compared with non-treated mast cells in plateau regions (about 1 min onward), the upregulation of intracellular Ca^{2+} by licarin A in activated mast cells is a novel finding. Although we do not have clear-cut evidence, our findings

potentially show that licarin A has priming effects on Ca^{2+} transporters.

The question of why licarin A subtly inhibited histamine release from DNP-HSA-stimulated RBL-2H3 cells despite elevated intracellular Ca^{2+} levels remains. To clarify the mechanism, protein expression levels of selected signalling molecules were analysed by immunoblotting. Licarin A modestly inhibited phosphorylated PKC α/β II, an isoform of Ca^{2+} -dependent protein kinase C. Previous reports showed that phosphorylation of PKC β induces morphological changes in antigen-stimulated RBL-2H3 cells, leading to degranulation.^[27,28] Although we do not have direct evidence of a relationship between increased intracellular Ca^{2+} levels and the unchanged histamine release in DNP-HSA-stimulated RBL-2H3 cells treated with licarin A, this compound's activity may be due, at least partly, to the regulation of some downstream pathways involving phosphorylated PKC α/β II after the change in intracellular Ca^{2+} .

Reports by Murakami *et al.* have shown that licarin A depresses the phosphorylation of I κ B α and transcriptional activity of NF κ B, and inhibits activator protein-1 binding to the 12-tetradecanoylporbol-13-acetate response element sequence in lipopolysaccharide-stimulated RAW 264.7 cells.^[8,25] Similarly, our studies revealed that licarin A downregulates the expression of phosphorylated p65 NF κ B in DNP-HSA-stimulated RBL-2H3 cells. In antigen-stimulated mast cells, activation of PKC-IKK-I κ B-NF κ B signal transduction induces TNF- α production.^[29] Our previous study demonstrating the inhibitory effect of dexamethasone, an NF κ B inhibitor, on Ca^{2+} ionophore-stimulated RBL-2H3 cells is in agreement.^[22] Thus, licarin A might, at least partly, suppress TNF- α production and secretion via downregulation of PKC-IKK-I κ B-NF κ B signal transduction.

We reported that certain natural compounds suppress the production/release of chemical mediators via inhibition of phosphorylated p38 MAPK.^[22,30] Licarin A modestly depressed the phosphorylation of p38 MAPK. Previously, we found that TNF- α levels in conditioned medium from DNP-HSA-stimulated RBL-2H3 cells treated with 10 μM SB202190, a commercial p38 MAPK inhibitor, were reduced by 12 ± 2.1 %. COX-2 protein expression was also reduced substantially. These results agree with a report by Gonzalez-Espinosa *et al.* using bone marrow-derived mast cells.^[31] Furthermore, Kim *et al.* reported that the inhibition of p38 MAPK gave rise to reduced histamine release from DNP-HSA-stimulated RBL-2H3 cells.^[32] Unfortunately, the p38 MAPK inhibitor we employed only marginally reduced histamine release from DNP-HSA-stimulated RBL-2H3 cells. Whether this difference in the magnitude of inhibition of histamine release is due to the use of a different inhibitor is unclear, but p38 MAPK plays an important role in the pro-

duction and secretion of chemical mediators. Based on these results along with our previous reports, licarin A appears to reduce the production and secretion of chemical mediators via its actions on the p38 MAPK pathway.

In mast cells, a variety of stimuli, such as *c-kit* and substance P, induce physiological reactions via JNK, including proliferation, TNF- α production and arachidonic acid release.^[33–35] JNK might be controlled by at least three signalling pathways.^[36,37] JNK, similarly to p38 MAPK, plays an important role in activated mast cells. Further, certain compounds derived from natural products^[38,39] or extracts^[40,41] have inhibitory effects on the phosphorylation of JNK, leading to the suppression of chemical mediator production. However, licarin A has no apparent effect on the phosphorylation of JNK.

In antigen-stimulated mast cells, signal transduction pathways underpinning degranulation and pro-inflammatory molecule production are extremely complex.^[42,43] In this study, licarin A attenuated the levels of each signalling molecule, with the exception of intracellular Ca^{2+} . Whether the activation of the p38 MAPK pathway after antigen stimulation depends on PKC α/β II or not remains controversial. We postulated two possible mechanisms by which licarin A may inhibit chemical mediator production and secretion from antigen-stimulated RBL-2H3 cells. First, licarin A may act on individual signal molecules, which downregulate chemical mediators. Second, there may be unidentified master regulator molecules upstream of the signalling pathways investigated, and licarin A may modulate these. We hope to distinguish between the potential pharmacological effects of licarin A in future studies. Additionally, licarin A appears to have wide inhibitory effects against activated mast cells from the subacute to late phases of immediate hypersensitivity; thus, it might be a candidate compound for the production of anti-allergy drugs.

Conclusions

This study is the first report to show that licarin A possesses novel pharmacological actions, including the inhibition of histamine release, and PGD2 and TNF- α secretion/production in antigen-stimulated RBL-2H3 cells. These pharmacological actions might be attributable to the suppression of some signalling pathways involved in PKC α/β II and p38 MAPK. Elucidation of the precise pharmacological mechanisms remains problematic; however, it appears that licarin A might be useful in the treatment of allergy. It has been shown that the bioactivity of natural products in *in vitro* assays is not necessarily recapitulated *in vivo*; therefore, further investigations into the *in vivo* effects of licarin A, in addition to delineating its mechanisms of action, are warranted.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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