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Research Paper

Effect of natsudaidain isolated from *Citrus* plants on TNF-α and cyclooxygenase-2 expression in RBL-2H3 cells

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Abstract

Objectives Flavonoids inhibit the activity of chemical mediators released from mast cells. Our aim was to investigate the effects of natsudaidain, a polymethoxyflavone isolated from *Citrus* plants, on mast cells.

Methods We investigated the inhibitory effects of natsudaidain, which is a polymethoxyflavone isolated from *Citrus* plants, on histamine release, tumour necrosis factor-α production and cyclooxygenase-2 expression in Ca ionophore-stimulated rat basophilic leukemia cells (A23187-stimulated RBL-2H3 cells) by spectrofluorometric, ELISA and immunoblotting methods.

Key findings The percent of histamine release from A23187-stimulated RBL-2H3 cells pretreated with natsudaidain at 5, 25 and 50 μM was not changed as compared with non-treated A23187-stimulated cells. At 100 and 200 μM, natsudaidain pretreatment resulted in slightly reduced histamine release (% histamine release, 89.8 ± 3.5% and 71.5 ± 5.6% at 100 and 200 μM). Thus, natsudaidain hardly affects histamine release from RBL-2H3 cells, except at high concentrations. On the other hand, natsudaidain dose-dependently inhibited tumour necrosis factor-α protein and mRNA levels in A23187-stimulated RBL-2H3 cells; a concentration of 6.8 μM was required for a 50% reduction. In addition, all concentrations of this compound that we tested also inhibited cyclooxygenase-2 protein expression. The mRNA levels of cyclooxygenase-2 in A23187-stimulated RBL-2H3 cells treated with natsudaidain were also markedly decreased. The phosphorylated-p38 MAPK protein levels in A23187-stimulated RBL-2H3 cells treated with natsudaidain were lower than in the non-treated cells.

Conclusions These findings suggest that natsudaidain inhibits tumour necrosis factor-α and cyclooxygenase-2 production by suppressing p38 MAPK phosphorylation but not p65 NFκB phosphorylation, and that natsudaidain might alleviate inflammatory diseases.

Keywords cyclooxygenase-2; mast cells; natsudaidain; tumour necrosis factor-α

Introduction

Mast cells are important effector cells in allergic inflammation, innate immunity and chronic inflammation, in which they secrete diverse chemical mediators, such as histamine and prostaglandins (PGs), and a range of multifunctional cytokines, including interleukin (IL)-6, IL-8, IL-13 and tumour necrosis factor-α (TNF-α), as well as numerous chemokines.[1,2] These chemical mediators induce inflammatory reactions, such as immune cell recruitment, airway contraction and the augmentation of venular permeability.

To date, the effects of natural products on chemical mediator release from mast cells have been examined by using rat peritoneal mast cells, mouse bone marrow-derived mast cells and rat basophilic leukaemia cells (RBL-2H3) treated with stimulators such as compound 48/80 and Ca ionophore A23187.[3–5] RBL-2H3 cells are rat mucosal type mast cells, and the reaction of these cells against stimulators, such as IgE antibody and A23187, is similar to that of normal mast cells.[6,7] We have also previously reported that phenylpropanoids and phytoquinoids isolated from *Illicium* plants inhibited chemical mediators from RBL-2H3 cells.[8] Flavonoids, which are contained in various plants and natural foods, especially flavonols such as quercetin, kaempferol and fisetin, strongly inhibit histamine release from mast cells.[9–11] In addition, these flavonols inhibit IL-6 or

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TNF-α release from mouse bone marrow-derived cultured mast cells and rat peritoneal mast cells.[12,13]

Previous reports have shown that *Citrus* plants possess anti-inflammatory, anticancer and antiallergic effects.[14–16] We also reported that coumarins from *Citrus* plants exhibit inhibitory effects on tumour promotion in mouse skin.[17,18]

Natsudaidain and nobiletin are polymethoxyflavones isolated from *Citrus* plants. Although few reports have examined the effect of nobiletin on mast cells, nobiletin inhibits protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are induced in the inflammatory state, in human fibroblasts and RAW264.7 cells, suggesting that nobiletin mediates anti-inflammatory effects.[19,20] Since the structure of natsudaidain closely resembles that of nobiletin, natsudaidain is assumed to have anti-inflammatory effects similar to those of nobiletin. However, the effect of natsudaidain on chemical mediators in mast cells is less clear.

