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Short communication

Inhibition of TPA-induced cyclooxygenase-2 expression and skin inflammation in mice by wogonin, a plant flavone from Scutellaria radix

Byoung Kyu Park, Moon Young Heo, Haeil Park, Hyun Pyo Kim *

College of Pharmacy, Kangwon National University, Chunchon 200-701, South Korea

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Abstract

Wogonin (5,7-dihydroxy-8-methoxyflavone), isolated from Scutellaria radix, was previously reported to inhibit the expression and activity of the enzyme cyclooxygenase-2 in lipopolysaccharide (LPS)-stimulated cells of a mouse macrophage cell line, RAW 264.7. Here, in order to find in vivo effects, inhibition by wogonin of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cyclooxygenase-2 expression and anti-inflammatory activity in vivo were investigated. When applied topically to the dorsal skin of mice, wogonin at doses of 50–200 μg/site/treatment (total of five treatments in 3 days) inhibited cyclooxygenase-2 expression and prostaglandin E_2 production induced by multiple treatments with TPA. At 200 μg/site/treatment, wogonin caused a 55.3% reduction of prostaglandin E_2 production on the dorsal skin compared with an increased production in the TPA-treated control group. The same compound significantly inhibited mouse ear edema induced by TPA in both preventive (58.1% inhibition) as well as curative treatment (31.3% inhibition) schedules at 200 μg/ear/treatment. Inhibition of neutrophil infiltration was also observed. Therefore, wogonin may be beneficial for cyclooxygenase-2-related skin disorders. © 2001 Published by Elsevier Science B.V.

Keywords: Flavonoid; Wogonin; Cyclooxygenase; Skin inflammation

1. Introduction

Cyclooxygenases convert arachidonic acid to prostanoids, which provoke various biological effects. In contrast to the homeostatic function of small amounts of prostanoids synthesized by cyclooxygenase-1 (a constitutive isoform), cyclooxygenase-2, a recently identified inducible isoform of cyclooxygenase, produces large quantities of prostanoids (Needleman and Isakson, 1997). Since prostanoids (e.g. prostaglandin E_2) are deeply involved in many pathological conditions, especially inflammation-related diseases, evaluation of the modulatory activity on cyclooxygenase-2 of natural products may be important to find new anti-inflammatory agents.

Many flavonoids of plant origin show anti-inflammatory activity in vivo (Harborne and Williams, 2000). In addition to their antioxidative properties, it is thought that flavonoids inhibit inflammatory responses via modulation of the activity of enzymes associated with arachidonic acid metabolism such as phospholipase A_2, cyclooxygenase and/or lipoxygenase, depending on their chemical structure (Middleton and Kandaswami, 1994). Recently, several investigations have demonstrated that certain flavonoids, especially flavones and flavonols, inhibit the production of inflammatory mediators such as prostaglandin E_2/nitric oxide (NO) by down-regulation of cyclooxygenase-2/inducible NO synthase (iNOS; NOS type 2) expression (Liang et al., 1999; Kim et al., 1999; Chen et al., 2000; Cheon et al., 2000; Raso et al., 2001). It is now thought that flavonoids may exert anti-inflammatory activity, at least in part, by modulation of the activity of enzymes involved in arachidonic acid metabolism and/or in part by modulation of the level of expression of inducible enzymes involved in the inflammatory process.

Among the flavonoid derivatives, wogonin (5,7-dihydroxy-8-methoxyflavone) is one of the most potent inhibitors of cyclooxygenase-2 and inducible NO synthase expression in the lipopolysaccharide (LPS)-stimulated mouse macrophage cell line, RAW 264.7 (Kim et al., 1999; Wakabayashi and Yasui, 2000; Chi et al., 2001a). Moreover, the same compound inhibits cyclooxygenase-2 in the homogenate of aspirin-pretreated RAW 264.7 cells (IC_{50} = 46 μM) without affecting cyclooxygenase-1 activity (Chi et al., 2001a). Although some other flavonoids,

* Corresponding author. Tel.: +82-33-250-6915; fax: +82-33-255-9271.

E-mail address: hpkim@cc.kangwon.ac.kr (H.P. Kim).

