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Original Article

Koboku, an extract of magnolia bark, inhibits leukotriene synthesis in rat basophilic leukemia-1 cells

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ABSTRACT

To determine anti-allergic effects of Koboku, a Chinese herbal medicine, we investigated its inhibitory action on the production of cysteinyl leukotrienes (LT) and LTB_4, which are important chemical mediators in the pathogenesis of allergic diseases. A23187-stimulated synthesis of cysteinyl LT and LTB_4 was measured by HPLC in the absence or presence of various concentrations of Koboku in rat basophilic leukemia-1 (RBL-1) cells. In a dose-dependent manner, Koboku inhibited synthesis of cysteinyl LT and LTB_4 by up to 92.3 and 100%, respectively. Immunoglobulin E-mediated release of cysteinyl LT and LTB_4, which was measured by specific radioimmunoassay (RIA) was also inhibited by Koboku in RBL-2H3 cells. Sites of inhibition of Koboku in the metabolic pathway of LT synthesis were phospholipase A_2 (PLA_2) and 5-lipoxygenase (5-LO), but not LTC_4 synthase or LTA_4 hydrolase. We conclude that the anti-allergic effect of Koboku may be attributable, at least in part, to the inhibition of LT synthesis.

Key words: cytosolic phospholipase A_2, Koboku, leukotriene, leukotriene A_4 hydrolase, leukotriene C_4 synthase, 5-lipoxygenase, rat basophilic leukemia-1 cell, rat basophilic leukemia-2H3 cell.

INTRODUCTION

Koboku, the bark of *Magnolia obovata* Thunberg (Magnoliaceae), is a component of Chinese herbal folk medicines[1] being used for treating allergic diseases, including bronchial asthma. Freeze-dried granules of *Magnoliae* cortex extract, which are produced under pharmaceutically controlled methods, are officially registered as Koboku in the Pharmacopoeia of Japan. The compound is now commercially available. Cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4), arachidonate 5-lipoxygenase metabolites, are major chemical mediators that are released from mast cells by immediate-type allergic reactions[2] and from eosinophils in allergic inflammation.[3,4] Cysteinyl leukotrienes (LT) appear to be important in producing smooth muscle constriction[5,6] and have other properties that affect the inflammatory processes, such as the induction of mucus secretion[7] or the accumulation of inflammatory cells.[8] The present study examined the possible inhibitory effects of Koboku on the *in vitro* production of cysteinyl LT and LTB_4 and further clarified its sites of inhibition in the metabolic pathway of LT in rat basophilic leukemia-1 (RBL-1) cells, which produce a large amount of cysteinyl LT and LTB_4.

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METHODS

Materials

Rat basophilic leukemia-1 cells were obtained from Dainippon Pharmaceutical Co. (Tokyo, Japan) and RBL-2H3 cells were kindly provided by K Maeyama (Department of Pharmacology, Tohoku University, Japan). Other materials used include anti-biotic–anti-mycotic liquid (a mixture of penicillin G, streptomycin and amphotericin B), fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM; both from Gibco Oriental, Tokyo, Japan), arachidonic acid (AA), LTB_4, LTC_4, LTD_4 and LTE_4 (all from Funakoshi, Tokyo, Japan), LTA_4 methyl ester (Ultrafine Chemical, Manchester, England), A23187, glutathione (reduced form), bovine γ-globulin

and dithiothreitol (Sigma Chemical Co., St Louis, MO, USA), adenosine 5′-triphosphate (ATP, monosodium salt; from Oriental Yeast Co., Tokyo, Japan), Norit A and magnolol (Wako, Osaka, Japan) and β-eudesmol (Nihon Sanso, Tokyo, Japan). 5,6,8,9,11,12,14,15-[^{3}H]-Arachidonic acid, [5,6,8,9,11,12,14,15–^{3}H]-LTB_4 (5.92–8.88 TBq/mmol) and [14,15,19,20–^{3}H]-LTC_4 (3.7–8.88 TBq/mmol) were obtained from Amersham Japan (Tokyo, Japan), purified rat myeloma IgE was obtained from Zymed (San Francisco, CA, USA) and sheep antiserum to rat IgE was obtained from Bethyl (Montgomery, TX, USA). Antisera to LTB_4 and LTC_4 were kindly provided by Dr Daniel Tai (University of Kentucky, Lexington, KY, USA). Koboku freeze-dried granules were obtained from Tsumura Co. (Tokyo, Japan). Other materials were reagent grade and were obtained from commercial sources.

