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Effect of wogonin, a plant flavone from Scutellaria radix, on the suppression of cyclooxygenase-2 and the induction of inducible nitric oxide synthase in lipopolysaccharide-treated RAW 264.7 cells

Yeon Sook Chi, Bong Sun Cheon, Hyun Pyo Kim*

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

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Abstract

Plant flavonoids show anti-inflammatory activity both *in vitro* and *in vivo*. Some flavonoids, such as flavone derivatives, have been reported previously to inhibit nitric oxide (NO) production by suppressing inducible nitric oxide synthase (iNOS) expression. In this investigation, the effects of wogonin, a potent inhibitor of NO production among the flavonoids tested, on cyclooxygenase-2 (COX-2) induction and activity were elucidated further in connection with iNOS, using a mouse macrophage cell line, RAW 264.7. Wogonin inhibited NO and prostaglandin E_2 (PGE_2) production from lipopolysaccharide-induced RAW cells with IC_{50} values of 31 and 0.3 μM, respectively. When added after the induction of iNOS and COX-2, wogonin inhibited the formation of PGE_2 (IC_{50} = 0.8 μM), but not the production of NO. Wogonin inhibited COX-2 activity directly (IC_{50} = 46 μM) from the homogenate of aspirin-pretreated RAW cells, as determined by measuring [^{14}C]PGE_2 formation from [^{14}C]arachidonic acid. However, it did not inhibit iNOS or phospholipase A_2 activity. Western blotting showed that wogonin suppressed the induction of both iNOS and COX-2. Prednisolone also suppressed the induction of iNOS and COX-2. Whereas RU-486 (a steroid receptor antagonist) reversed the suppressive activity of prednisolone, it did not affect the suppressive activity of wogonin, suggesting that the suppressive activity of wogonin is not mediated by binding to a steroid receptor. Results from the present study demonstrated that wogonin is a direct COX-2 inhibitor, as well as an inhibitor of iNOS and COX-2 induction. Wogonin may be a potential agent for use in the treatment of inflammatory diseases.

Keywords: Flavonoid; Wogonin; Nitric oxide; Prostaglandin; Inducible nitric oxide synthase; Cyclooxygenase-2; RAW 264.7

1. Introduction

Plant flavonoids affect inflammatory processes in mammals and possess both anti-inflammatory and immunomodulatory activities *in vitro* and *in vivo*. These anti-inflammatory and immunomodulatory actions have been reported at the cellular level to involve, in part, inhibition of COX/LO and protection from oxidation [1,2]. In addition to the direct inhibition of AA-metabolizing enzymes, recent investigations also revealed that certain flavonoids, mainly flavones such as wogonin and tectorigenin, inhibit the induction of iNOS (NOS type 2) expression in macrophages, thus reducing NO production [3–7]. These previous studies indicated that flavonoids might express anti-inflammatory activity by modulating the expression and activity of enzymes that participate in inflammatory responses, suggesting that flavonoids may be general inhibitors of inflammation-induced cell activation. Indeed, other investigators have shown recently that several flavonoids, such as apigenin and tectorigenin, inhibit the induction of COX-2, an inducible isoform of COX [8,9]. The mechanism for the inhibition of enzyme expression was reported, at least in part, as due to the suppression of NFκ-B activation, possibly by inhibition of IκB kinase activity [9]. Although these previous studies demonstrated that certain flavonoids inhibit the expression of COX-2 enzyme without directly inhibiting its activity, it is not clear whether other flavonoids directly

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

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

Abbreviations: COX, cyclooxygenase; LO, lipoxygenase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PG, prostaglandin; AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride; AA, arachidonic acid; SNAP, (±)-*S*-nitro-*N*-acetylpenicillamine; RU-486, mifepristone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LPS, lipopolysaccharide; BH_4, (6*R*)-5,6,7,8-tetrahydrobiopterin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and PLA_2, phospholipase A_2.

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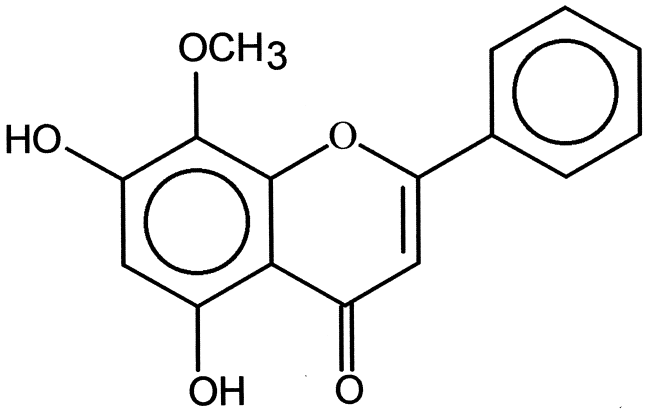

Fig. 1. Chemical structure of wogonin.

affect COX-2 activity and/or expression. In addition, although NO has been controversially reported to affect the level of COX activity and/or COX-2 expression [10,11], whether the suppressive effect of flavonoids on COX-2 induction is an independent process or an indirect phenomenon, via reduced production of NO, is yet to be answered. Therefore, in the present study, the effects of wogonin, one of the most potent flavonoid inhibitors of iNOS induction [3], on COX-2 induction and activity were further elucidated in connection with iNOS activity. It was found that wogonin potently inhibited COX-2 induction and activity, thus reducing PG synthesis. It was also determined that the down-regulation of COX-2 by wogonin was independent of the down-regulation of iNOS.

