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Short communication

Wogonin inhibits inducible prostaglandin E_2 production in macrophages

Ichiro Wakabayashi *, Kenichi Yasui

Department of Hygiene and Preventive Medicine, School of Medicine, Yamagata University, Iida-Nishi 2-2-2, Yamagata 990-9585, Japan

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Abstract

Effects of 5,7-dihydroxy-8-methoxyflavone (wogonin) on cyclooxygenase-2 (COX-2)-mediated prostaglandin E_2 production in macrophages were investigated. Stimulation with lipopolysaccharide (LPS; 1 μg/ml) greatly increased prostaglandin E_2 production in RAW 264.7 murine macrophages. The stimulated prostaglandin E_2 production was abolished in the presence of indomethacin (1 μM) or cycloheximide (2 μM), suggesting that the increased production of prostaglandin E_2 by LPS reflects the inducible synthesis of prostaglandin E_2 by COX-2. Wogonin (0.1–50 μM) concentration-dependently inhibited inducible prostaglandin E_2 production. Wogonin at concentrations as low as 0.5 μM directly attenuated enzymatic activity of COX-2. The protein expression of COX-2 was depressed by wogonin at concentrations of 10 μM and more. These results suggest that wogonin decreases inducible prostaglandin E_2 production in macrophages by inhibiting both COX-2 activity and COX-2 expression. The former action requires much lower doses of wogonin. These wogonin actions may explain, in part, its anti-inflammatory action.

Keywords: Wogonin; Flavonoid; Cyclooxygenase; Prostaglandin; Macrophage

1. Introduction

Prostaglandin E_2 is an inflammatory mediator, converted from arachidonic acid by cyclooxygenase. It is known that there are two isoforms of cyclooxygenase: a constitutively expressed form (cyclooxygenase-1) and an inducibly expressed form (cyclooxygenase-2 (COX-2)) (Mitchell et al., 1995). In a variety of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators such as lipopolysaccharide (LPS), resulting in the release of a large amount of prostaglandin E_2 at inflammatory sites. COX-2 has been reported to be expressed in clinical conditions such as rheumatoid arthritis and osteoarthritis (Sano et al., 1992; Crofford et al., 1994), and also in animal models of inflammation (Seibert et al., 1994; Vane et al., 1994). A recent study has shown that endogenous arachidonic acid is converted to prostaglandin only by COX-2 in response to inflammatory stimuli (Shitashige et al., 1998). Since prostaglandin E_2 produced by COX-2 at inflammatory sites modulates inflammatory responses, e.g. edema, fever and pain, COX-2 inhibitors have recently received much attention as effective drugs which suppress inflammation with few gastrointestinal side effects (Crofford et al., 2000).

Flavonoids comprise a group of low molecular weight, naturally occurring plant products. They conduct a variety of biological activities such as anti-inflammatory, antioxidant, antiviral, antitumor and antiallergic actions (Pathak et al., 1991). 5,7-Dihydroxy-8-methoxyflavone (wogonin) is a flavonoid from the root of *Scutellaria baicalensis* Georgi, which is a traditional medicinal herb in China and has anti-inflammatory and smooth muscle-relaxing actions. Previous studies (Hope et al., 1983; Kimura et al., 1987) have indicated that this kind of flavonoid inhibits lipoxygenase but not cyclooxygenase (cyclooxygenase-1), resulting in decreased production of inflammatory mediators such as leukotrienes, which may in part explain the anti-inflammatory action of wogonin. Moreover, quercetin, another flavonoid, was reported to inhibit phospholipase A2 in neutrophils (Lee et al., 1982).

COX-2 and nitric oxide (NO) synthase are known to be induced simultaneously by activators such as cytokines and LPS (Mitchell et al., 1995). Some flavonoid compounds, including wogonin, have been reported to inhibit inducible NO production through the depression of an expression of inducible NO synthase (iNOS) (Kobuchi et al., 1997; Kim et al., 1999; Manjeet and Ghosh, 1999;

* Corresponding author. Tel.: +81-23-628-5252; fax: +81-23-642-7378.