Measurement of cysteinyl LT and LTB_4 synthesis in intact cells

The production of cysteinyl LT (LTC_4, LTD_4 and LTE_4) and LTB_4 was measured in intact RBL-1 cells by methods previously described.[9] Cells were grown in DMEM supplemented with antibiotic–antimycotic liquid and 10% FBS, then distributed into 3.5 cm diameter petri dishes at a density of 2×10^6 per 2 mL. Various concentrations of Koboku were added to the medium. Cells were incubated for 10 min prior to exposure to 10^{-5} mol/L A23187 for 15 min at 37°C. The culture medium (2 mL) was then removed and mixed with 3.5 mL ice-cold methanol. After centrifugation (1000 g, 15 min), the supernatants were applied to a pretreated Sep Pak C18 cartridge (Waters Associates, Milford, MA, USA) for purification and concentration. A portion (90 μL) of the eluted sample was then injected into a high-performance liquid chromatography (HPLC) column for assay of LT.

The HPLC system consisted of a computer control multi pump and a UV-8010 absorbance detector (TOSO, Tokyo, Japan). A Novapak C18 column (Waters Associates; 0.39×15 cm) was used with acetonitrile/methanol/water/acetic acid (31:17.08:51.24:0.68 (v/v)) for assay of LT. The solvent was adjusted to pH 4.5 with NH_4OH. The flow rate was 0.8 mL/min and elution was monitored at 280 nm. Retention times for LTC_4, LTD_4, LTE_4, 6-trans-LTB_4 diastereoisomers (12-epi-6-trans LTB_4 and 6-trans LTB_4) and LTB_4 were approximately 6, 8, 9, 11 and 14 min, respectively. Identification of these metabolites was based on comigration with authentic standards and specificity of UV absorbance. Quantification of LT was based on the peak area ratios with respect to authentic standards.

Measurement of cysteinyl LT and LTB_4 synthesis in intact RBL-2H3 cells by radioimmunoassay

Although RBL-1 cells produce a large amount of cysteinyl LT and LTB_4 after stimulation with A23187, these cells have no high-affinity IgE receptors on their surface. Therefore, we used RBL-2H3 cells expressing high-affinity IgE receptors on their cell surface in this particular study in which the inhibitory action of Koboku on IgE-mediated synthesis of LT was examined. Rat basophilic leukemia-2H3 cells produce a smaller amount of LTB_4 and a much smaller amount of cysteinyl LT by IgE-mediated stimulation than do RBL-1 cells stimulated with A23187. We therefore used a specific radioimmunoassay (RIA) for the measurement of cysteinyl LT and LTB_4 in this study.[10]

