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Effects of Methyl Gallate on Arachidonic Acid Metabolizing Enzymes: Cyclooxygenase-2 and 5-Lipoxygenase in Mouse Bone Marrow-Derived Mast Cells

Se Jong Kim*, Meihua Jin[1]*, Eunkyung Lee[1], Tae Chul Moon[1], Zhejiu Quan[1], Ju Hye Yang[1], Kun Ho Son[2], Kil-Ung Kim[3], Jong Kun Son[1], Hyeun Wook Chang[1]

Research Institute of Natural Product Kyongbuk Provincial A. T. A, Uisung 769-803, Korea, [1]College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea, [2]Department of Food and Nutrition, Andong National University, Andong 760-749, Korea, and [3]College of Agriculture and Life Science, Kyungpook National University Daegu 702-701, Korea

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Methyl gallate (MG) is a medicinal herbal product that is isolated from *Paeonia lactiflora* 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 an IC_{50} values of 17.0 μM. This compound also found inhibited the COX-2-dependent conversion of the exogenous arachidonic acid to PGD_2 in a dose-dependent manner with an IC_{50} values of 19.0 μM, using a COX enzyme assay kit. However, at concentrations up to 80 μM, MG did not inhibit COX-2 protein expression in BMMC, indicating that MG inhibits COX-2 activity directly. Furthermore, MG consistently inhibited the production of leukotriene C_4 (LTC_4) in a dose dependent manner, with an IC_{50} value of 5.3 μM. These results demonstrate that MG has a dual cyclooxygenase-2/5-lipoxygenase inhibitory activity, which might provide the basis for novel anti-inflammatory drugs.

Key words: *Paeonia lactiflora*, Methyl gallate, Inflammation, Cyclooxygenase-2, 5-Lipoxygenase, Bone marrow-derived mast cells

INTRODUCTION

The root of *Paeonia lactiflora* Pall. Paeoniaceae) used commonly to treat the common cold, menstrual dysfunction, leukorrhea, uterine bleeding and abdominal pain (Dan and Andrew, 1986; Kim, 2001) as well as being utilized as an anti-pyretic and anti-inflammatory agent (Lin *et al.*, 1999; Yasusda *et al.*, 1999; Chou *et al.*, 2003). It was recently reported that the root of *P. lactiflora* has anti-oxidant and antimutagenic activity (Kim *et al.*, 2002; Lee *et al.*, 2002). MG is known as one of major antioxidants (Westenburg *et al.*, 2000; Galato *et al.*, 2001; Cho *et al.*, 2003; Sohi *et al.*, 2003; Lee *et al.*, 2005) from some plants. In addition, this compound has a variety of biological effects including antiplatelet activity (Lim *et al.*, 2004), protection of DNA damage from oxidative stress (Hsieh *et al.*, 2004), protection from lung injury induced by phosgene (Sciuto *et al.*, 2001), attenuation of diabetic oxidative stress (Cho *et al.*, 2004), antiapoptotic activity (Sohi *et al.*, 2003), protection of human umbilical vein endothelial cells from oxidative stress (Whang *et al.*, 2005), suppressed TH2 cytokines (Kato *et al.*, 2001), antiviral (Kane *at al.*, 1988) and anti-tumor activity (Bailey *et al.*, 1986). However, the underlying mechanisms for its anti-inflammatory activity are not completely understood. As part of an ongoing investigation into anti-inflammatory compounds from *P. lactiflora*, methyl gallate (MG) was isolated from the MeOH extract of *P. lactiflora*, and examined.

Mast cells are one of the most important effector cells in the allergic response. When activated, they release a number of biologically active molecules. These include histamine, serotonin, proteoglycans and serine proteases through exocytosis, membrane-derived lipid mediators (e.g. eicosanoids) through the activation of the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways, and the *de*

*Se Jong Kim and Meihua Jin contributed equally to this work.
Correspondence to: Hyeun Wook Chang, College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea
Tel: 82-53-810-2811, Fax: 82-53-810-4654
E-mail: hwchang@yu.ac.kr

novo synthesis of various cytokines (Stevens *et al.*, 1989; Yamaguchi *et al.*, 1999; Murakami *et al.*, 2001).