E-mail address: wakabaya@med.id.yamagata-u.ac.jp (I. Wakabayashi).

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Wakabayashi, 1999). Moreover, a recent study has shown that apigenin and its related flavonoids (kaempferol and genistein) attenuate inducible production of both prostaglandin E_2 and NO due to the inhibition of nuclear factor-κB (NF-κB) activation (Liang et al., 1999). However, it has not been determined whether flavonoids from *S. baicalensis* Georgi affect inducible prostaglandin production. We thus investigated in the present study the effects of wogonin on COX-2-mediated prostaglandin E_2 production in RAW 264.7 macrophages.

2. Materials and methods

2.1. Cell culture

RAW 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum, 4 mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37°C under 5% CO_2–95% air. The cells were spread in 24-well culture plates and cultured until they reached confluency. The confluent cells were used for the assays. COX-2 was induced in RAW cells by incubation with LPS (1 μg/ml) for 24 h. In the preliminary experiments, wogonin up to 50 μM did not affect the viability of RAW macrophages evaluated by Trypan Blue dye exclusion test.

2.2. Measurement of prostaglandin E_2

RAW cells were cultured in 24-well plates. Aliquots of culture medium under each experimental condition were sampled and stored at −20°C until the assay. The prostaglandin E_2 concentration of the aliquot was measured with enzyme immunoassay using a commercial kit (prostaglandin E_2 EIA system, Amersham Life Science) within a month after sampling. The amount of prostaglandin E_2 production was expressed as the absolute value of prostaglandin E_2 (pg) per protein level (mg) in each well, or the percentage of LPS-stimulated prostaglandin E_2 release in the control treated with vehicle, which was calculated by subtracting the basal prostaglandin E_2 release from the total release.

2.3. Measurement of COX-2 activity

COX-2 activity was determined in intact RAW macrophages by measuring the accumulation of prostaglandin E_2 in the conditioned medium containing arachinonic acid. The cells cultured in 24-well plates were stimulated with LPS (1 μg/ml) for 24 h. Then, the medium in each well was removed and replaced with 0.5 ml of Dulbecco's Modified Eagle's Medium containing wogonin, indomethacin or vehicle, and incubated for 30 min. Then, arachidonic acid (1 μM) was added to the medium and further incubated at 37°C for 60 min. Next, aliquots of culture medium were sampled, and the prostaglandin E_2 concentration of the aliquot was measured as described above. After subtracting the prostaglandin E_2 level without LPS stimulation in each condition, which reflects the cyclooxygenase-1-mediated prostaglandin E_2 synthesis, inducible prostaglandin E_2 production converted from exogenous arachidonic acid was calculated, and the COX-2 activity was expressed as a percentage of the control level incubated with a vehicle instead of wogonin or indomethacin.

2.4. Protein separation and Western blotting

RAW cells were cultured in 24-well plates until reaching a confluency. Following incubation with the requisite treatments, the culture medium was removed, and the cells were washed twice in cold phosphate-buffered saline (PBS), then solubilized in hot (70°C) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The samples were dispersed by repeated passage through a 25G needle and then transferred to eppendorf tubes. The samples were boiled for 10 min and then stored at −80°C until analysis. Aliquots (20 μg protein) were subjected to SDS-PAGE on 7.5% polyacrylamide slab gels and then blotted onto polyvinylidene difluoride membrane. Polyvinylidene difluoride blots were blocked for 1 h in 150 mM NaCl, 20 mM Tris [*tris*-buffered saline (TBS), pH 7.5], containing 5% non-fat milk, and then incubated overnight at 4°C with anti-(mouse COX-2) IgG (1:250 dilution) (Transduction Laboratory) in TBS containing 5% non-fat milk. The membrane was then washed in TBS and incubated with goat anti-mouse alkaline phosphatase-conjugated antibody (1:3000 dilution) for 2 h. After further washing with TBS, blots were detected by the enhanced chemiluminescence method using an immuno-blot assay kit (Immune-Blot Assay Kit, Bio-Rad).