Rat basophilic leukemia-2H3 cells were distributed into 24-well plates at a density of 10^6 cells/mL. Rat IgE (0.1 μL) was added to each well and the cells were incubated for 6 h at 37°C. The medium was then discarded and, after the addition of fresh DMEM (300 μL), cells were exposed to various concentrations of Koboku and were incubated for 10 min prior to stimulation with anti-IgE (0.1 μL) for 30 min at 37°C. The medium was recovered and stored at –70°C until measurements of cysteinyl LT and LTB_4 were performed. Radioimmunoassay for cysteinyl LT and LTB_4 was performed as previously described.[10] Briefly, 100 μL of each sample was incubated at 4°C for 10 h with 100 μL each of anti-LTC_4 antiserum, [^{3}H]-LTC_4 and 50 mmol/L sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and γ-globulin (1 mg/mL). Free [^{3}H]-LTC_4 was separated by centrifugation at 1000 g for 10 min after the addition of 0.5 mL γ-globulin-coated charcoal (Norit A) to the assay mixture. The resulting supernatant was transferred to a scintillation vial containing 5 mL scintillation fluid and the radioactivity was measured with a liquid scintillation counter (model LS7500; Beckman, Irvine, CA, USA). The amount of LTC_4 was calculated from a standard curve. The final dilution of anti-LTB_4 antiserum was 1:2000. The minimal detectable amount of LTC_4 was 8 pg per assay tube. The percentages of cross-reactivity of the anti-LTC_4 anti-serum with LTB_4, LTD_4 and LTE_4 and were 0.1, 139 and 3.2%, respectively. Therefore, the production of LTC_4 measured by RIA in the present study represents the sum of LTC_4 and LTD_4 levels, which are

expressed as cysteinyl LT. For the RIA of LTB_4, the same procedures were followed with the use of anti-LTB_4 antiserum (final dilution 1:2000) and [^{3}H]-LTB_4 instead of anti-LTC_4 antiserum and [^{3}H]-LTC_4, respectively. The minimal detectable amount of LTB_4 was 32 pg per assay tube. The percentages of cross-reactivity of anti-LTB_4 antiserum with LTC_4, LTD_4 and LTE_4 were <0.1%; those with 20OH-LTB_4 and 20COOH-LTB_4 were <5%.

Assay of PLA_2 activity in intact cells

Cells were seeded into a 24-well plate at a density of 5×10^5/mL and were incubated for 16 h at 37°C with [^{3}H]-AA (1.11 kBq; 0.15 pmol). The supernatant was discarded and was replaced with 1 mL fresh DMEM without FBS. Cells were incubated for 30 min at 37°C and were then exposed to Koboku for 10 min and to 10^{-5} mol/L A23187 for 5 min. Radioactivity released into the medium was counted with a type 2250CA liquid scintillation counter (Packard, Meriden, CT, USA). The inhibitory actions of magnolol or β-eudesmol on PLA_2, both of which are components in Koboku, were also examined by the same assay procedures.

Assay of 5-lipoxygenase activity

The production of cysteinyl LT and LTB_4 was measured with cell lysate as the enzyme source and free AA as the substrate. Cells were washed and were suspended at a density of 10^8/mL in a solution containing (in mmol/L): NaCl 137; KCl 2.6; sodium phosphate 0.36; HEPES 10 (pH 7.5); EDTA 1; dithiothreitol 1. The cell suspension was sonicated (Branson (Plainview, NY, USA) Model W200-P, level 4) on ice three times for 15 s each time. Two microliters of 0.1 mol/L AA were added to 198 μL reaction buffer containing cell lysate (50 μL), 3 mmol/L glutathione, 0.1 mmol/L ATP and 2 mmol/L $CaCl_2$, and the reaction mixture was incubated at 37°C for 5 min either in the absence or in the presence of Koboku. The reaction was stopped by adding 2 mL ice-cold methanol. After centrifugation, the supernatant was applied to a pretreated Sep Pak C18 cartridge. Leukotrienes were separated and measured as described for intact cell experiments.

Assay of LTC_4 synthase and LTA_4 hydrolase

To evaluate the action of Koboku on the activity of LTC_4 synthase and LTA_4 hydrolase without the influence of 5-LO, the production of cysteinyl LT and LTB_4 was measured in intact RBL-1 cells after the addition of LTA_4-free acid as a substrate into the incubation medium. Twenty microgram LTA_4-free acid, prepared as previously described,[11] were added to the medium and the mixture was incubated at 37°C for 5 min in the absence or presence of Koboku. Produced cysteinyl LT and LTB_4 were assayed by HPLC as described earlier.

Statistical analysis

Data are presented as the mean±SEM. Statistical analysis was performed by one-way analysis of variance. The significance of the differences between groups was evaluated by Sheffé's multiple comparison method. A level of $P<0.05$ was accepted as statistically significant.