Prostagladins (PGs) elicit a variety of important biological responses. Among them are their ability to induce pain, fever and the symptoms associated with various inflammatory responses. Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce pain and inflammatory swelling by blocking PGs synthesis at the COX stage. However, most NSAIDs used clinically also inhibit the production of the PGs that are not only associated with the inflammatory processes but are also involved in maintaining normal physiological processes. The main limitation in using NSAIDs is their side effects, which include gastrointestinal ulcerogenic activity and kidney dysfunction. These effects limit their long-term therapeutic value (Vane *et al.*, 1971; Whittle *et al.*, 1980). The enzyme responsible for PGs synthesis exists in two isoforms, COX-1 (constitutive isoform) and COX-2 (inducible form) (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 inhibit the COX-2 and 5-LOX reactions simultaneously might enhance their individual anti-inflammatory effects and reduce the undesirable side effects associated with NSAIDs (Fiorucci *et al.*, 2001). This study describes for the first time a new biological action of MG on the metabolic enzymes associated with the arachidonic cascade.

MATERIALS AND METHODS

Materials

Methyl gallate (MG) was isolated from the leaves and stems *Paeonia lactiflora* Pallas. The isolated compound was chemically and structurally identified according to the previous reports (Shoyama *et al.*, 1990). Its chemical structure is shown in Fig. 1. MG 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 BMMC

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 IL-3. After 3 weeks, >98% of the cells were found to be BMMC checked by the previously described procedure (Murakami *et al.*, 1999).

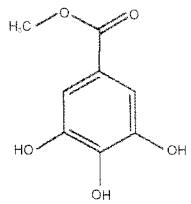

Fig. 1. Chemical structure of methyl gallate

Determination of prostaglandin D_2 (PGD_2)

For measuring inhibitory activity on COX-2 by MG, BMMC suspended at a cell density of 5×10^5 cells/mL in enriched medium were preincubated with aspirin (10 μg/mL) for 2 h in order to irreversibly inactivate preexisting COX-1. After washing, BMMC were activated with *c-kit* 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 MG previously dissolved in dimethylsulfoxide (DMSO). All reactions were stopped by centrifugation at 120 g at 4°C for 5 min. The supernatant and cell pellet were immediately frozen in liquid N_2 and stored at -80°C for further analysis. Concentrations of PGD_2 in the supernatant were measured using PGD_2 assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to manufacturer's instruction. In this assay conditions, COX-2-dependent phases of PGD_2 generation reached approximately 1.8 ng/10^6 cells. All data was the arithmetic mean of triplicate determinations.

LTC_4 determination

BMMC suspended in enriched medium at cell density of 1×10^6 cells/mL were pretreated with MG for 15 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 were determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to manufacturer's instruction. Under the conditions employed, and LTC_4 reached approximately 600 pg/10^6 cells. All data was the arithmetic mean of triplicate determinations.

Effect of MG on the COX-2 enzyme activity

COX-2 activities were measured using the colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to manufacturer's instruction. 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 lyzed 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) 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 root of *P. lactiflora* contains as its bioactive constituents oxypaeoniflorin, paeoniflonin, albiflorin, benzoyl albiflorin, gallic acid, methyl gallate, penta galloylglucose, paeonol and benzoic acid (Wu *et al.*, 1996; Lee *et al.*, 2005). As part of an ongoing study aimed at developing new COX-2/5-LOX dual inhibitors from natural products, the methanol extract of *P.lactiflora* was found to strongly inhibit the late phase of PGD_2 generation as well as LTC4 generation by BMMC. Murakami *et al.* reported that BMMC shows biphasic PGD_2 biosynthetic responses over time, as well as 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. 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 combinations of cytokines and LPS (Murakami *et al.*, 1994). This cell model also appears to be suitable for examining the effect of 5-LOX inhibitors, because 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 (Moon *et al.*, 1999; Lee *et al.*, 2004; Son *et al.*, 2005, 2006). When BMMCs are activated with a combination of KL, IL-10 and LPS in the presence or absence of MG, the COX-2-dependent phase of PGD_2 generation is inhibited in a dose-dependent manner with an IC_{50} value of approximately 17.0 μM (Fig. 2A). A colorimetric COX (ovine) inhibitor screening assay kit was used to determine if MG inhibits COX-2 dependent PGD_2 generation *via* the direct inhibition of COX-2 enzyme activity. MG inhibits the COX-2

