Inhibition of Prostaglandin Production by a Structurally-Optimized Flavonoid Derivative, 2',4',7-Trimethoxyflavone and Cellular Action Mechanism

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Flavonoids possess anti-inflammatory activity in vitro and in vivo. In order to find the anti-inflammatory flavone derivatives having optimum chemical structures, various flavones were previously synthesized and evaluated for their inhibitory activity of prostaglandin E2 (PGE2) production from lipopolysaccharide (LPS)-treated mouse macrophage cell line, RAW 264.7 cells. Through this screening procedure, 2',4',7-trimethoxyflavone (TMF) was selected for further pharmacological study. From the present investigation, it was found that TMF potently inhibited PGE2 production from LPS-treated RAW cells with an IC50 of 0.48 μM, compared to the IC50 values of 0.07 and 1.09 μM for NS-398 and wogonin. TMF, however, did not inhibit cyclooxygenase-2 (COX-2) activity or COX-2 expression level. Instead, TMF was proved to be a phospholipase A2 (PLA2) inhibitor. The IC50 values of TMF against secretory PLA2-IIA (sPLA2-IIA) and cytosolic PLA2 (cPLA2) were 70.5 and 70.4 μM, respectively. At doses of 10—250 μg/ear, TMF also showed in vivo anti-inflammatory activity by topical application against mouse croton oil-induced ear edema assay, suggesting a potential for new anti-inflammatory agent.

Key words flavonoid; 2',4',7-trimethoxyflavone; prostaglandin; phospholipase; anti-inflammatory activity

Flavonoids from plant origin possess anti-inflammatory activity in vitro and in vivo. The several action mechanisms have been proposed to explain their in vivo anti-inflammatory activity.1,2) Many flavonoids exert anti-oxidative and free radical scavenging activities. Some derivatives modulate cellular activities of the inflammation-associated cells such as mast cells and lymphocytes. In particular, varieties of flavonoid derivatives including quercetin inhibit the activity of arachidonic acid (AA)-metabolizing enzymes; phospholipase A2 (PLA2), cyclooxygenases (COX) and/or lipoxygenases (LOX).3–5) Recent investigations have also proved that certain flavonoids, especially flavone derivatives, have capacity to down-regulate proinflammatory enzyme expression such as COX-2 (an inducible isoform of COX) and inducible nitric oxide synthase (iNOS).6,7) Since the reaction products of these proinflammatory enzymes including prostaglandins (PG) and NO are crucially involved in inflammation, several attempts have been made to derive the optimal chemical structures of flavonoids showing significant inhibition of these proinflammatory enzyme activity and/or expression. For instance, various flavone derivatives have been synthesized and their inhibitory activity of PGE2 production from bacterial lipopolysaccharide (LPS)-treated macrophages were compared in order to find flavonoids acting on PLA2 and/or COX-2.8,9) Through this screening procedure, 2',4',7-trimethoxyflavone (TMF) was found to possess a potent inhibitory activity of PGE2 production. Therefore, in the present investigation, this compound is selected for further pharmacological study and cellular action mechanism is elucidated. In vivo anti-inflammatory activity is also studied to examine the potential as new anti-inflammatory agent.

MATERIALS AND METHODS

**Chemicals** [1-14C]AA (54.6 mCi/mmol) and 1-acyl-2-[1-

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**Fig. 1.** Chemical Structures of TMF and Wogonin
acetic acid was added a catalytic amount of iodine and the reaction mixture was refluxed for 4—6 h. The solution was cooled to room temperature and poured into saturated aqueous sodium thiosulfate solution. The resulting precipitate was filtered, washed with cold water, and crystallized from dichloromethane—methanol to give TMF as a solid: mp 138—139 °C. 1H-NMR (200 MHz, CDCl3) δ 8.09—8.14 (1H, d, J = 8.6 Hz, H-5), 7.84—7.89 (1H, d, J = 8.8 Hz, H-6), 7.06 (1H, s, H-3), 6.90—6.98 (2H, m, H-6, 8), 6.55—6.65 (2H, m, H-5’, 3’). 3.92 (3H, s, —OCH3), 3.91 (3H, s, —OCH3) and 3.87 (3H, s, —OCH3); 13C-NMR (50 MHz, CDCl3) δ 177.8 (C-4’), 163.3 (C-7’), 162.5 (C-2’), 159.8 (C-4’), 158.9 (C-2’), 157.5 (C-9), 129.7 (C-5), 126.4 (C-6’), 117.1 (C-10), 113.5 (C-6), 113.1 (C-5’), 110.6 (C-3), 104.6 (C-8), 99.7 (C-1’), 98.3 (C-3’), 55.2 (—OCH3), 55.0 (—OCH3), and 54.9 (—OCH3).