2. Materials and methods

2.1. Chemicals

AMT was purchased from Tocris Cookson Ltd. [1-^{14}C]AA (54.6 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H]AA (100 Ci/mmol), and l-[2,3,4,5-^{3}H]arginine monohydrochloride (58 Ci/mmol) were obtained from NEN. NS-398, SNAP, and RU-486 were obtained from Biomol. AA (99%), MTT, LPS (*Escherichia coli* 0127:B8), NADPH, FAD, and BH_4 were purchased from the Sigma Chemical Co. Prednisolone was obtained from the Upjohn Co. DMEM and other cell culture reagents, including FBS, were products of Gibco BRL. Dowex-50W X8 cation exchanger and a protein assay kit were purchased from Bio-Rad Laboratories. TLC plates (20 × 20 cm) were products of the Merck Co. Wogonin (Fig. 1) was isolated from Scutellaria radix and structurally identified according to a previous report [12]. The purity of wogonin was >95% (w/w).

2.2. RAW 264.7 cell culture and measurement of NO and PGE_2 concentrations

RAW 264.7 cells (obtained from the American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO_2 at 37° and activated with LPS according to previously described procedures [3]. Briefly, cells were cultured in 96-well plates (2×10^5 cells/well). After preincubation for 2 hr, test compounds including wogonin and LPS (1 μg/mL) were added and incubated for 24 hr, unless otherwise specified. Test compounds including wogonin were dissolved in DMSO on the day of experiment and diluted with serum-free DMEM into appropriate concentrations. The final concentration of DMSO was adjusted to 0.1% (v/v). Control groups also received the same amount of DMSO. Cell viability was assessed by the MTT assay based on experimental procedures described previously [13]. For determination of NO concentration, the stable conversion product of NO, nitrite (NO_2^-), was measured using the Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4]. Optical density was measured with a microplate reader (Spectra Max, Molecular Devices) at 550 nm. The PGE_2 concentration in the medium was measured using an enzyme immunoassay (EIA) kit (Cayman Chemical Co.) according to the recommendation of the manufacturer. To determine the effects of wogonin on NO and PGE_2 production after the induction of iNOS and COX-2, cells were incubated with LPS (1 μg/mL) for 24 hr and washed thoroughly three times with serum-free DMEM. Then test compounds were added without LPS, and cells were incubated for another 24 hr. NO and PGE_2 concentrations were measured in the medium as described above. To check the effect of wogonin on COX-2 activity induced by exogenously added AA, cells pre-activated with LPS for 24 hr were washed thoroughly, and AA (100 μM) and wogonin were added. Following a 15-min incubation, PGE_2 concentration was measured using an EIA kit.

2.3. iNOS and COX-2 activity assay

To isolate the iNOS-containing enzyme fraction, RAW 264.7 cells in 100-mm culture dishes were activated with LPS (1 μg/mL) for 20 hr. Cells were scraped, collected in serum-free DMEM, and washed twice with serum-free DMEM. After homogenization with a Teflon homogenizer, the sample was centrifuged at 15,000 *g* for 30 min at 4°, and the supernatant was used as a source for iNOS. iNOS activity was measured by the formation of [^{3}H]citrulline from [^{3}H]arginine following a previously described procedure [3]. Briefly, the reaction mixture (170 μL) [1 mM NADPH, 10 μM FAD, 1 mM dithiothreitol, 100 μM BH_4, 0.2 mM EGTA, [^{3}H]arginine (0.5 μCi/mL), and 50 μL homogenate in 50 mM HEPES buffer (pH 7.5)] was incubated at 37° for 1 hr. The reaction was terminated by the addition of 200 μL of ice-cold stop buffer (50 mM morpholinoethanesulfonic acid, pH 5.5, and 5 mM EDTA). The sample was passed through Dowex-50W X8 resin, and the radioactivity of the eluate was counted using a liquid scintillation counter (LKB model 1209).