RESULTS

Measurement of cysteinyl LT and LTB_4 synthesis in intact cells

Calcium ionophore-stimulated cells produced LTC_4, LTD_4, LTE_4 and LTB_4 (Fig. 1a). The production of LTC_4, LTD_4 and LTE_4 contributed to total cysteinyl LT production. A dose–response curve of the effects of A23187 on LT-production is shown in Fig. 1b. As the production of cysteinyl LT and LTB_4 were increased in a dose-dependent manner by A23187, we used a concentration of 10^{-5} mol/L A23187 throughout the studies. As shown in Fig. 2a,b, the production of cysteinyl LT and LTB_4 was dose-dependently and significantly inhibited by Koboku.

Effect of Koboku on the IgE-mediated release of cysteinyl LT and LTB_4

The IgE-loaded RBL-2H3 cells synthesized cysteinyl LT and LTB_4 following stimulation with anti-IgE. The production of cysteinyl LT was completely inhibited by Koboku at 100 μg/mL (Fig. 3a). The production of LTB_4 was dose-dependently and significantly inhibited by Koboku, as shown in Fig. 3b.

Effects of Koboku on PLA_2 activity

The average incorporation of [^{3}H]-AA into RBL-1 cells was approximately 75% after 16 h (data not shown). At 100 μg/mL, Koboku significantly inhibited the release of [^{3}H]-AA from A23187-stimulated RBL-1 cells by 57%

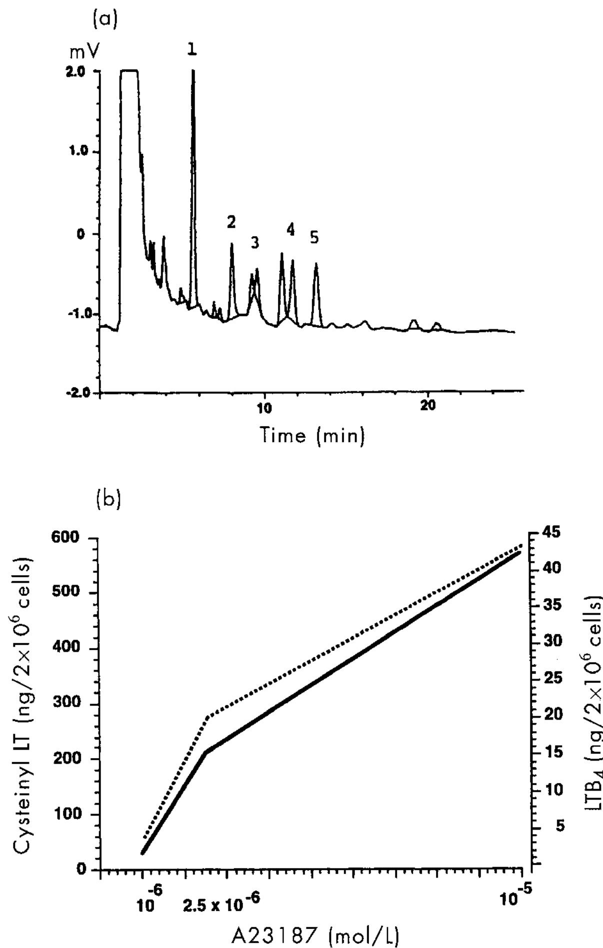

Fig. 1 Reverse-phase (RP) HPLC profiles. (a) Representative RP-HPLC profiles of lipoxygenase metabolites from 2×10^6 cells stimulated with calcium ionophore A23187 (10^{-5} mol/L) for 15 min at 37°C. 1, leukotriene (LT) C_4; 2, LTD_4; 3, LTE_4; 4, 6-trans-LTB_4 diastereoisomers; 5, LTB_4. (b) Dose-response curve of LT production stimulated with A23187 from 10^{-6} to 10^{-5} mol/L. (—), production of cysteinyl LT; (-----), production of LTB_4.