In this study, we investigated the effects of natsudaidain on histamine and TNF-α release from rat basophilic leukaemia cells (RBL-2H3) stimulated with a Ca ionophore (A23187). In addition, we evaluated the effects of this compound on COX-2 production in A23187-stimualted RBL-2H3 cells.

Materials and Methods

Plant material and natsudaidain

Fresh leaves of *C. madurensis* Loulreiro (MUY0032) were collected from the National Institute of Fruit Tree Science, Okitsu, Shizuoka. A voucher specimen was deposited in the Faculty of Pharmacy of Meijo University. Natsudaidain was isolated from *C. madurensis* Loulreiro.

The 85% ethanolic extracts were subjected to preparative TLC (benzene–acetone (10 : 1), hexane–acetone (4 : 1)) to obtain 3-hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone as a pale yellow oil. The purity was corroborated as being ≥ 99% by measurements of melting point, IR, UV, MS, high-resolution MS and ^{1}H NMR spectra.[21] The structure of natsudaidain is shown in Figure 1.

The sample was dissolved in dimethyl sulfoxide (DMSO) and was added to culture medium to give a final DMSO concentration of 0.1% v/v; DMSO at this concentration has no significant effect on the growth of the cell line tested (data not shown).

Materials

Minimum Eagle's medium (MEM), CelLytic M-cell lysis reagent and anti-β actin antibody were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Mouse COX-2 antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Antibodies to p38, phosphorylated-p38 MAPK and p65, and phosphorylated-p65 NFκB were purchased from Cell Signaling Technology (Beverly, MA, USA). Western blotting detection reagents (ECL plus) were purchased from Amersham Biosciences (Little chalfont, UK).

Cell culture and cell viability

RBL-2H3 cells obtained from Tohoku University were grown in MEM supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin at 100 U/ml and streptomycin at 100 μg/ml under 5% CO_2 at 37°C. Cell viability was measured by MTT assay.

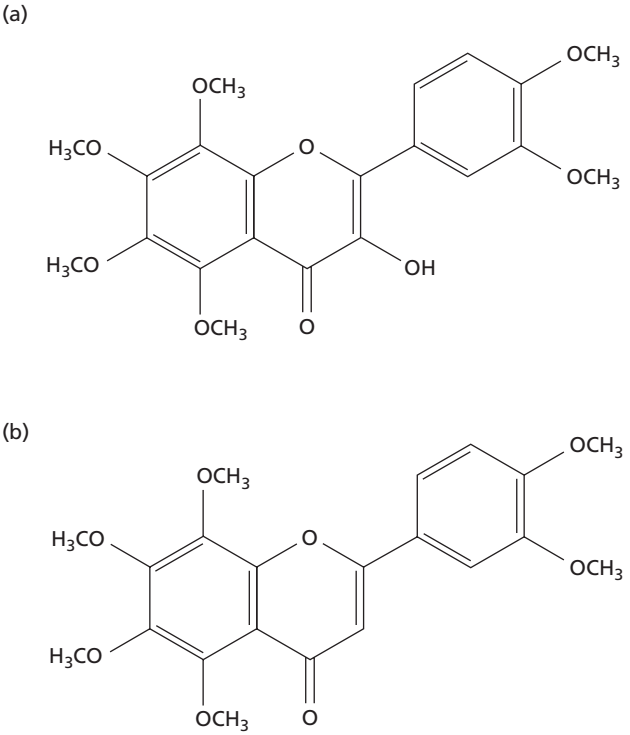

Figure 1 Structures of polymethoxyflavones isolated from *Citrus* plants: (a) natsudaidain; (b) nobiletin.

