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Naturally Occurring Biflavonoid, Ochanflavone, Inhibits Cyclooxygenases-2 and 5-Lipoxygenase in Mouse Bone Marrow-derived Mast Cells

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Ochnaflavone is a medicinal herbal product isolated from Lonicera japonica that inhibits cyclooxygenase-2 (COX-2) dependent phases of prostaglandin D_2 (PGD_2) generation in bone marrow-derived mast cells (BMMC) in a concentration-dependent manner with IC_{50} values of 0.6 μM. Western blotting probed with specific anti-COX-2 antibodies showed that the decrease in quantity of the PGD_2 product was accompanied by a decrease in the COX-2 protein level. In addition, this compound consistently inhibited the production of leukotriene C_4 (LTC_4) in a dose dependent manner, with an IC_{50} value of 6.56 μM. These results demonstrate that ochnaflavone has a dual cyclooxygenase-2/5-lipoxygenase inhibitory activity. Furthermore, this compound strongly inhibited degranulation reaction in a dose dependent manner, with an IC_{50} value of 3.01 μM. Therefore, this compound might provide a basis for novel anti-inflammatory drugs.

Key words: *Lonicera japonica*, Ochnaflavone, Biflavonoid, Cyclooxygenase-2, 5-Lipoxygenase, Bone marrow-derived mast cells

INTRODUCTION

Flavonoids, one of abundant classes of plant constituents, are known to be nature's tender drug showing various biological/pharmacological activities such as anticancer, antibacterial, antiviral, anti-inflammatory, immunomodulatory activities (Middleton *et al.*, 1992). Numerous studies have demonstrated that the anti-inflammatory activity of certain flavonoids might be contributed by inhibiting enzyme activity involved in arachidonic acid cascade related enzymes such as phospholipaseA_2 (PLA_2), cyclooxygenase (COX) and lipoxygenases (LOXs). Recently, it was found that some flavonoids suppressed the inducible enzyme expression such as COX-2 and inducible nitric oxide synthase (iNOS), thereby reducing the production of prostaglandins (PGs) and nitric oxide (NO) (Baek *et al.*, 1999; Chi *et al.*, 2001). Among the flavonoids derivatives, biflavonoids are structurally unique flavonoid-dimmers connected by a C-O-C or C-C bond. Previously, we and other groups already reported their biological function *in vitro* and *in vivo*. Especially, ochanflavone and several other biflavones were found to be groups IIA secrertory phospholipase A_2 ($sPLA_2$-IIA) inhibitor (Chang *et al.*, 1994; Gil *et al.*, 1997).

Mast cells are one of the most important effector cells in allergic response (Galli *et al.*, 1996). When activated, mast cells release a number of biologically active molecules, including histamine, serotonin, proteoglycans and serine proteases through exocytosis, membrane-derived lipid mediators, such as eicosanoids, through the activation of the COX-2 and 5-LOX pathways, and preformed and de novo synthesized various cytokines (Stevens *et al.*, 1989; Yamaguchi *et al.*, 1999; Murakami *et al.*, 2001). PGs elicit

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a variety of important biological responses. Among these properties are their ability to induce pain, fever and the symptoms associated with inflammatory responses. Non-steroidal anti-inflammatory drugs (NSAIDs) reduce the pain and inflammatory swelling by blocking PGs synthesis at the COX stage. However, most NSAIDs which have been used clinically inhibit the production of the PGs that are not only associated with the inflammatory processes but are also involved in maintaining the normal physiological processes. The main limitation in using NSAIDs is their side effects, including gastrointestinal ulcerogenic activity and kidney dysfunction, which limits their therapeutic value of their safe and long-term use (Vane *et al.*, 1971; Whittle *et al.*, 1980). The enzyme responsible for PGs synthesis exists as two isoforms, COX-1 (constitutive isoform) and COX-2 (inducible form) (Maier *et al.*, 1990; O'Banion *et al.*, 1992). Arachidonic acid can also be converted to leukotrienes (LTs) by the action of 5-LOX.

Therefore, the development of dual inhibitors that can simultaneously inhibit COX-2/5-LOX and degranulation reaction might enhance their individual anti-inflammatory effects and reduce the undesirable side effects that are associated with NSAIDs. This study describes for the first time a new biological function of ochnaflavone for arachidonic cascade metabolism enzymes and degranulation reaction.

MATERIALS AND METHODS

Materials

Ochnaflavone (apigenin-3'-O-C-4-apigenin dimmer) was isolated from *Lonicera japonica*. The isolated compound was chemically and structurally identified according to the previous reports (Son *et al.*, 1992). Its chemical structure is shown in Fig. 1. ochnaflavone used in this study showed a single spot on TLC and was prepared by dissolving in dimethyl sulfoxide (DMSO) and final concentrations of DMSO were adjusted to 0.1% (v/v) in culture media. Control with DMSO alone was run in all cases.

