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

Jong Keun SON,^a Min Jung SON,^a Eunkyung LEE,^a Tae Chul MOON,^a Kun Ho SON,^b Cheorl-Ho KIM,^{*,c} Hyun Pyo KIM,^d Sam Sik KANG,^e and Hyeun Wook CHANG^{*,a}

^a College of Pharmacy, Yeungnam University; Gyongsan 712–749, Korea: ^b Department of Food and Nutrition, Andong National University; Andong 760–749, Korea: ^c Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University and National Research Laboratory for Glycobiology; Kyungju City, Kyungbuk 780–714, Korea: ^d College of Pharmacy, Kangwon National University; Chuncheon 200–701, Korea: and ^e Natural Products Research Institute, Seoul National University; Seoul 110–460, Korea.

Received August 3, 2005; accepted September 13, 2005

Ginkgetin, a biflavone from *Ginkgo biloba* leaves, was previously reported to be a phospholipase A₂ inhibitor and this compound showed the potent antiarthritic activity in rat adjuvant-induced arthritis as well as analgesic activity. This investigation was carried out to find effects on cyclooxygenase-2 (COX-2) *in vitro* effect. Ginkgetin inhibits COX-2 dependent phases of prostaglandin D₂ (PGD₂) generation in bone marrow-derived mast cells (BMMC) in a concentration-dependent manner with IC₅₀ values of 0.75 μ M. Western blotting probed with specific anti-COX-2 antibodies showed that the decrease in quantity of the PGD₂ product was accompanied by a decrease in the COX-2 protein level. In addition, this compound consistently inhibited the production of leukotriene C₄ (LTC₄) in a dose dependent manner, with an IC₅₀ value of 0.33 μ M. These results demonstrate that ginkgetin has a dual cyclooxygenase-2/5-lipoxygenase inhibitory activity. Furthermore, this compound also inhibited degranulation reaction in a dose dependent manner, with an IC₅₀ value of 6.52 μ M. Therefore, this compound might provide a basis for novel anti-inflammatory agents.

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

Flavonoids are widely distributed polyphenol compounds in the plant kingdom. Some flavonoids 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 lipoxygenases (LOXs). Recently, it was found that some flavonoids suppressed the inducible enzyme expression such as COX-2^{3,5,6)} 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.^{12–14)} 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) 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.^{15,16)} The enzyme responsible for PGs synthesis exists as two isoforms, COX-1 (constitutive isoform) and COX-2 (inducible form).^{17,18)} 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 ginkgetin for arachidonic cascade metabolism enzymes and degranulation reaction in mast cells.

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-

* To whom correspondence should be addressed. e-mail: hwchang@yu.ac.kr; chkimbio@mail.dongguk.ac.kr

Fig. 1. Chemical Structure of Ginkgetin

otics 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 5×10^5 cells/ml in enriched medium were preincubated with aspirin (10 μ 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 $120 \times g$ at 4 °C for 5 min. The supernatant and cell pellets were immediately frozen in liquid N₂ and stored at -80 °C for further analysis. PGD₂ 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 PGD₂ generation reached 1.6 ng/10⁶ cells. All data was the arithmetic mean of triplicate determinations.

LTC₄ 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 EIA. LTC₄ 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 LTC₄ reached approximately 350 pg/10⁶ 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 μ l 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 μ l of 0.2 M Glycine-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: $[S/(S+P)] \times 100$, 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 β -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, 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 ochonaflavone, amentoflavone and ginkgetin were found to be inhibitors of group sPLA₂ from rat platelet,¹⁰⁾ and similar data results were also obtained with morelloflavone.¹¹⁾ 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, A₂₃₁₈₇²¹⁾ and also inhibited lymphocyte proliferation induced by T- or B-cell mitogen.¹⁾ 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 PGD₂ biosynthetic responses over time, in addition to COX-1-dependent immediate and COX-2-dependent delayed responses. The immediate PGD₂ generation occurring within 2 h is associated with the coupling of COX-1 and the delayed PGD₂ 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 LTC₄ 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 PGD₂ generation was inhibited in a dose-dependent manner with an IC₅₀ value of approximately 0.75 μ M (Fig. 2A), while in the presence of 10 μ M of this compound did not inhibit COX-1 dependent phase of PGD₂ generation (data not shown).

Fig. 2. Effect of Ginkgetin on COX-2 Dependent PGD₂ Generation (A) and COX-2 Protein and GAPDH Expression (B)

BMMC were preincubated for 30 min with the indicated concentration of ginkgetin 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 ginkgetin. PGD₂ 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 : 3000. The procedure was described in Materials and Methods in detail.

Fig. 3. Effect of Ginkgetin on the Generation of LTC₄

BMMC were pre-incubated for 30 min with the indicated concentrations of ginkgetin and then stimulated with 100 ng/ml of KL for 15 min. LTC₄ released into the supernatant was quantified by EIA kit. All data was the arithmetic mean of triplicate determinations.