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including several catechins, quercetin (flavonol) and sanggenon D, also inhibit cyclooxygenase-2, they inhibit cyclooxygenase-1 more strongly than cyclooxygenase-2, as evidenced by comparison of their IC_{50} values, showing no selectivity toward cyclooxygenase-2 (Noreen et al., 1998; Chi et al., in press). Thus, in addition to inhibition of cyclooxygenase-2 expression, a selective inhibition of cyclooxygenase-2 is a unique property of wogonin among the flavonoids so far tested. These previous results indicate the therapeutic potential of wogonin against cyclooxygenase-2-related inflammatory disorders. In fact, several previous studies have demonstrated the in vivo anti-inflammatory activity of wogonin following its oral administration in animal models of inflammation including arthritic inflammation (Kubo et al., 1984; Lin and Shieh, 1996). However, in vivo evidence of cyclooxygenase-2 down-regulation by wogonin has not yet been demonstrated. Furthermore, there has been no report showing its anti-inflammatory potential against chronic skin inflammation. To answer these questions, the effects of wogonin on chronic skin inflammation in mice were studied using multiple treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

2. Materials and methods

2.1. Materials

Wogonin, 5,7-dihydroxy-8-methoxyflavone, was isolated from the methanol extract of *Scutellaria baicalensis* Georgi according to the previously described procedure (You et al., 1999). The purity was $>95\%$ based on high-pressure liquid chromatographic analysis. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), prednisolone and indomethacin were purchased from Sigma (St. Louis, MO).

2.2. Animals

Male ICR mice (specific pathogen-free, 18–22 g) were obtained from Charles-River, Japan. Animals were maintained with Purina laboratory chow and water ad libitum in our animal facility, at least 7 days prior to experiments under the conditions of 21 ± 1 °C, 40–60% relative humidity and 12-h/12-h (light/dark) cycle.

2.3. TPA-induced skin inflammation and Western blotting of cyclooxygenase-2

To examine the cyclooxygenase-2 down-regulating potential of wogonin in vivo, wogonin was topically applied to the dorsal skin of mice according to a modified procedure of the original method (Stanley et al., 1991). In brief, the dorsal hair of mice was cut and removed by application of Nair®. Each group comprised three mice. After 24 h (day 1), TPA dissolved in acetone (3 μg/10 μl) was applied to the dorsal area (approximately 15×15 mm). Wogonin dissolved in oil-based vehicle (50–200 μg/10 μl vehicle/treatment) was topically smeared on the same site at 2 and 12 h after TPA treatment. On day 2, TPA was reapplied, and wogonin was applied at 2 and 12 h later. On day 3, TPA was applied and, 2 h later, wogonin was applied to the same area. Six hours after the last treatment with wogonin, mice were killed by cervical dislocation. The dorsal skin was removed and divided into two parts. For Western blotting, one part of the dorsal skin was homogenized with a Polytron (Kinetica, Swiss) in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride and 0.2 U/ml aprotinin. Protein concentration was determined by Bio-Rad protein assay. The same amounts of protein were denatured in sample buffer and subjected to electrophoresis on an 8% Tris–glycine gel. After blotting with a PVDF membrane, cyclooxygenase-1/cylooxygenase-2 bands were visualized with cyclooxygenase-1 (No-160109, Cayman Chem., Ann Arbor, MI) or cyclooxgenase-2 antibody (No-160116) and 3,3′-diaminobenzidine reagents according to the previously described procedure (Chi et al., 2001a). In order to measure prostaglandin E_2 concentration, three biopsies (4-mm punch) were obtained from the remaining part of the dorsal skin. The combined biopsies were homogenized in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 μM indomethacin. After addition of ethanol, the sample was centrifuged at $1500 \times g$ for 10 min. Then 50 mM citrate buffer (pH 3.5) was added to the supernatant, which was then centrifuged again at $2500 \times g$ for 10 min. The resulting supernatant was applied to a 6-ml Sep-Pak C_{18} cartridge (Waters Associate, USA) pre-activated with 5 ml methanol and then with 5 ml distilled water. The cartridge was rinsed with 5 ml distilled water, followed by 5 ml hexane. The final eluate was obtained with 5 ml ethyl acetate containing 1% methanol and dried under N_2 stream. After the residue was dissolved in a small amount of assay buffer, prostaglandin E_2 concentration was measured with an enzyme-linked immunoabsorbant assay (EIA) kit (Cayman Chem.) according to the manufacturer's instructions.