Preparation and activation of bone marrow-derived mast cells (BMMC) and PGD_2 determination

Bone marrow cells from male Balb/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of interleukin (IL)-3. After 3 weeks > 98% of the cells were found to be BMMC when checked by the previously described procedure (Bingham *et al.*, 1996) To measure inhibitory activity on COX-2 by ochnaflavone, cells suspended at a cell density of 5×10^5 cells/mL in enriched medium were preincubated with aspirin (10 μg/mL) for 2 h to irreversibly

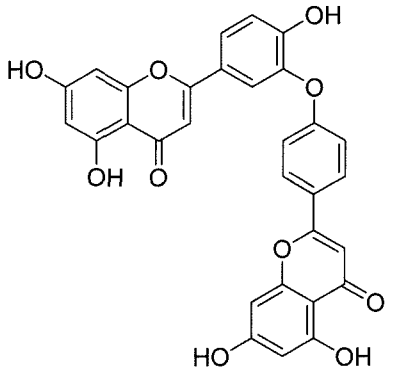

Fig. 1. Chemical structure of ochnaflavone

inactivate preexisting COX-1. After washing, BMMC were activated with *c-kt* ligand (KL, 100 ng/mL), IL-10 (100 U/mL) and LPS (100 ng/mL) at 37°C for 8 h in the presence or absence of ochanflavone previously dissolved in dimethylsulfoxide (DMSO). All reactions were stopped by centrifugation at 120 g at 4°C for 5 min. The supernatant and cell pellets were immediately frozen in liquid N_2 and stored at -80°C for further analysis. PGD_2 was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer's instruction. Under the conditions employed, COX-2-dependent phases of PGD_2 generation reached 1.6 ng/10^6 cells. All data was the arithmetic mean of triplicate determinations.

LTC_4 determination

BMMC suspended in enriched medium at a cell density of 1×10^6 cells/mL were pretreated with ochnaflavone for 30 min at 37°C and stimulated with KL (100 ng/mL). After 20 min of stimulation, the supernatants were isolated for further analysis by EIA. LTC_4 was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer's instruction. Under the conditions employed, 5-LOX dependent LTC_4 reached approximately 500 pg/10^6 cells. All data was the arithmetic mean of triplicate determinations.

Assay of β-hexosaminidase release

β-hexosaminidase (β-HEX), a marker of mast cell degranulation was quantitated by spectrophotometric analysis of the hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma Chemical Co.). Briefly, after harvesting supernatant, cells were lysed in the same volume of medium by freeze and thaw three times. 10 mL of the BMMC lysate or supernatant samples were mixed with 50 mL of β-HEX substrate solution (1.3 mg/mL *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 100 mM sodium citrate, pH 4.5) in each well of 96-well plates and then incubated at 37°C for 60 min. The reaction

was stopped by adding 140 mL of 0.2 M Glycin-NaOH (pH 10.7). The absorbance at 410 nm was measured in a microplate reader. The percentage of β-HEX released into the supernatant was calculated by the following formula: [S / (S + P)] × 100, where S and P are the β-HEX contents of supernatant and cell pellet.

SDS-PAGE/Immunoblot analysis

After activation with KL, IL-10 and LPS, BMMC were washed once with 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (PBS) and lysed in PBS containing 0.1% SDS and 10 mM b-mercaptoethanol at 1×10^7 cells/mL. The lysate (1×10^5 cells equivalent) was applied to 10% SDS-polyacrylamide gels. After running the gel, the protein bands were blotted onto nitrocellulose membranes (Schleicher and Schull, Dassel, Germany) using a semi-dry blotter (MilliBlot-SDE system, Millipore, Bedford, MA) according to the manufacturer's instructions. Membranes were then washed once with 10 mM Tris-buffered saline (TBS, pH 7.2) containing 0.1% tween-20 (TBS-T), and then blocked for 1 h in TBS-T containing 3% skim milk. After washing the membranes with TBS-T, an antibody directed against COX-2 was added at a dilution of 1:3,000 -5,000 in TBS-T. After incubation for 2 h followed by washing three times, membranes were treated for 1 h with horseradish peroxide-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA) (diluted to 1:7,000) in TBS-T. The protein bands were visualized using an enhanced chemiluminesence (ECL) system (Amersham Corp., Newark, NJ, U.S.A.).