Cell stimulation

RBL-2H3 cells (1×10^6 cells/well) were preincubated with natsudaidain at defined concentrations. Pretreated cells were stimulated with A23187 (2 μM) and were then cultured at 37°C for 3 h (for mRNA measurement) and 8 h (for protein measurement). For signalling analysis, cells were cultured with natsudaidain at 25 μM at 37°C for 30 min. Cells were then stimulated with A23187 (2 μM) at 37°C for 5, 15, 30 and 60 min.

Histamine release assay

RBL-2H3 cells (1×10^5 cells/well) were cultured for 30 min in MEM containing specific concentrations of the test compounds. Cells were then stimulated with 2 μM A23187 for 30 min. Histamine contents were determined by HPLC coupled with post-column derivatisation fluorometry, as described previously.[8]

TNF-α release assay

TNF-α levels in culture supernatants were determined by using a rat TNF-α ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Immunoblotting analysis

The expression of COX-2, NFκB and MAPK proteins was determined by immunoblotting analysis. Briefly, after

stimulation, the cells were lysed in CelLytic M-Cell Lysis reagent (Sigma-Aldrich Co.) according to the manufacturer's protocol and mixed with SDS–PAGE sample buffer, then the samples were boiled for 10 min. Samples were subjected to SDS–PAGE in a 5–20% gradient gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Co., MA, USA). The PVDF membrane was incubated with 5% skimmed milk for 1 h at room temperature. Then, the PVDF membrane was incubated with anti-COX-2, and phosphorylated-p65, p65 NFκB, phosphorylated-p38 and p38 MAPK antibodies overnight at 4°C and then with HRP-conjugated species-specific anti-mouse and anti-rat IgG antibodies (Cell Signaling Technology) for 1 h at room temperature. The blots were probed with the ECL plus Western blot detection system according to the manufacturer's instructions.

Quantitation of TNF-α and COX-2 mRNAs

Total RNA was isolated from RBL-2H3 cells by using an RNeasy mini kit (Qiagen K.K., Tokyo, Japan) and was then reverse-transcribed to prepare cDNA using a High-capacity cDNA Archives kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed with 20 ng of cDNA with SYBR Premix EX taq (Takara Co., Otsu, Japan) by using an ABI-9500 (Applied Biosystems). PCR conditions were 10 s at 95°C and then 40 cycles of 95°C for 5 s and 60°C for 34 s, followed by a standard melting curve analysis. The number of copies in each real-time PCR reaction was normalised against a housekeeping gene (GAPDH).

The TNF-α and COX-2 mRNA coding sequences derived from Gen-Bank were used in the Primer 3 program (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) to design primers for the real-time PCR assays. The following primers were used: TNF-α, forward primer 5′-GCATGATCC-GAGATGTGGAA-3′ and reverse primer 5′-ACGAG-CGGGAATGAGAAGAG-3′; COX-2, forward primer 5′-CCCATGTCAAAACCGTGGTG-3′ and reverse primer 5′-CTGTGTTTGGGGTGGGCTTC-3′; and GAPDH, forward primer 5′-TGCCACTCAGAAGACTGTGG-3′ and reverse primer 5′-GGATGCAGGGATGATGTTCT-3′.

Statistical analysis

Differences in various treatments were statistically examined by using the Kruskal–Wallis test. Individual differences between treatments were examined with the Dunn's test by using the SAS GLM procedure. Results were expressed as mean ± SD. $P < 0.05$ was considered statistically significant as compared with RBL-2H3 cells in response to A23187 (positive control).