2.4. TPA-induced ear edema assay

To measure anti-inflammatory (anti-edema) activity in the chronic skin inflammation model, the TPA-induced ear edema assay (multiple treatment with TPA) was carried out using a similar treatment schedule as described above. Each group comprised six mice. TPA and wogonin were applied topically to the ears of mice instead of the dorsal skin in the preventive study. Prednisolone was used as a reference compound. At 12-h intervals, ear thickness was measured using a dial thickness gauge (Lux Scientific Instrument, USA), and the increase in thickness in the control group was regarded as edematous inflammation. To measure myeloperoxidase activity, an index of neutrophil infiltration, mice were killed after a final measurement of ear thickness (60 h) and biopsies were obtained with a punch (4 mm). Each biopsy was homogenized in

1.5 ml of 0.5% hexadecyltrimethylammonium bromide solution (pH 5.4) and the homogenate was centrifuged essentially following the published procedure (De Young et al., 1989). The supernatant (60 μl) was mixed with 70 μl of myeloperoxidase assay substrate kit (Sigma) and the color developed was measured in a microplate reader (Spectra Physics) at 620 nm. In order to determine the curative effect in the same animal model, TPA was applied to the ears of mice three times on three consecutive days (0, 24 and 48 h). Without further treatment with TPA, wogonin or prednisolone was applied topically to the ears of mice 60 and 84 h after the initial treatment with TPA. The ear thickness was measured at 12- or 24-h intervals. The myeloperoxidase assay was carried out at the end of the experiments as described above.

3. Results

3.1. Effects of wogonin on TPA-induced cyclooxygenase-2 expression and prostaglandin E_2 production in the dorsal skin of mice

As shown in Fig. 1, multiple treatment with TPA of the dorsal skin of mice drastically increased cyclooxygenase-2

