Ginkgetin, a Biflavone from *Ginkgo biloba* Leaves, Inhibits Cyclooxygenases-2 and 5-Lipoxygenase in Mouse Bone Marrow-Derived Mast Cells

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Flavonoids are widely distributed polyphenol compounds in the plant kingdom. Some flavonoid derivatives were previously reported to possess anti-inflammatory, immunomodulatory activities in vitro and in vivo.1–4 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 phospholipase A2 (PLA2), cyclooxygenase (COX) and lipoxygenase (LOXs). Recently, it was found that some flavonoids suppressed the inducible enzyme expression such as COX-25,6 and inducible nitric oxide synthase (iNOS), thereby reducing the production of prostaglandins (PGs) and nitric oxide (NO).2,7–9 Among the flavonoids derivatives, biflavonoids are structurally unique flavonoid-dimers 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 secretory phospholipase A2 (sPLA2-IIA) inhibitor.10,11 Mast cells are one of the most important effector cells in allergic response. 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 and 5-LOX pathways; and preformed and de novo synthesized various cytokines.2–14 PGs elicit a variety of important biological responses. Among these properties are their ability to induce pain, fever and the symptoms associated with inflammatory responses. Nonsteroidal anti-inflammatory drugs (NSAIDs) 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 ginkgetin for arachidonic cascade metabolism enzymes and degranulation reaction in mast cells.

Key words Ginkgo biloba; ginkgetin; biflavonoid; cyclooxygenase-2; 5-lipoxygenase; bone marrow-derived mast cell

MATERIALS AND METHODS

Materials Ginkgetin was isolated from leaves of *Ginkgo biloba* L. (Ginkgoaceae) and identified according to the previously described method.19 Ginkgetin was dissolved 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 PGD2 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, antibi-
ototics 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. 20) To measure inhibitory activity on COX-2 by ginkgetin, cells suspended at a cell density of $5 \times 10^5$ cells/ml in enriched medium were preincubated with aspirin (10 $\mu$g/ml) for 2 h to irreversibly inactivate preexisting COX-1. After washing, BMMC were activated 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 ginkgetin 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 ginkgetin 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 ELA. 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 350 pg/10$^6$ cells. All data was the arithmetic mean of triplicate determinations.

### 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 β-mercaptoethanol at 1×10$^7$ cells/ml. The lysate (1×10$^6$ 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, U.S.A.) 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 1:3000—5000 in TBS-T. After incubation for 2 h followed by washing three times, membranes were treated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA, U.S.A.) (diluted to 1:7000 in TBS-T). The protein bands were visualized using an enhanced chemiluminescence (ECL) system (Amersham Corp., Newark, NJ, U.S.A.).

### RESULTS AND DISCUSSION

Biflavonoids such as ochnaflavone, amentoflavone and ginkgetin were found to be inhibitors of group sPLA$_2$ from rat platelet, 20) and similar data results were also obtained with morelloflavone. 21) In addition, ginkgetin and its isomer isoginkgetin, inhibited the release of arachidonic acid metabolites from rat peritoneal macrophages stimulated by phorbol ester or calcium ionophore, A$_23187$ 21) and also inhibited lymphocyte proliferation induced by T- or B-cell mitogen. 23) Furthermore, previously we also reported that ginkgetin and bilobetin inhibited tumor necrosis factor-α (TNF-α) production and COX-2 as well as iNOS protein expression in Raw264.7 cell line. 3) Taken together, data from the above studies strongly suggest that ginkgetin 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. 22) This cell model also appears 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. 23) 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. 24, 25) When the BMMC were activated with a combination of KL, IL-10 and LPS in the presence or absence of ginkgetin, the COX-2-dependent phase of PGD$_2$ generation was inhibited in a dose-dependent manner with an IC$_{50}$ value of approximately 0.75 μM (Fig. 2A), while in the presence of 10 μM of this compound did not inhibit COX-1 dependent phase of PGD$_2$ generation (data not shown).
The inhibitory effect of the ginkgetin on PGD₂ 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 and COX-2 protein expression was inhibited in a dose-dependent manner by ginkgetin.

Arachidonic acid can also be converted to leukotrienes (LTs) by the action 5-LOX in BMMC. The inhibition of 5-LOX is believed to be the ideal treatment for allergic diseases and asthma.²⁶ Therefore, the inhibitory activity of ginkgetin on the generation of LTC₄ in the BMMC was examined. Fig. 3 shows that the BMMC stimulated with KL for 15 min produced approximately 350 pg/ml LTC₄, and preincubation of the BMMC with ginkgetin resulted in the dose-dependent suppression of this LTC₄ biosynthesis with an IC₅₀ value of 0.33 μM.

Mast cells are one of the most important effector cells in allergic response. 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 FcεRI 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 leads to release of a number of biologically active molecules, including histamine, serotonin, proteoglycans and neutral proteases. Among these molecules, histamine is one of the most important chemical mediators in the pathologic allergic reaction.²⁷ 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 ginkgetin on the degranulation reaction in the BMMC was examined. As shown in Fig. 4, ginkgetin caused the dose-dependent inhibition of β-Hex release with an IC₅₀ value of 6.52 μM.

The present study showed that ginkgetin 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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REFERENCES