Results and Discussion

Natsudaidain did not inhibit histamine release from A23187-stimulated RBL-2H3 cells (Figure 2). However, a slight but significant reduction in histamine release from RBL-2H3 cells was seen at 200 μM (% histamine release, 71.5 ± 5.6%, $P < 0.05$). These levels of natsudaidain had no effect on cell viability (data not shown). Kobayashi and Tanabe[22] previously reported that nobiletin at concentrations of 100 and 500 μM inhibited histamine release from RBL-2H3 cells. Although the mechanism for the inhibitory effects of natsudaidain on histamine release is not evident, our results indicate that high concentrations of natsudaidain tend to inhibit histamine release from RBL-2H3 cells. However, this inhibitory effect of natsudaidain was very low compared with that of fisetin (a flavonol) at a concentration of 25 μM. Thus, our results show that the inhibitory activity of natsudaidain on histamine release from mast cells is negligible as compared with other flavonols. We focused on the effect of natsudaidain at low concentrations rather than at high concentrations on TNF-α and COX-2 from RBL-2H3 cells.

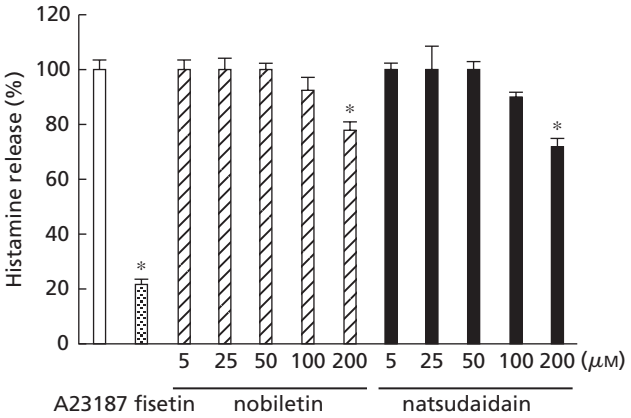

Figure 2 Dose-dependent effects of natsudaidain on histamine release from RBL-2H3 cells. RBL-2H3 cells were pretreated with various concentrations of natsudaidain (5, 25, 50, 100 and 200 μM: black bar), nobiletin (5, 25, 50, 100 and 200 μM: hatched bar), or fisetin (25 μM: stippled bar) for 30 min. Then, the cells were stimulated with A23187 (2 μM) for 30 min. Histamine release is presented as the means ± SD of three independent experiments. *$P < 0.05$ vs A23187-stimulated alone (white bar).

Natsudaidain treatment dose-dependently decreased the TNF-α protein levels in culture supernatant (Figure 3a). The concentration of natsudaidain required for a 50% reduction was 6.8 μM; thus, natsudaidain inhibited TNF-α secretion to the same extent as nobiletin (the concentration of 50% reduction, 10.2 μM). Expression levels of TNF-α mRNA were also dose-dependently reduced (Figure 3b), although TNF-α mRNA levels in RBL-2H3 cells pretreated with 1 μM natsudaidain were unchanged as compared with RBL-2H3 cells stimulated with A23187 alone. These data showed that natsudaidain inhibits TNF-α secretion from RBL-2H3 cells via suppression of mRNA transcription. Previous reports have found that flavonoids and coumarins inhibit TNF-α release from mast cells.[23–25] However, this is the first report to show that natsudaidain suppresses TNF-α production in mast cells, suggesting that natsudaidain is beneficial for the suppression of TNF-α release from activated mast cells.

When RBL-2H3 cells were pretreated with natsudaidain at concentrations of 5, 25 or 50 μM, COX-2 protein expression