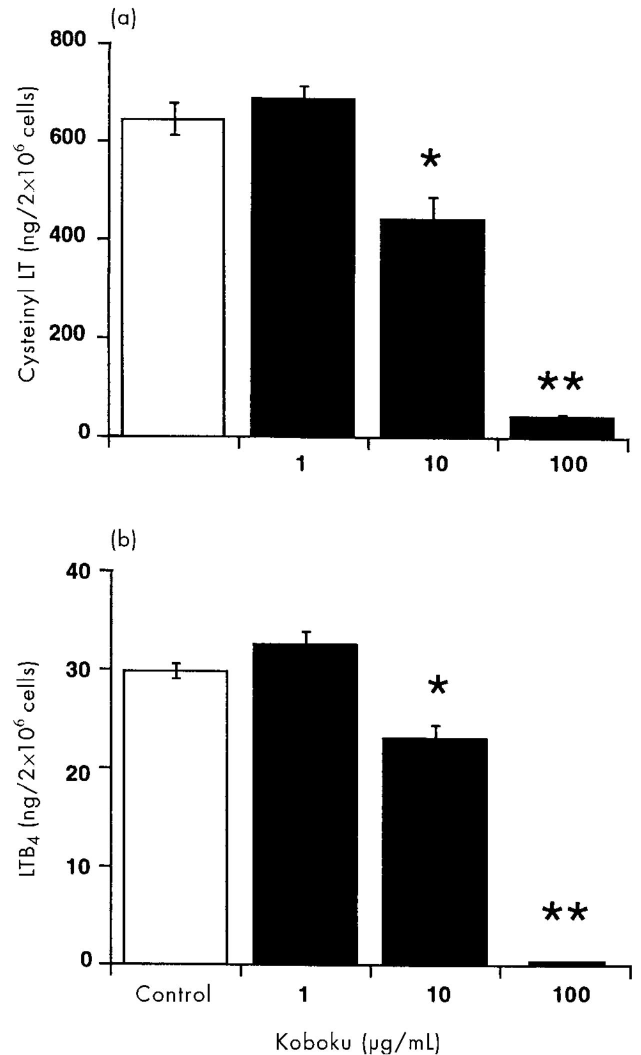

Fig. 2 Effect of Koboku on A23187-stimulated synthesis of (a) cysteinyl leukotrienes (LT) and (b) LTB_4 in RBL-1 cells. Cells were stimulated with 10 μmol/L A23187 for 15 min in the absence (□) or presence (■) of various concentrations of Koboku. The production of LT was measured by HPLC. Data are the mean±SEM ($n=3$). *$P<0.05$, **$P<0.01$ versus control.

(Fig. 4). β-Eudesmol showed no inhibition of PLA_2 activity at 10^{-5} mol/L. Magnolol significantly inhibited release by 28.4% at a concentration of 10^{-5} mol/L (Table 1).

Effects of Koboku on 5-LO

To examine the actions of Koboku on 5-LO, we incubated cell lysates with AA as the substrate for 10 min in the absence or presence of Koboku. The production of cysteinyl LT and LTB_4 was inhibited by Koboku (100 μg/mL) by 52 and 40%, respectively (Fig. 5).

Effects of Koboku on LTA_4 hydrolase and LTC_4 synthase

Koboku (100 μg/mL), which significantly inhibited A23187-stimulated production of LT in previous studies, showed no significant inhibition of the synthesis of cysteinyl LT and LTB_4 when cells were incubated with LTA_4-free acid as the common substrate of LTA_4 hydrolase and LTC_4 synthase (data not shown).