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

Fig. 4. Effect of Ginkgetin on the Release of β-Hexosaminidase

BMMC were pre-incubated for 30 min with the indicated concentrations of ginkgetin and then stimulated with 100 ng/ml of KL for 15 min. β-Hexosaminidase released into the supernatant cell lysate was measured. The procedure was described in Materials and Methods in detail. All data was the arithmetic mean of triplicate determinations.

disorders by virtue of both their ability to be activated through Fc_εRI 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.

Acknowledgment This work was supported by a Korean Research Foundation Grant (KRF-2004-005-E00003).

REFERENCES

- 1) Lee S. J., Choi J. H., Son K. H., Chang H. W., Kang S. S., Kim H. P., *Life Sci.*, **57**, 551–558 (1995).
- 2) Kim H. K., Son K. H., Chang H. W., Kang S. S., Kim H. P., *Planta Med.*, **65**, 465–467 (1999).
- 3) Baek S. H., Yun S. S., Kwon T. K., Kim J. R., Chang H. W., Kwak J. Y., Kim J. H., Kwun K. B., *Shock*, **12**, 473–478 (1999).
- 4) Kwak W. J., Han C. K., Chang H. W., Kang S. S., Park B. K., Kim H. P., *Planta Med.*, **68**, 316–321 (2002).
- 5) Kim H. P., Mani I., Iversen L., Ziboh V. A., *Prostaglandins Leukot. Essent. Fatty Acids*, **58**, 17–24 (1998).
- 6) McMurray R. W., Hardy K. J., *Am. J. Med. Sci.*, **323**, 181–189 (2002).
- 7) Sadowska-Krowicka H., Mannick E. E., Oliver P. D., Sandoval M., Zhang X. J., Eloby-Childress S., Clark D. A., Miller M. J., *Proc. Soc. Exp. Biol. Med.*, **217**, 351–357 (1998).
- 8) Cheon B. S., Kim Y. H., Son K. S., Chang H. W., Kang S. S., Kim H. P., *Planta Med.*, **66**, 596–600 (2000).
- 9) Kim S. J., Park H., Kim H. P., *Arch. Pharm. Res.*, **27**, 937–943 (2004).
- 10) Chang H. W., Baek S. H., Chung K. W., Son K. H., Kim H. P., Kang S. S., *Biochem. Biophys. Res. Commun.*, **205**, 843–849 (1994).
- 11) Gil B., Sanz M. J., Terencio M. C., Gunasegaran R., Paya M., Alcaraz M. J., *Biochem. Pharmacol.*, **53**, 733–740 (1997).

- 12) Stevens R. L., Austen K. F., *Immunol. Today*, **10**, 381—386 (1989).
- 13) Yamaguchi M., Sayama K., Yano K., Lantz C. S., Noben-Trauth N., Ra C., Costa J. J., Galli S. J., *J. Immunol.*, **162**, 5455—5465 (1999).
- 14) Murakami M., Kudo I., *Adv. Immunol.*, **77**, 163—194 (2001).
- 15) Vane J. R., *Nature: New Biol.*, **231**, 232—235 (1971).
- 16) Whittle B. J., Higgs G. A., Eakins K. E., Vane J. R., *Nature (London)*, **284**, 271—273 (1980).
- 17) Maier J. A., Hla T., Maciag T., *J. Biol. Chem.*, **265**, 10805—10808 (1990).
- 18) O'Banion M. K., Winn V. D., Young D. A., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4888—4892 (1992).
- 19) Kang S. S., Kim J. S., Kwak W. J., Kim K. H., *Kor. J. Pharmacogn.*, **21**, 111—120 (1990).
- 20) Murakami M., Kambe T., Shimbara S., Kudo I., *J. Biol. Chem.*, **274**, 3103—3115 (1999).
- 21) Lee S. J., Son K. H., Chang H. W., Kang S. S., Kim H. P., *Arch. Pharm. Res.*, **20**, 535—538 (1997).
- 22) Murakami M., Matsumoto R., Austen K. F., Arm J. P., *J. Biol. Chem.*, **269**, 22269—22275 (1994).
- 23) Murakami M., Austen K. F., Arm J. P., *J. Exp. Med.*, **182**, 197—206 (1995).
- 24) Moon T. C., Murakami M., Kudo I., Son K. H., Kim H. P., Kang S. S., Chang H. W., *Inflam. Res.*, **48**, 621—625 (1999).
- 25) Lee S. H., Son M. J., Ju H. K., Lin C. X., Moon T. C., Choi H. G., Son J. K., Chang H. W., *Biol. Pharm. Bull.*, **27**, 786—788 (2004).
- 26) Fiorucci S., Meli R., Bucci M., Cirino G., *Biochem. Pharmacol.*, **62**, 1433—1438 (2001).
- 27) Kitamura Y., *Arerugi*, **54**, 45—47 (2005).
- 28) Kim H. K., Son K. H., Chang H. W., Kang S. S., Kim H. P., *Planta Med.*, **65**, 465—467 (1999).