And wogonin was isolated from the methanol extract of Scutellaria radix as described previously.10 The purity of the flavonoids was >95% (w/w). RAW 264.7 Cell Culture and Measurement of PGE2 Concentration RAW 264.7 cells (mouse macrophage cell line, American Type Culture Collection) were routinely cultured with DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO2 at 37 °C according to the previously described procedures.11,12 Briefly, cells were plated in 96-well plates (2 x 104 cells/200 µl/well). After pre-incubation for 2 h, test compounds including TMF and LPS (1 µg/ml) were added and incubated for 24 h, unless otherwise specified. Test compounds were dissolved in DMSO and diluted with serum-free DMEM into appropriate concentrations. Final concentrations of DMSO in the culture never exceeded 0.1% (v/v). Cell viability was assessed with MTT assay as described previously.12 PGE2 concentration in the medium was measured using a commercial EIA kit (Cayman Chemical, Co.) according to the manufacturer’s recommendation. In order to determine the direct inhibitory activity against COX-2, cells were incubated with LPS (1 µg/ml) for 24 h to induce COX-2 and completely washed with serum-free DMEM three times. Then, test compounds were added with arachidonic acid (100 µM), but without LPS, and cells were incubated for another 30 min. From the medium, PGE2 concentrations were measured as described above. In each experiment, wogonin (COX-2 inhibitory flavone) was used as a reference flavonoid.11

Western Blot Analysis For measuring the expression level of COX-2, Western blotting was used. In brief, RAW cells were cultured in 6-well plates (5 x 105 cells/well) in the presence or absence of LPS (1 µg/ml) with/without test compounds for 20 h. After preparing cell homogenate, the supernatant was obtained by centrifugation at 15000 g for 30 min. Using Tris–glycine gel (4—15%, Novex Lab.), electrophoresis was carried out and bands were blotted to PVDF membranes. The same amount of protein (10 µg) was loaded on each lane. COX-2 antibody (No-160116, Cayman Chem.) was incubated and bands were visualized by treatment of secondary antibody and DAB reagent (Vector Lab.). Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis RAW cells were cultured in 6-well plates (5 x 105 cells/well) in the presence or absence of LPS with/without test compounds for 5 h. After preparing cell homogenate, total RNA was extracted using RNeasy mini kit (Qiagen) according to the supplier’s protocol. The concentration of RNA content was determined at 260 nm and 280 nm. cDNAs were synthesized using RT reaction at 42 °C, 5 min and 99 °C, 5 min in Gene Cycler thermal cycler (Bio-Rad). Primers were synthesized on the basis of the repeated mouse cDNA sequence for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The primer sequences used for PCR were as follows: COX-2: sense, 5’-ACT CAC TCA GTT TGT GTA GTC ATT C-3’, antisense, 5’-TTT GAT TAG TAC TGT AGG GTT AAT G-3’, 583 bp; G3PDH: sense, 5’-TGA AGG TCG GTG TGA ACG GAT TTG GC-3’, antisense, 5’-CAT GTA GGC CAT GAG TGC CAC-3’, 983 bp. The amplification was performed at 94 °C for 15—60 s, 50—68 °C for 30—60 s, and 72 °C for 45—90 s with 25—30 cycles under saturation, in 25 µl reaction mixture. After amplification, 5 µl of the reaction mixture was analyzed on 1.5% agarose gel electrophoresis and the bands were visualized by ethidium bromide staining for 10 min.

Measurement of PLA2 Activity in [14C]AA-Prelabeled RAW Cells In order to label AA into membrane lipids, [1-14C]AA (0.05 µCi/well) was added to RAW cells (5 x 105 cells/well in 24-well plates) and incubated for 24 h as described previously.13 After complete washing with phosphate-buffered saline containing 0.25% (v/v) fatty acid-free bovine serum albumin, the cells were activated with LPS (1 µg/ml) with/without test compounds. The medium was collected at 5 h and radioactivity released (AA and metabolites) in the medium was counted.