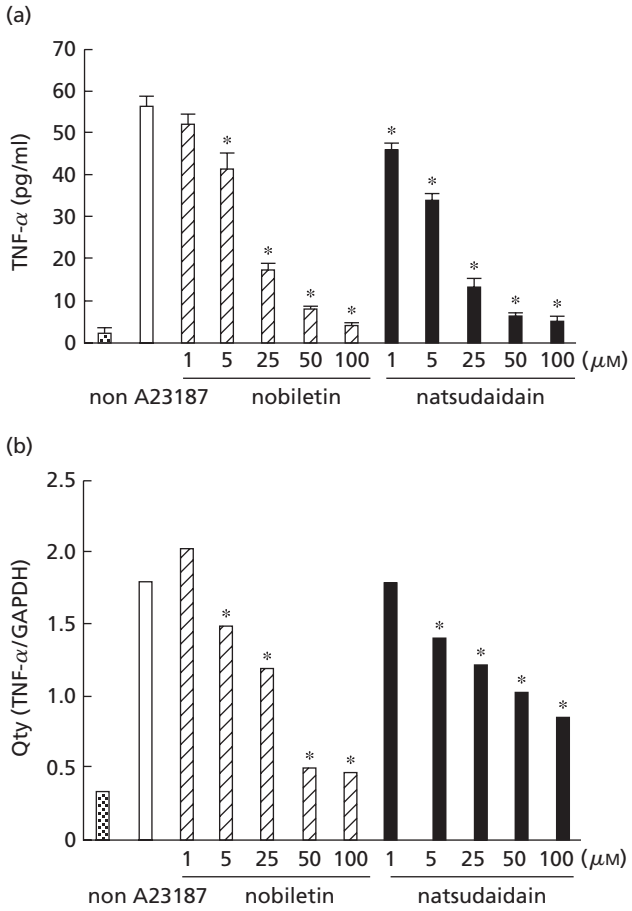

Figure 3 Effect of natsudaidain on TNF-α expression. (a) RBL-2H3 cells were pretreated with various concentrations of test compound (1, 5, 25, 50 and 100 μM) for 30 min. Cells were then stimulated with A23187 (2 μM) and cultured for 8 h. (b) RBL-2H3 cells were pretreated with various concentrations of test compounds (1, 5, 25, 50 and 100 μM) for 30 min. Cells were then stimulated with A23187 (2 μM) and cultured for 3 h. TNF-α protein and mRNA levels are presented as the means ± SD of three independent experiments. *$P < 0.05$ vs A23187 stimulation alone (white bar).

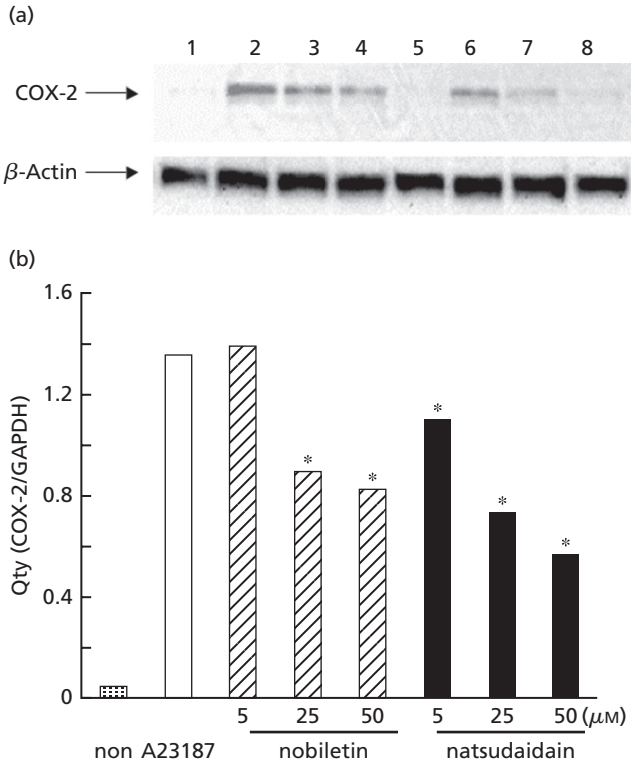

Figure 4 Effect of natsudaidain on COX-2 expression. (a) RBL-2H3 cells were pretreated with various concentrations of test compound (5, 25 and 50 μM) for 30 min. A23187 (2 μM)-stimulated cells were then cultured for 8 h. Lanes show untreated cells (lane 1), A23187-treated cells (lane 2), nobiletin-treated cells (5, 25 and 50 μM) +A23187 (lanes 3, 4 and 5) and cells treated with natsudaidain (5, 25 and 50 μM) +A23187 (lanes 6, 7 and 8). (b) RBL-2H3 cells were pretreated with various concentrations of test compound (5, 25 and 50 μM) for 30 min. Cells were then stimulated with A23187 (2 μM) and cultured for 3 h. COX-2 mRNA levels are presented as the means ± SD of three independent experiments. *$P < 0.05$ vs A23187 stimulation alone white bar.