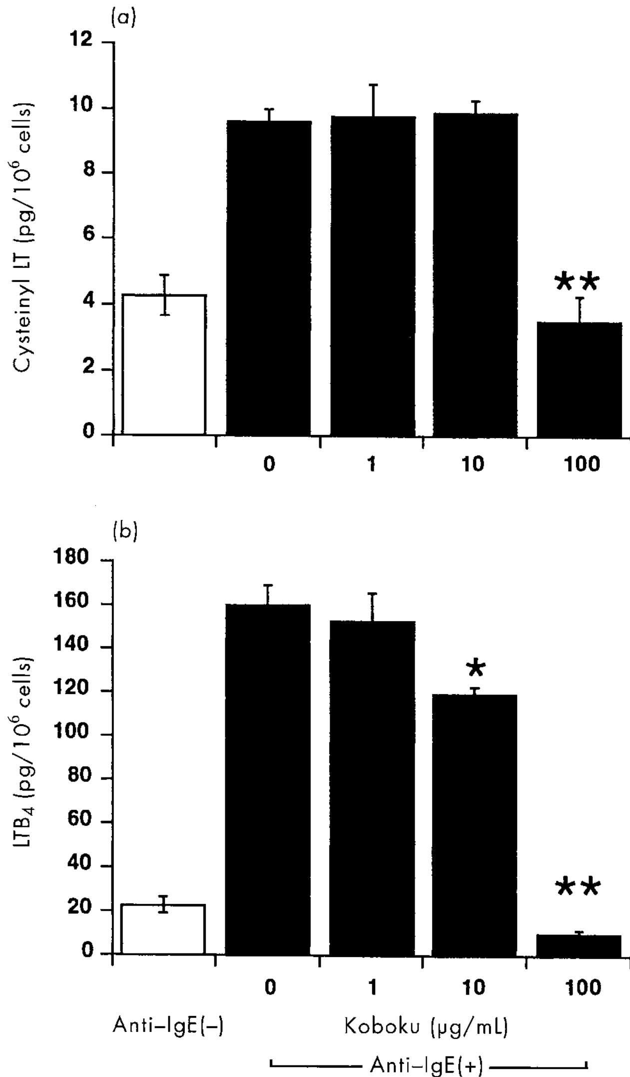

Fig. 3 The action of Koboku on the IgE-mediated production of (a) cysteinyl leukotrienes (LT) and (b) (LTB_4). The IgE-loaded 2H3 cells were stimulated with anti-IgE for 30 min in the presence of various concentrations of Koboku (■). The production of cysteinyl LT and LTB_4 was then measured by using specific RIA, as described in Methods. Data are the mean±SEM ($n = 4$). *$P<0.05$, **$P<0.01$ versus control (no Koboku treatment group; □).

Table 1. Inhibitory action of magnolol or β-eudesmol on the release of [^{3}H]-arachidonic acid from A23187-stimulated cells

Concentration (mol/L)	Radioactivity (c.p.m.) β-eudesmol	Magnolol
–	2023±58	2068±25
10^{-7}	1904±46	1853±105
10^{-6}	2007±39	1927±45
10^{-5}	2053±57	1481±113*

Data are the mean±SEM ($n = 4$; *$P = 0.0018$).

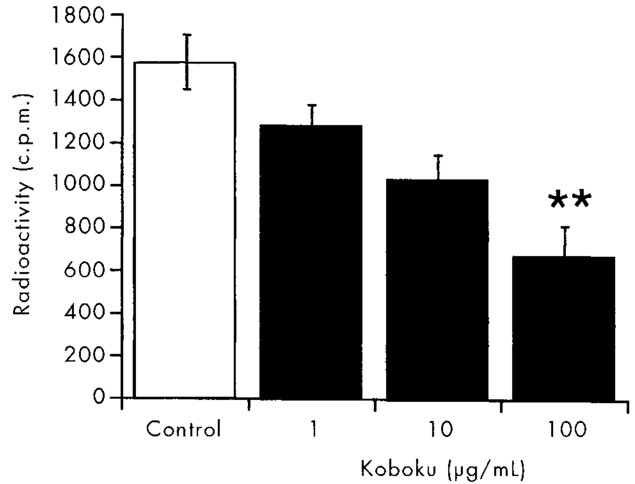

Fig. 4 Action of Koboku on phospholipase A_2 activity. [^{3}H]-Arachidonic acid was incorporated into cell membranes and was stimulated with A23187 in the absence (□) or presence (■) of Koboku. Released radioactivity in the medium was counted. Data are the mean±SEM ($n = 4$). **$P<0.01$ versus control.