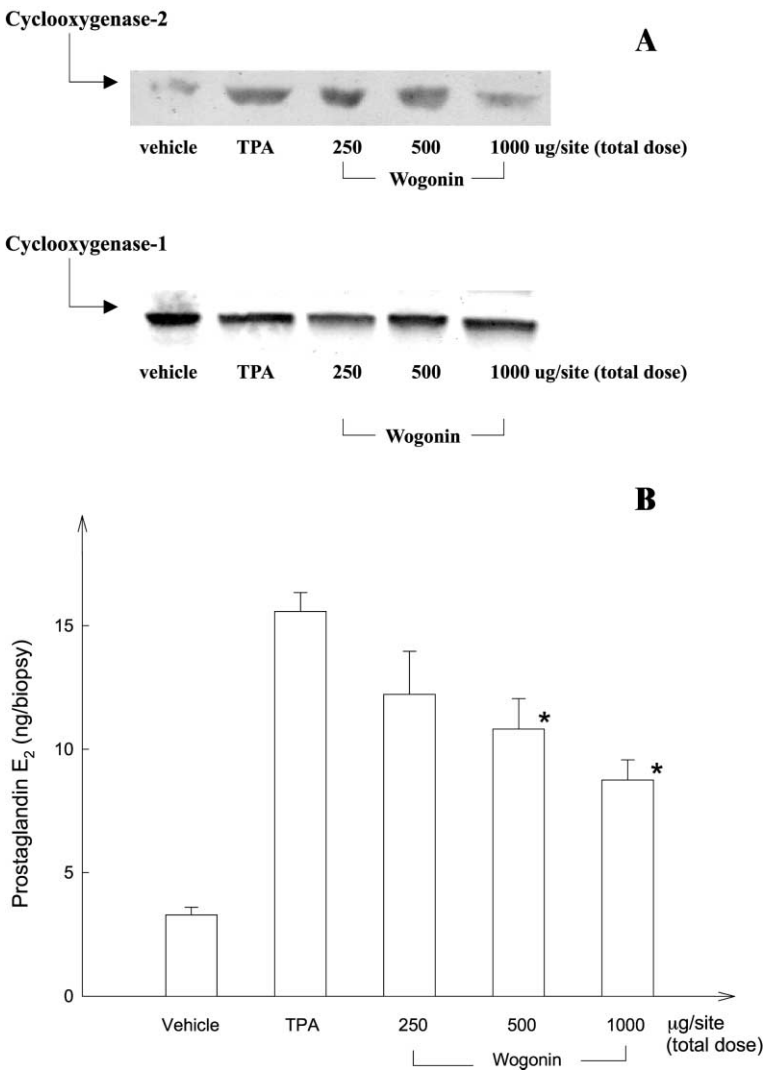

Fig. 1. Effects of wogonin on TPA-induced cyclooxygenase-2 expression and prostaglandin E_2 production in mice. (A) Effects on cyclooxygenase-1/cyclooxygenase-2 expression (Western blotting). TPA was applied topically to the dorsal area of mice three times for three consecutive days and wogonin was applied five times at each dose of 50, 100 and 200 μg/site/treatment as described in Section 2. At day 3, mice were killed and a homogenate of the skin was prepared. The same amount of protein (10 μg) was loaded on each lane. Experimental results for one of three mice were shown. Note: Cyclooxygenase-2 expression was visibly inhibited by the treatment with wogonin, while cyclooxygenase-1 expression was not affected. (B) Effects on prostaglandin E_2 concentration in the dorsal skin. From the dorsal skin of each mouse, three biopsies (4-mm punch) were obtained. In the homogenate of the combined biopsies of each mouse, prostaglandin E_2 concentration was measured using an EIA kit as described in Section 2. Prostaglandin E_2 concentration of the TPA-treated group was 15.6 ± 0.8 ng/biopsy. The data are presented as arithmetic means $\pm$ S.D. ($n = 3$). *: $P < 0.05$, significantly different from TPA-treated group by unpaired Student's *t*-test.

Table 1
Inhibition of TPA-induced ear edema by wogonin

Preventive effect

Compounds	Total dose (μg/ear)	Increase in ear thickness (mm)					Myeloperoxidase activity (OD/min/biopsy)
		12 h	24 h	36 h	48 h	60 h	
Control	–	–	0.01 ± 0.01[a]	0.01 ± 0.01	–	–	0.203 ± 0.070
TPA	–	0.13 ± 0.03	0.21 ± 0.03	0.16 ± 0.03	0.23 ± 0.04	0.31 ± 0.04	1.777 ± 0.311
Prendisolone	250	0.09 ± 0.01*	0.08 ± 0.02*	0.05 ± 0.02*	0.09 ± 0.03*	0.07 ± 0.02*	0.381 ± 0.020*
	500	0.06 ± 0.02*	0.05 ± 0.02*	0.03 ± 0.01*	0.05 ± 0.02*	0.05 ± 0.02*	0.148 ± 0.067*
Wogonin	250	0.12 ± 0.03	0.13 ± 0.02*	0.11 ± 0.02*	0.18 ± 0.03*	0.16 ± 0.02*	0.657 ± 0.393*
	500	0.11 ± 0.05	0.16 ± 0.03*	0.12 ± 0.05*	0.18 ± 0.07	0.16 ± 0.07*	0.638 ± 0.174*
	1000	0.10 ± 0.02*	0.12 ± 0.05*	0.09 ± 0.03*	0.14 ± 0.06*	0.13 ± 0.08*	0.410 ± 0.182*

Curative effect

Compounds	Total dose (μg/ear)	Increase in ear thickness (mm)				Myeloperoxidase activity (OD/min/biopsy)
		72 h	84 h	96 h	120 h	
TPA	–	0.28 ± 0.05	0.23 ± 0.06	0.19 ± 0.04	0.16 ± 0.03	0.489 ± 0.150
Prendisolone	100	0.22 ± 0.02*	0.17 ± 0.03*	0.12 ± 0.02*	0.09 ± 0.03*	0.218 ± 0.123*
	200	0.21 ± 0.03*	0.15 ± 0.03*	0.11 ± 0.04*	0.07 ± 0.03*	0.156 ± 0.132*
Wogonin	100	0.26 ± 0.06	0.21 ± 0.06	0.12 ± 0.03*	0.12 ± 0.02*	0.171 ± 0.060*
	200	0.25 ± 0.04	0.21 ± 0.04	0.14 ± 0.03*	0.12 ± 0.02*	0.175 ± 0.036*
	400	0.23 ± 0.04	0.17 ± 0.05	0.14 ± 0.04*	0.11 ± 0.03*	0.229 ± 0.092*