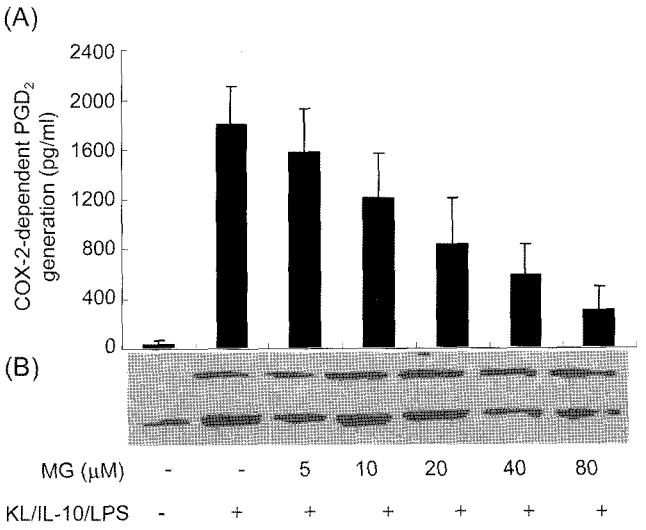

Fig. 2. Effect of MG COX-2 dependent PGD2 generation and COX-2 protein expression in the BMMC. (A) BMMC were incubated with *c-kit* ligand (KL, 100 ng/mL), IL-10 (100 U/mL) and LPS (100 ng/mL) at 37°C for 8 h in the presence or absence of indicated concentration of MG. PGD_2 released into the supernatant was quantified by EIA kit. (B) Samples were processed by SDS-PAGE and transferred to a nitrocellulose filter. The immunoblot was then probed with anti-COX-2 antibody.

dependent PGD_2 generation in a concentration-dependent manner with an IC_{50} value of 19.0 mM (Fig. 3). In addition to the direct inhibition of the COX-2 enzyme activity, the inhibitory activity of MG on the delayed generation of PGD_2 by BMMC might involve the regulation of COX-2 protein expression. This possibility was excluded from result showing that MG does not affect COX-2 protein expression (Fig. 2B). This clearly shows that the inhibition of PGD_2 production by MG in BMMCs was not due to a reduction in the COX-2 expression level. Arachidonic acid can also be converted into leukotriens (LTs) by the action of 5-lipoxygenase (5-LOX) in BMMC. The inhibition of 5-

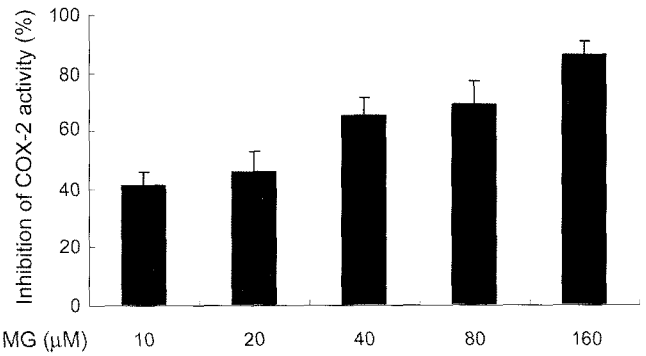

Fig. 3. Effect of MG on COX-2 enzyme activity. PGD_2 generation from endogenous arachidonic acid was assessed by the colorimetric COX (ovine) inhibitor screening assay kit. All data was the arithmetic mean of triplicate determinations.

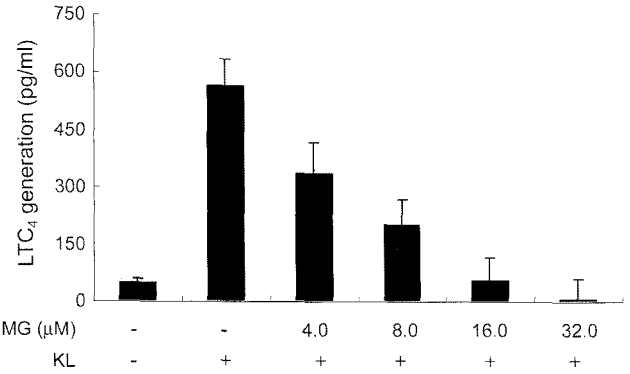

Fig. 4. Effect of MG on the generation of LTC_4. BMMC were pre-incubated for 30 min with the indicated concentrations of MG and then stimulated with 100 ng/mL of KL for 15 min. LTC_4 released into the supernatant was quantified by EIA kit. All data was the arithmetic mean of triplicate determinations.

LOX is believed to be the ideal treatment for various allergic diseases and asthma (Piepr, 1983). Therefore, the inhibitory activity of MG on the generation of LTC_4 in BMMC was examined. Fig. 4 shows that the BMMC stimulated with KL for 15 min produced ~500 pg/mL LTC_4. Preincubating the BMMC with MG resulted in the dose-dependent suppression of this LTC_4 biosynthesis with an IC_{50} value of 5.3 μM.

In conclusion, the anti-inflammatory effects of MG might be partly due to the dual inhibition of COX-2/5-LOX. Therefore, MG might be a useful biochemical and pharmacological tool for examining the role of COX-2/5-LOX dual inhibitors under certain physiological and pathological conditions.

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