2.5. Protein determination

The cells were solubilized in 1 N NaOH. After neutralization with 2 N HCl, the protein concentration of the cell lysates was determined using Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

2.6. Statistics

The data were expressed as the mean with standard error. Statistical analysis was performed with analysis of variance followed by Dunnett's post-hoc test. P values of less than 0.05 were considered significant.

2.7. Chemicals

Cycloheximide (Sigma, St. Louis, MO, USA) was dissolved in PBS to make a stock solution of 1 mM and kept at 4°C. LPS (Sigma) was dissolved in PBS to make a stock solution of 100 μg/ml and kept at −20°C. Indomethacin (Wako, Osaka, Japan) was dissolved in equimolar solution

of Na_2CO_3 to make a solution of 1 mM just before use, and diluted with PBS. Wogonin (Wako) was dissolved in ethanol to make a stock solution of 10 mM. The concentration of each drug was expressed as the final concentration in the well.

3. Results

3.1. Effects of wogonin on prostaglandin E_2 production

LPS stimulation resulted in a great increase of prostaglandin E_2 production in RAW cells. The stimulated prostaglandin production was abolished by coincubation with indomethacin (1 μM) and cycloheximide (2 μM) (Fig. 1A). Wogonin (0.1–50 μM) concentration-dependently inhibited LPS-stimulated prostaglandin E_2 production (Fig. 1B). The degree of inhibition by wogonin at 10 μM was comparable to those by indomethacin and cycloheximide (Fig. 1A).

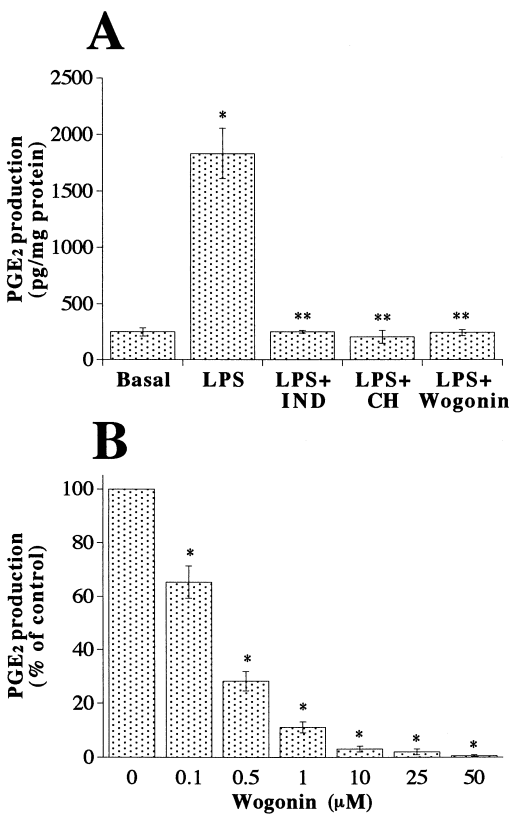

Fig. 1. (A) Effects of indomethacin, cycloheximide and wogonin on lipopolysaccharide (LPS)-stimulated prostaglandin E_2 production in RAW cells. The cells were stimulated with LPS (1 μg/ml) for 24 h. During LPS stimulation, the cells were coincubated with indomethacin (IND, 1 μM), cycloheximide (CH, 2 μM) or wogonin (10 μM) for 24 h. Basal, the basal prostaglandin E_2 production without LPS stimulation. *: Significantly different from the basal level; **: significantly different from the LPS-stimulated control $n = 5$. (B) Concentration-dependent effects of wogonin on LPS-stimulated prostaglandin E_2 production in RAW cells. The cells were stimulated with LPS (1 μg/ml) for 24 h. During LPS stimulation, the cells were coincubated with various concentrations of wogonin for 24 h. The net production of prostaglandin E_2 by cyclooxygenase-2 was calculated by subtracting the basal production from the accumulated prostaglandin E_2 production during LPS stimulation, and was expressed as the percentage of the control level (incubation with a vehicle of wogonin). Asterisks denote significant differences compared to the control ($n = 5$).