RESULTS AND DISCUSSION

The flow or whole plants of *Lonicera japonica* have been used as an antidote, diuretics, tonics, antipyresis and anti-inflammatory agents. We previously reported the anti-inflammatory and the analgesic activity of the water extract of *L. japonica* and its *n*-butanol (*n*-BuOH) fraction. Furthermore, we isolated major anti-inflammatory constituents from BuOH fraction of this plant (Lee *et al.*, 1995). There are diverse families of flavonoids including chalcone and flavone dimmer. Among them, certain biflavonoids were reported to possess diverse biological activities. For example, ochnaflavone, amentoflavone and ginkgetin were found to be inhibitors of group secretory phospholipase A_2 ($sPLA_2$) from rat platelet, and similar data results were also obtained with morelloflavone (Chang *et al.*, 1994). In addition, biflavonoid such as ochnaflavone, ginkgetin and its isomer isoginkgetin, inhibited the release of archidonic acid metabolites from rat peritoneal macrophages stimulated by phorbol ester or calcium ionophore, A_{23187} (Lee *et al.*, 1997) and also inhibited lymphocyte proliferation induced by T- or B-cell mitogen (Lee *et al.*, 1995). Furthermore, previously we also reported that gingketin and bilobetin inhibited tumor necrosis factor-α (TNF-α) production and COX-2 as well as iNOS protein expression in RAW264.7 cell line (Baek *et al.*, 1999). Taken together, data from the above studies strongly suggest that ochnaflavone may affect mast cells activation.

Murakami et al. reported that BMMC exhibit biphasic PGD_2 biosynthetic responses over time, in addition to COX-1-dependent immediate and COX-2-dependent delayed responses. The immediate PGD_2 generation occurring within 2 h is associated with the coupling of COX-1 and the delayed PGD_2 generation, which occurs after several hours of culturing (during 2-10 h), is associated with the de novo induction and function of COX-2 after stimulation with particular cytokines and LPS combinations (Murakami *et al.*, 1994).

This cell model also appear to be suitable for assessing the effect of 5-LOX inhibitors, since the immediate LTC_4 generation elicited by the IgE-dependent or cytokine-initiated stimulus occurs in BMMC through 5-LOX (Murakami *et al.*, 1995). Therefore, the BMMC system is useful for screening selective COX-1/COX-2 or 5-LOX and COX-2/5-LOX dual inhibitors from various sources (Lee *et al.*, 2004). When the BMMC were activated with a combination of KL, IL-10 and LPS in the presence or absence of ochnaflavone, the COX-2-dependent phase of

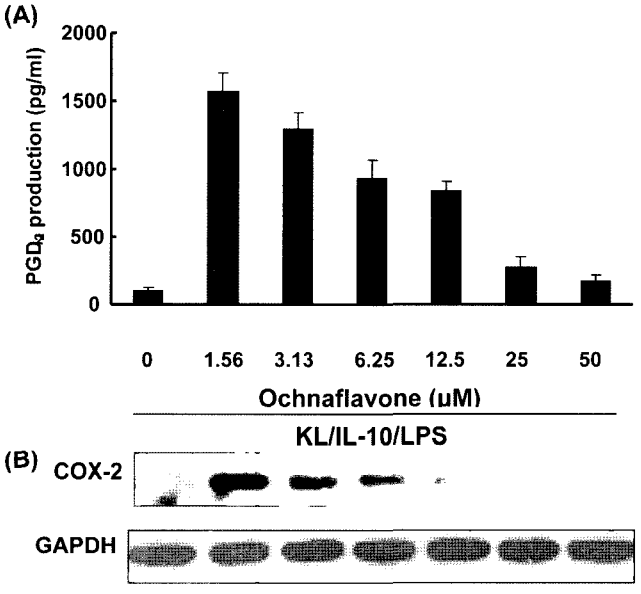

Fig. 2. Effect of ochnaflavone on COX-2 dependent PGD_2 generation (A) and COX-2 and COX-1 protein expression (B). BMMC were pre-incubated for 30 min with the indicated concentration of ochnaflavone and then stimulated with KL (100 ng/mL), IL-10 (100 U/mL) and LPS (100 ng/mL) at 37°C for 8 h in the presence or absence of ochnaflavone. PGD_2 released into the supernatant was quantified by EIA kit. Samples were processed by SDS-PAGE and transferred to a nitrocellulose filter. The immunoblot was then probed with anti-COX-2 at a dilution 1:3,000. The procedure is described in MATERIALS AND METHODS in detail.

PGD_2 generation was inhibited in a dose-dependent manner with an IC_{50} value of approximately 0.63 μM (Fig 2A), while in the presence of 10 μM of this compound did not inhibited COX-1 dependent phase of PGD_2 generation (data not shown).

The inhibitory effect of the ochnaflavone on PGD_2 production was examined to determine if it is a direct effect of the COX-2 protein or if this inhibition is mediated by some other mechanism. As shown in Fig. 2B, the COX-2 protein was not detected in unstimulated BMMC, whereas combination of KL, IL-10 and LPS strongly induced the formation of detectable COX-2 protein.COX-2 protein expression was inhibited in a dose-dependent manner by ochnaflavone.