The data presented are for one of two sets of separate experiments.
[a] $n = 12$.
* $P < 0.05$, significantly different from TPA-treated group in each column by unpaired Student's *t*-test.

expression. The prostaglandin E_2 concentration also increased to 15.6 ± 0.8 ng/biopsy from the basal level of 3.3 ± 0.3 ng/biopsy ($n = 3$). Under the same experimental conditions, wogonin clearly down-regulated cyclooxygenase-2 expression induced by TPA, while cyclooxygenase-1 level was not affected at doses of 50–200 μg/site/treatment. Furthermore, the prostaglandin E_2 concentration in the area was significantly decreased by treatment with wogonin (55.3% reduction at 200 μg/site/treatment). It was also observed that the erythema formation induced by TPA was almost completely abolished by wogonin at the doses tested (data not shown).

3.2. Effects of wogonin on TPA-induced ear edema in mice

Table 1 shows the anti-inflammatory activity of wogonin against TPA-induced ear edema in mice. In the preventive study, the thickness of ears increased with TPA treatment, but a slight decrease in thickness was observed after the second treatment with TPA (36 h), which might reflect short-term feedback adaptation of the skin to multiple treatment with TPA. Wogonin significantly inhibited the thickness of ears at all times up to 60 h. The thickness was reduced by 48.4%, 48.4% and 58.1% for 250, 500 and 1000 μg/ear of wogonin (60 h), respectively. Prednisolone had more potent activity in the same model (83.9% reduction at 500 μg/ear). Myeloperoxidase activity also decreased significantly in prednisolone- and wogonin-treated groups. Even with the curative treatment schedules, wogonin inhibited ear edema (31.3% reduction at 400 μg/ear at 120 h) as well as myeloperoxidase activity.

4. Discussion

In the present investigation, multiple treatment with TPA of ICR mouse skin provoked cyclooxygenase-2 induction and increased prostaglandin E_2 production. Similar results were previously observed, showing that topical treatment with TPA of mouse skin increased cyclooxygenase-2 expression without changing cyclooxygenase-1 expression, which led to an increased production of prostaglandin E_2 at the site (Muller-Decker et al., 1995; Zhao et al., 1999). These biological changes were inhibited by a specific cyclooxygenase-2 inhibitor, SC-58125 (Muller-Decker et al., 1995). Under our experimental conditions, it was clearly demonstrated that wogonin inhibited cylooxygenase-2 expression and that this down-regulation of cyclooxygenase-2 was related to the reduced production of prostaglandin E_2 in the animal skin. The present in vivo results extend our previous in vitro findings for wogonin in RAW 264.7 cells (Chi et al., 2001a). In addition, wogonin dose-dependently inhibited TPA-induced mouse ear edema in both preventive and curative treatment schedules. Neutrophil infiltration was also reduced in both models, indicating that wogonin inhibited edematous inflammation as well as neutrophil infiltration regardless of its application time. These results may be important in the search for new

potential therapeutic agents since prostanoids, especially prostaglandin E_2, are associated with chronic skin inflammatory diseases such as atopic dermatitis (Fogh and Kragballe, 2000).

Several flavonoids of plant origin down-regulate cyclooxygenase-2 in cell culture. However, silymarin (flavonolignan) may be the only flavonoid derivative to down-regulate cyclooxygenase-2 in vivo in SENCAR mouse epidermis (Zhao et al., 1999). Many plant extracts in which flavonoids are main constituents have been traditionally used as topical anti-inflammatory agents in Asia. Further study of different types of flavonoids will give us a better understanding of the in vivo mechanism of action of these plant-derived chemicals.

In conclusion, wogonin may have potential as a topical anti-inflammatory agent against cyclooxygenase-2-related skin disorders, both as preventive and/or curative treatment. This is the first report showing in vivo evidence of cyclooxygenase-2 down-regulation by wogonin.

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