Discussion

Our results show that Koboku inhibits the A23187-stimulated production of cysteinyl LT (LTC_4, LTD_4, LTE_4) and LTB_4 in intact RBL-1 cells. Koboku also inhibited the IgE-mediated release of cysteinyl LT and LTB_4 in RBL-2H3 cells, which have high-affinity IgE-receptors on their cell surface.

Following the increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) induced by A23187 and IgE receptor-mediated stimulation, AA is released from membrane phospholipids by PLA_2, the first step enzyme. There are at least three different types of PLA_2.[12] Of these, a high molecular weight cytosolic PLA_2 ($cPLA_2$) is mainly responsible for AA metabolism.[13] Cytosolic PLA_2 translocates to the perinuclear membrane, the site of LT synthesis.[14] Phospholipase A_2 activity, as reflected by the release of [^{3}H]-AA, was significantly and dose-dependently inhibited by Koboku. The second step enzyme, 5-LO, is also translocated from the cytosol to the perinuclear membrane following an increase in $[Ca^{2+}]_i$.[15] Koboku inhibited the production of cysteinyl LT and LTB_4 when AA was used as the substrate in the cell-free study, but it did not inhibit the production of cysteinyl LT and LTB_4 when LTA_4-free acid was used as the substrate. These results indicate that Koboku inhibits 5-LO as well as PLA_2. The possible sites of inhibition by Koboku in the arachidonate–LT synthetic pathway are illustrated in Fig. 6.

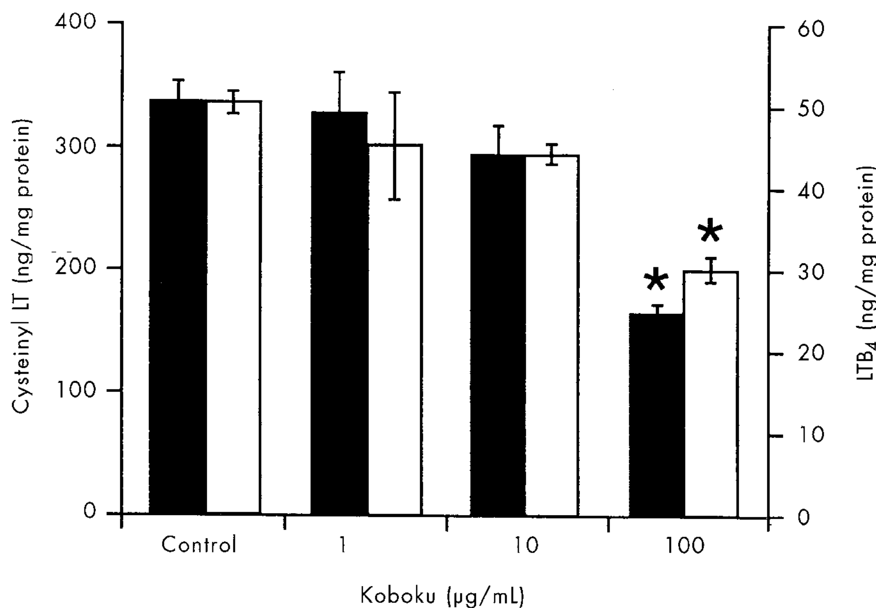

Fig. 5 Inhibitory action of Koboku on the production of leukotrienes (LT) in a cell-free assay. Cell lysate (as an enzyme source) was incubated with free arachidonic acid (10^{-1} mol/L) as the substrate in the absence (control) or presence of Koboku. (■), cysteinyl LT; (□), LTB_4. Data are the mean±SEM ($n = 3$). *$P<0.05$ versus control.