PLA2 Enzyme Assay The cDNAs for human sPLA2-IIA and cPLA2 were cloned into the expression vector and were transfected into human embryonic kidney 293 cells (HEK293 cells) using LipofecAMINE PLUS as described previously.14 The culture supernatants were used as enzyme sources. The standard reaction mixture (200 µl) contained 100 mM Tris–HCl (pH 9.0), 6 mM CaCl2, 1% BSA and 2.5 µM radiolabeled 1-acyl-2-[1-14C]arachidonoyl-sn-glycerol phosphoethanolamine. The reaction was started by an addition of aliquot of culture medium as enzyme source and carried out at 37 °C for 20 min. [14C]Arachidonic acid released was extracted by the method described previously,15 and radioactivity was counted. Under this condition, an enzyme mixture in the control experiment hydrolyzed a portion of approximately 10% from the total radioactivity labeled. TMF was dissolved in DMSO and added to the enzyme assay tubes at less than 2% (v/v) of the final volume. Control experiments showed that DMSO at concentrations up to 2% had no effect on the enzyme activity.

In Vivo Anti-inflammatory Activity (Croton Oil-Induced Mouse Ear Edema Assay) Male ICR mice (specific pathogen-free, 18—22 g) were purchased from Orient Co. (Korea) and maintained in an animal facility (KNU) under conditions of 20—22 °C, 40—60% humidity and 12 h/12 h light/dark cycle at least 7 d prior to experiment. Animals were fed with Purina lab. chow and water ad libitum. In order to provoke edematous inflammation, 2.5% croton oil in acetone (25 µl) was topically applied to right ears of mice essentially following the previously described procedure.16 Five hours later, the ear thickness increased was measured using an engineering gauge (Mitutoyo Co., Japan). Test compounds dissolved in acetone (20 µl/ear) were smeared to the same site of ears 30 min before croton oil treatment.
**Statistical Analysis** Experimental values were represented as arithmetic mean ± S.D. The unpaired Student’s t-test was used to determine the statistical significance. All experiments were performed at least twice and they gave the similar results.

RESULTS

By LPS treatment, PGE$_2$ production and COX-2 expression increased greatly from RAW 264.7 cells for 24 h incubation. On this experimental condition, TMF potently inhibited PGE$_2$ production from LPS-induced RAW cells concentration-dependently when added simultaneously with LPS (Fig. 2A). The IC$_{50}$ values were 0.07, 1.09 and 0.48 μM for NS-398 (selective COX-2 inhibitor), wogonin and TMF, respectively. To examine direct COX-2 inhibitory activity, RAW cells were pre-incubated with LPS for COX-2 induction, and TMF and AA were added. In this condition, however, TMF showed only a weak inhibition of PGE2 production, while NS-398 and wogonin did strongly inhibit PGE2 production (Fig. 2B). The IC$_{50}$ values were 0.35, 7.13 and >100 μM for NS-398, wogonin and TMF, respectively. These results suggest that TMF inhibits PGE$_2$ production from LPS-induced RAW cells possibly by down-regulating COX-2 induction, but not mainly by direct COX-2 inhibition. In order to check COX-2 expression level, Western blotting technique was employed. LPS treatment induced COX-2 expression. However, COX-2 level was not reduced by the treatment of TMF (10, 100 μM), whereas wogonin (100 μM) used as a reference flavonoid strongly inhibited COX-2 expression as expected (Fig. 2C). RT-PCR analysis also verified the above finding, in that TMF did not reduce mRNA level of COX-2, but wogonin (100 μM) reduced considerably.

All these results have clearly shown that TMF strongly inhibited PGE$_2$ production from LPS-treated macrophages. Nonetheless, this inhibition was not mainly due to COX-2 inhibition or COX-2 down-regulation. On the other hand, it was speculated that TMF might reduce PGE$_2$ production from LPS-treated RAW cells by blocking AA release through PLA$_2$ inhibition. To examine this possibility, RAW cells were labeled with $[^{14}$C]-AA for 24 h. After complete washing, LPS and TMF were simultaneously added, and the released radioactivity (AA and metabolites) was measured as PLA$_2$ activity. As demonstrated in Table 1, TMF significantly inhibited AA release from RAW cells over 5—100 μM, whereas wogonin (10, 100 μM) exhibited almost no effect. More direct evidence of PLA$_2$ inhibitory action of TMF was obtained by the enzyme assay. Using culture supernatant of the transfected cells as an enzyme source, TMF was found to inhibit sPLA$_2$-IIA as well as cPLA$_2$ (Fig. 3). The IC$_{50}$ values of TMF were revealed to be 70.5 and 70.4 μM against sPLA$_2$-IIA and cPLA$_2$, respectively. When the expression level was examined from the homogenate of LPS-treated RAW cells by RT-PCR analysis, it was found that cPLA$_2$ mRNA was constitutively expressed and the expression level was not changed by the presence/absence of LPS with/without TMF (10—100 μM) (data not shown). All these findings strongly suggest that TMF is a PLA$_2$ inhibitor and may reduce PGE$_2$ production from LPS-treated RAW cells by PLA$_2$ inhibition, at least partly by cPLA$_2$ inhibition. During the present study, TMF did not significantly reduce the viability of RAW cells