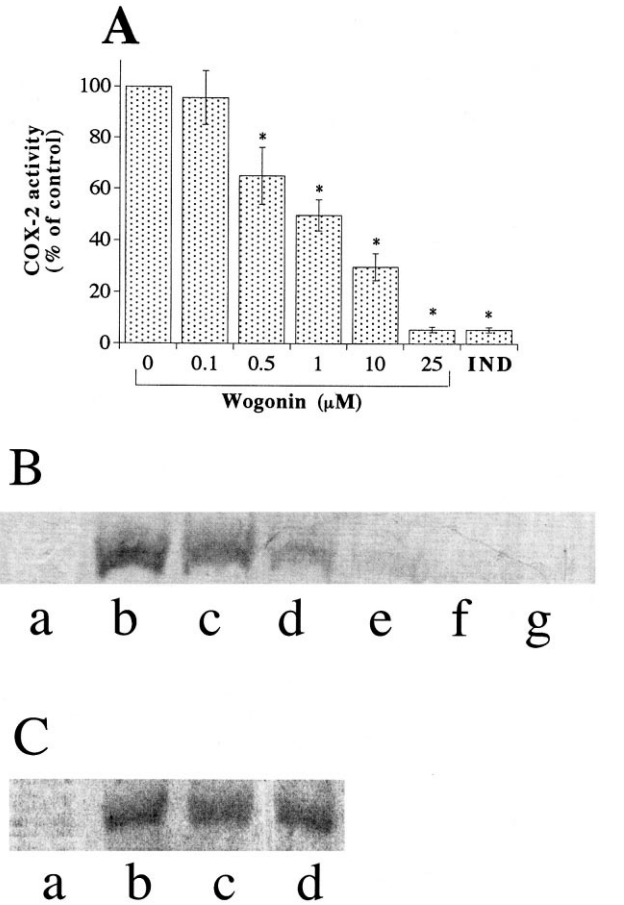

Fig. 2. (A) Effects of wogonin on cyclooxygenase-2 (COX-2) activity in RAW cells. The cells, after being stimulated with LPS (1 μg/ml) for 24 h, were pretreated with wogonin (0.1–25 μM), indomethacin (IND, 1 μM) or their vehicles for 30 min, and then further incubated with arachidonic acid (1 μM) for 60 min. The net production of prostaglandin E_2 by COX-2 during arachidonic acid incubation was obtained by subtracting the prostaglandin E_2 production without LPS stimulation, and the COX-2 activity was expressed as the percentage of the control level incubated with a vehicle instead of wogonin or indomethacin. Asterisks denote significant differences compared to the control ($n = 4$). (B) Effects of wogonin on protein expression of COX-2 in RAW macrophages: (a) basal (without LPS stimulation); (b) LPS (1 μg/ml) + vehicle; (c) LPS + wogonin (5 μM); (d) LPS + wogonin (10 μM); (e) LPS + wogonin (25 μM); (f) LPS + wogonin (50 μM); (g) LPS + cycloheximide (2 μM). (C) Effect of wogonin on the protein level of COX-2 after expression. The RAW cells were incubated with wogonin or vehicle for 90 min after COX-2 had been induced by stimulation with LPS (1 μg/ml) for 24 h. (a) Basal (without LPS); (b) LPS + vehicle; (c) LPS + wogonin (1 μM); (d) LPS + wogonin (25 μM).

3.2. Effects of wogonin on COX-2 activity

Fig. 2A shows the COX-2 activity in RAW cells. Wogonin (0.5–25 μM) concentration-dependently inhib-

ited it. The degree of the maximum inhibition by wogonin (25 μM) was comparable to that by indomethacin.

3.3. Effects of wogonin on COX-2 protein expression

Fig. 2B shows protein expression of COX-2 evaluated by Western blotting. Under basal conditions, COX-2 expression was not detectable in RAW cells (Fig. 2B, lane a). LPS stimulation for 24 h caused substantial expression of COX-2 (Fig. 2B, lane b). The COX-2 expression was concentration-dependently suppressed by coincubation with wogonin at concentrations of 10 μM and more (Fig. 2B, lanes c–f). Cycloheximide (2 μM) indeed completely inhibited the COX-2 expression (Fig. 2B, lane g). On the other hand, wogonin (1, 25 μM) did not affect the protein level of COX-2 in RAW cells, after COX-2 had been expressed by 24-h stimulation with LPS (Fig. 2C).