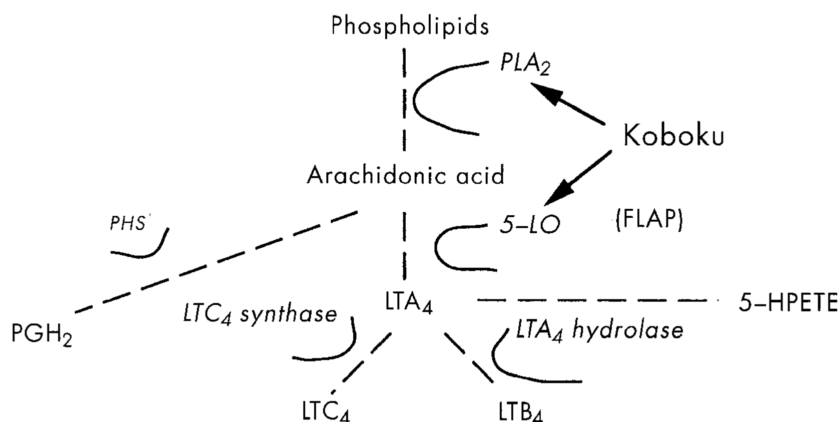

Fig. 6 Arachidonate 5-lipoxygenase (5-LO) metabolic pathway; arrows indicate the possible inhibitory sites of action of Koboku. PHS, prostaglandin H_2 synthase; PGH_2, prostaglandin H_2; LTC_4, LTB_4, LTA_4, leukotriene C_4, B_4 and A_4, respectively; PLA_2, phospholipase A_2; FLAP, five lipoxygenase activating protein; 5-HPETE, 5-hydroxyperoxy-eicosotetranoic acid.

There have been no pharmacokinetic studies done with Koboku in humans. A standard dose of Chinese herbal medicine for patients is 7.5 g/day (divided into three doses). If 2.5 g Koboku is given and 100% of the compound is absorbed and is equally distributed throughout the aqueous compartments of the body of a 50 kg adult, the calculated concentration of Koboku in the body fluids would be approximately 80 µg/mL. Magnolol is a major component of Koboku and is approximately 5% (w/w) of Koboku. When the concentration of magnolol is estimated by the simulated calculation of Koboku in the body fluid, the concentration of magnolol is found to be approximately 4 µg/mL ($=1.5\times10^{-5}$ mol/L). Thus, the serum concentrations of Chinese herbal medicines and their components possibly reach the concentrations that were examined in the present study, although this is a simple simulation and there is no definitive evidence as to the actual concentrations reached in the human body.

Koboku contains bioactive chemicals such as β-eudesmol, α-pinene, β-pinene, camphene, limonene, magnocurarine, magnoflorine, magnolianin, magnolol and honokiol. Honokiol, a diphenyl compound, reportedly inhibits 5-LO activity but not PLA_2 activity.[16] This suggests that the inhibitory action of Koboku on LT synthesis is, in part, attributable to honokiol. We examined the inhibitory action of β-eudesmol and magnolol, which are commercially available components of Koboku, on PLA_2 activity. Magnolol at a concentration of 10^{-5} mol/L, but not β-eudesmol, inhibited PLA_2 activity by 28%, suggesting that magnolol is responsible for the inhibition of PLA_2 by Koboku. Other unidentified

substances may also be responsible for inhibitory action. PLA_2 inhibition contributes to the overall inhibition of LT synthesis. Koboku inhibition of 5-LO activity, as measured by cysteinyl LT and LTB_4 production using AA as the substrate, was 52 and 42%, respectively. Inhibition of PLA_2 activity at the same Koboku concentration was 57%. Thus, Koboku demonstrated a synergistic inhibition of LT synthesis through its inhibition of two pivotal enzymes, PLA_2 and 5-LO. However, the detailed mechanism of this inhibition remains to be elucidated. Although we have demonstrated that Koboku and its components inhibit the synthesis of LT, which play important roles in the development of allergic diseases, there may be fundamental differences between *in vitro* studies and *in vivo* clinical effectiveness. In order to lessen the gap, further pharmacokinetic studies on Koboku in humans are needed.

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