Arachidonic acid can also be converted to leukotrienes (LTs) by the action of 5-LOX in BMMC. The inhibition of 5-LOX is believed to be the ideal treatment for inflammatory disease including allergic disease and asthma (Fiorucci *et al.*, 2001). Therefore, the inhibitory activity of ochnaflavone on the generation of LTC_4 in the BMMC was examined. Fig. 3 shows that the BMMC stimulated with KL for 15 min produced ~500 pg/mL LTC_4, and preincubation of the BMMC with ochnaflavone resulted in the dose-dependent suppression of this LTC_4 biosynthesis with an IC_{50} value of 6.56 μM.

Mast cells are one of the most important effector cells in many inflammatory reactions including allergy, asthma and anaphylactic shock. They have long been implicated in the pathology and mortality of anaphylaxis and other allergic disorders by virtue of both their ability to be activated through FcRI bound antigen-specific IgE and their concentration at surfaces that interface with the external environment. Mast cells may also be activated by various cytokines through each cytokine receptors. Activation through any of these receptors lead to release of a number of biologically active molecules, including histamine, serotonin, proteoglycans and neutral proteases, which are stored in cytoplasmic granules, through exocytosis.

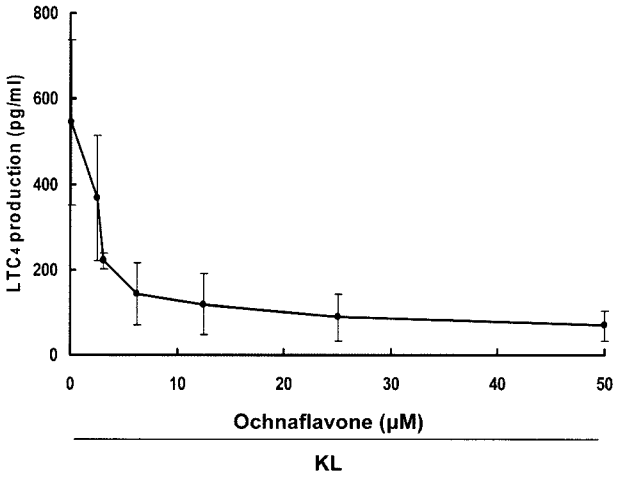

Fig. 3. Effect of ochnaflavone on the generation of LTC_4. BMMC were pre-incubated for 30 min with the indicated concentrations of ochnaflavone and then stimulated with 100 ng/mL of KL for 15 min. LTC_4 released into the supernatant was quantified by EIA kit. All data was the arithmetic mean of triplicate determinations.

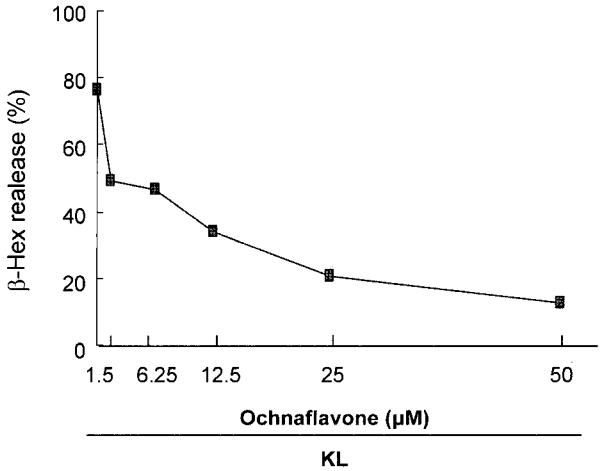

Fig. 4. Effect of ochnaflavone on the release of â-hexosaminidase. BMMC were pre-incubated for 30 min with the indicated concentrations of ochnaflavone and then stimulated with 100 ng/mL of KL for 15 min. â-hexosaminidase released into the supernatant cell lysate was measured. The procedure is described in MATERIALS AND METHODS in detail. All data was the arithmetic mean of triplicate determinations.

Among these molecules, histamine is one of the most important chemical mediators in the pathologic allergic reaction (Kitamura, 2005). When mast cells are activated by various stimuli, the release of histamine bears a close parallel to that of β-Hex, which is one of degranulation marker. Therefore, the inhibitory activity of ochanflavone on the degranulation reaction in the BMMC was examined. As shown in Fig. 4, ochnaflavone caused the dose-dependent inhibition of β-Hex release with an IC_{50} value of 3.01 μM.

The present study showed that ochnaflavone may be a useful biochemical and pharmacological tool for determining the role of COX-2/5-LOX dual inhibitors and/or antihistamine agents in certain physiological and pathological events.

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