4. Discussion

LPS stimulation for 24 h induced a great increase in prostaglandin E_2 release from the macrophages, which was abolished to the basal level in the presence of indomethacin, a nonselective cyclooxygenase inhibitor, or cycloheximide, an inhibitor of protein synthesis. Therefore, the amount of prostaglandin E_2 increased by LPS stimulation was synthesized via the COX-2 pathway. Wogonin (above several micromolar concentrations) has recently been shown to inhibit LPS-stimulated iNOS expression and subsequent NO production in RAW macrophages (Kim et al., 1999; Wakabayashi, 1999). In the present study, lower concentrations (submicromolar) of wogonin displayed inhibitory effects on prostaglandin E_2 production in response to LPS. Similarly to the inhibitory action on inducible NO production, wogonin at 10 μM and more depressed COX-2 protein expression. NF-κB is a major activator of inflammatory genes, including iNOS and COX-2 genes (Kotake et al., 1998), and has been reported to be inhibited by apigenin, another flavonoid compound (Liang et al., 1999). Thus, wogonin may inhibit both iNOS and COX-2 expression, possibly by disturbance of NF-κB activation. In addition to the effect on COX-2 expression, wogonin also inhibited COX-2 activity as evaluated by the conversion of exogenous arachidonic acid to prostaglandin E_2 in the cells after LPS stimulation. The concentrations of wogonin inhibiting COX-2 activity are much lower (at least 1/20) than those inhibiting COX-2 expression. The limited incubation time (total of 1.5 h) for wogonin in the enzyme activity assay may explain the slightly higher threshold concentration for the inhibition of COX-2 activity than that for the inhibition of prostaglandin E_2 production in the present study (Figs. 1B and 2A). Wogonin did not affect the protein level of COX-2 after COX-2 had been expressed by LPS stimulation (Fig. 2C). Therefore, wogonin action on COX-2 is not due to a decreased stability of COX-2 or COX-2 mRNA. Moreover, neither NO production nor the protein level of iNOS was affected by wogonin (1, 25 μM) after iNOS expression, while N^G-monomethyl-L-arginine, an iNOS inhibitor, strongly attenuated NO production even after iNOS expression (data not shown). These results suggest that wogonin does not alter the protein expression nonspecifically. Moreover, the wogonin action on COX-2 in the present study may not be due to a general anti-metabolic or catabolic effect. Wogonin at 1 μM inhibited inducible prostaglandin E_2 generation by 90%, and wogonin at 10 μM completely inhibited it, which was comparable to the maximum inhibition by indomethacin, a nonselective cyclooxygenase inhibitor. Thus, wogonin is a potent COX-2 inhibitor, and the anti-inflammatory action of wogonin may be partly due to the inhibition of inducible prostaglandin production. In a recent report by Liang et al. (1999), apigenin (4′,5,7-trihydroxyflavone) and related flavonoids suppressed COX-2 expression in RAW macrophages, while only (−)-epigallocatechin-3-gallate at a high concentration (25 μM) slightly inhibited COX-2 activity. The concentrations of these compounds for suppression of COX-2 expression are similar to those of wogonin, which suppressed COX-2 expression in the present study, and much higher than the concentrations of wogonin which inhibited COX-2 activity. Therefore, wogonin possesses an additional action (direct inhibition of COX-2 activity), which results in the potent inhibitory effect on LPS-induced prostaglandin E_2 production. To our knowledge, this is the first study showing a direct potent inhibitory action of flavonoids on COX-2.

Inducible prostaglandin production by COX-2 is involved in some inflammatory diseases (Crofford et al., 2000). The concentrations of wogonin which inhibit COX-2 activity are much lower compared to those which inhibit COX-2 and iNOS gene expression. Thus, wogonin and related compounds might be useful for the development of a new selective COX-2 inhibitor. In order to clarify the mechanism of wogonin inhibition of COX-2, further studies are needed on relationship between its structure and inhibitory action.

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