![Fig. 2. Effects on PGE$_2$ Production and COX-2 Expression from RAW 264.7 Cells](image-url)
Table 1. Inhibition of Arachidonate/Metabolites Release by TMF from LPS-Treated RAW 264.7 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>µM</th>
<th>Released cpm(a)</th>
<th>% Inhibition(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1208±121</td>
<td>—</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>2178±266</td>
<td>—</td>
</tr>
<tr>
<td>TMF</td>
<td>1</td>
<td>2332±323</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1628±102</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1212±93*</td>
<td>99.6</td>
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<tr>
<td></td>
<td>50</td>
<td>1218±199*</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1238±324*</td>
<td>98.1</td>
</tr>
<tr>
<td>Wogonin</td>
<td>10</td>
<td>2135±100</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2170±109</td>
<td>0.8</td>
</tr>
</tbody>
</table>

(a) n=3, (b) % inhibition=(1−compounds/LOS)×100, in which t denotes total release and s denotes spontaneous release of the control. * p<0.01, significantly different from the LPS-treated group.

Fig. 3. Inhibition of sPLA2-IIA and cPLA2 by TMF

Enzyme assay was carried out using the supernatant of the transfected cell lines according to the described procedure in Materials and Methods. sPLA2-IIA (○), cPLA2 (●). The arithmetic means from triplicate experiments were shown here.

DISCUSSION

The present investigation has clearly shown that TMF is a PLA2 inhibitor with considerable anti-inflammatory activity in vivo, and reduces PGE2 production from LPS-treated RAW cells, at least in part, by cPLA2 inhibition. To date, three major classes of PLA2 were found from mammalian systems at least; secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2) and calcium-independent PLA2 (iPLA2). Among these, the subtypes significantly involved in inflammatory disorders are sPLA2-IIA and cPLA2 (PLA2-IVα), which couple with COX-1 and COX-2 to generate prostanooids.11 TMF is found to inhibit sPLA2-IIA as well as cPLA2.

TMF potently inhibited PGE2 production from LPS-treated RAW cells at 0.1—1 µM ranges, whereas the IC50 values of the same compound against PLA2 enzymes were 70.4—70.5 µM. Although it is not clearly understood at present, the discrepancy of the concentrations affecting each system may be partly explained by the difference in sensitivities between cell culture system and intact enzyme. Other possible explanation is that TMF may also inhibit the cellular activation of PLA2. This flavone may inhibit PLC and/or mitogen-activated protein kinase (MAPK) pathway to prevent maximum activation of cPLA2 in cells.18 These mechanisms need to be elucidated further to clearly establish the detailed action mechanism of TMF.

The primary intention of our previous synthetic and screening studies was to find the optimum chemical structures based on the natural flavonoid molecules.8 From these efforts, TMF was found to be a most potent inhibitor of PGE2 production. Although this structure has not been reported as a plant constituent, TMF is certainly one of the flavone derivatives, which may share the property of natural bioflavonoids having less side effects. Among the natural flavonoids, some flavone derivatives were found to be inhibitors of PGE2 production from LPS-treated RAW cells through down-regulation of COX-2 induction.6 Especially, wogonin was revealed as a most potent inhibitor. The inhibitory action of wogonin was mediated in part by down-regulation of COX-2 and in part by COX-2 inhibition.11 Here, a synthetic flavone (TMF) showed potent inhibition of PGE2 production, being comparable to the inhibitory potency of wogonin or having even higher inhibitory potency. However, inhibition of PGE2 production by TMF was mainly due to PLA2 inhibition, but not due to the effect on COX-2. Although a variety of flavonoids were previously demonstrated as inhibitors of eicosanoid producing enzymes (COXs and LOXs), only a few derivatives were reported so far to be PLA2 inhibitory among naturally-occurring conventional flavonoids. They include quercetin and some other flavones/flavonols such as scutellarein and nepetin.22 Several prenylated flavonoids including abyssinone V and papyriflavonol A were also reported as PLA2 inhibitors.25,26 In this respect, the present finding of new PLA2 inhibitory flavonoid is meaningful. Furthermore, unlike COX inhibition, PLA2 inhibition may block all eicosanoid synthesis such as PGS and leukotrienes as well as platelet-activating factors to give broad impact on inflammatory and allergic disorders. Therefore, TMF as a PLA2 inhibitor is a potential anti-inflammatory agent possibly with broad spectrum of action.

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REFERENCES