was inhibited, as compared with A23187-stimulated RBL-2H3 cells (Figure 4a). In addition, this compound reduced COX-2 mRNA levels at all concentrations tested (reduction rate of 18.6, 45.6 and 58.1% at 5, 25 and 50 μM, respectively, against A23187-stimulated RBL-2H3 cells) (Figure 4b). These results demonstrate that natsudaidain inhibits COX-2 protein and mRNA as well as TNF-α. There are some previous reports that nobiletin inhibits COX-2 in other cells such as human synovial fibroblasts and keratinocytes.[19,20,26,27] However, the inhibitory activity of natsudaidain against COX-2 expression in mast cells has not been previously reported, and this study suggests that natsudaidain inhibits, at least in part, COX-2 mRNA transcription.

We used immunoblots to examine the time-dependent changes in p65 NFκB and p38 MAPK in A23187-stimulated RBL-2H3 cells after treatment with 25 μM natsudaidain. Natsudaidain treatment did not induce the reduction of phosphorylated-p65 NFκB protein levels as compared with A23187-stimulated cells (positive control) (Figure 5a). Also, Coward *et al.*[28] reported that p65 NFκB regulates TNF-α transcription. Based on these results, we concluded that natsudaidain does not suppress phosphorylated-p65 NFκB. We suspect that the inhibitory mechanisms of natsudaidain on TNF-α production are mediated by other signalling molecules. In contrast, natsudaidain treatment significantly decreased phosphorylated-p38 MAPK protein levels immediately after stimulation (Figure 5b). Some reports have demonstrated that the phosphorylation of p38 MAPK induced COX-2 and TNF-α expression.[29–32] These results show that natsudaidain partially regulates COX-2 and TNF-α production by suppressing the phosphorylation of p38 MAPK.

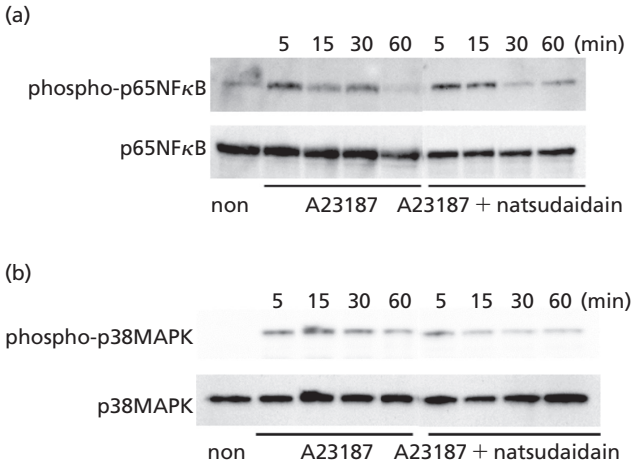

Figure 5 Effect of natsudaidain on the activation of NFκB and MAPK. To examine NFκB and MAPK activity, RBL-2H3 cells were pretreated with natsudaidain at a concentration of 25 μM for 30 min and then were stimulated with 2 μM A23187 for 5, 15, 30 and 60 min. The cells were then subjected to an immunoblot analysis by using anti phosphorylated-p65 (phospho-p65), p65 NFκB antibodies (a) and anti phosphorylated-p38 (phospho-p38), p38 MAPK antibodies (b). Similar results were obtained from three independent experiments.

Conclusion

This study suggests that natsudaidain, which is one of the polymethoxyflavonoids from *Citrus* plants, potently suppresses TNF-α and COX-2 production and that this suppression may be due at least in part to interference with p38 MAPK phosphorylation. Thus, natsudaidain may alleviate inflammatory diseases such as the late phase of type I allergy and chronic inflammation associated with fibrosis.

Conflict of interest

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

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