For the COX-2 activity assay, RAW 264.7 cells were pretreated with aspirin (250 μM) for 1.5 hr to inactivate COX-1 enzyme. After washing the cells three times with serum-free DMEM, LPS (1 μg/mL) was added to induce COX-2. Twenty hours later, cells were scraped and homogenized in 20 mM Tris–HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 330 mM sucrose, and 2 mM phenylmethylsulfonyl fluoride. After centrifugation, the supernatant obtained was used as the source for COX-2 enzyme. COX-2 activity was measured using [^{14}C]AA as a substrate according to a previously reported method [14]. The COX assay system contained the cell homogenate in 100 mM Tris–HCl buffer, pH 8.0, with 5 mM EDTA, 2 mM reduced glutathione, 50 mM l-tryptophan, and 2 μM hemoglobin with or without the test compounds. The reaction mixture (100 μL) including the homogenate (20 μg protein) was added to an Eppendorf tube containing [^{14}C]AA (0.01 μCi), and the mixture was incubated at 37° for 20 min. The reaction was terminated by adding 50 μL of ice-cold 0.15 N HCl. The reaction products were extracted with 900 μL of Folch solution, and the organic layer was dried under N_2. The products were separated twice by TLC using ethyl acetate:acetic acid (99:1) as a mobile phase. After autoradiography for 7 days, spots corresponding to PGE_2 were scraped and measured for radioactivity.

2.4. Separation of AA metabolites by HPLC

The extracted and dried metabolites prepared as above were also separated by HPLC (Shimatzu LC-10AT) connected to an on-line radioactivity flow detector (Packard 150TR). The sample solutions were run through an ODS-II reverse phase column (15 cm, Shinwa Chem.) at a flow rate of 1 mL/min. The metabolites were separated by gradient elution (solvent gradients A:B, 94:6 $\rightarrow$ 0:100; solvent A: 26% acetonitrile, 10% methanol, and 0.02% acetic acid in water; solvent B: 0.05% acetic acid in acetonitrile) for 50 min.

2.5. Western blot of iNOS and COX-2

For measuring the protein level of iNOS and COX-2, a western blotting technique was used [3]. RAW 264.7 cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 μg/mL) with or without the test compounds for 20 hr, unless otherwise indicated. After preparing the cell homogenate, the supernatant was obtained by centrifugation at 15,000 *g* for 30 min. Proteins were separated by Tris–glycine gel (4–15%, Novex Lab.) electrophoresis and were blotted to PVDF membranes. iNOS and COX-2 were detected by incubation with iNOS (N32030, Transduction Lab.) and COX-2 (No. 60116, Cayman Chemical Co.) antibodies. iNOS and COX-2 were visualized by exposure of the membranes to a horseradish peroxidase-conjugated secondary antibody and DAB reagent (Vector Lab.).

2.6. Measurement of PLA_2 activity in [^{3}H]AA-prelabeled RAW 264.7 cells

[^{3}H]AA (0.25 μCi/well) was added to RAW 264.7 cells (5×10^5 cells/well in 24-well plates) in DMEM supplemented with 10% FBS and 1% antibiotics and incubated for 24 hr. After complete washing with PBS containing 0.25% (w/v) fatty acid-free BSA, the cells were activated with LPS (1 μg/mL). The medium was collected at appropriate time intervals, and the radioactivity released into the medium was counted.

2.7. Statistical analysis

All values are presented as arithmetic means $\pm$ SD. Student's unpaired *t*-test was used to determine statistical significance. All experiments were performed at least twice and gave similar results.

3. Results

3.1. Inhibition by wogonin of NO and PGE_2 production from LPS-induced RAW 264.7 cells

When RAW 264.7 cells were exposed to LPS for 24 hr, the medium concentration of NO and PGE_2 was 59.2 $\pm$ 4.9 μM (32.3 $\pm$ 1.1 nmol/well/2 $\times$ 10^5 cells) and 34.9 $\pm$ 1.3 nM (2.46 $\pm$ 0.14 ng/well), respectively (N = 3). The basal medium concentrations, in the absence of LPS, were 1.0 $\pm$ 0.1 μM for NO and 3.1 $\pm$ 0.1 nM for PGE_2. As shown in Fig. 2, prednisolone (an anti-inflammatory steroid) potently reduced the production of PGE_2 ($IC_{50} < 0.1$ μM); it also reduced the production of NO but not as strongly. As expected, AMT (an iNOS inhibitor) dramatically inhibited NO production ($IC_{50} = 0.3$ μM) without inhibiting the synthesis of PGE_2. Wogonin inhibited the production of NO and PGE_2 in a concentration-dependent manner. The IC_{50} values of wogonin for NO and PGE_2 production were 31 and 0.3 μM, respectively. Wogonin inhibited PGE_2 synthesis more potently than NO production. In this experiment, no cytotoxic effects of prednisolone, AMT, or wogonin on RAW 264.7 cells in the presence or absence of LPS were observed at the concentrations tested. Figure 3 shows the effects of wogonin on the production of NO and PGE_2 during a 24-hr incubation period when iNOS and COX-2 were pre-induced with LPS. When added after activation with LPS, prednisolone reduced PGE_2 production by 37% (at 10 μM), a reduction that was not statistically significant. AMT strongly reduced NO production ($IC_{50} = 0.3$ μM) as expected, whereas its inhibition of PGE_2 production was weak (39% inhibition at 50 μM). Wogonin potently inhibited the production of PGE_2 in a concentration-dependent fashion ($IC_{50} = 0.8$ μM), but it did not inhibit NO production. To exclude PLA_2 activity, AA was exogenously added to RAW 264.7 cells, which were pre-activated with LPS. After a

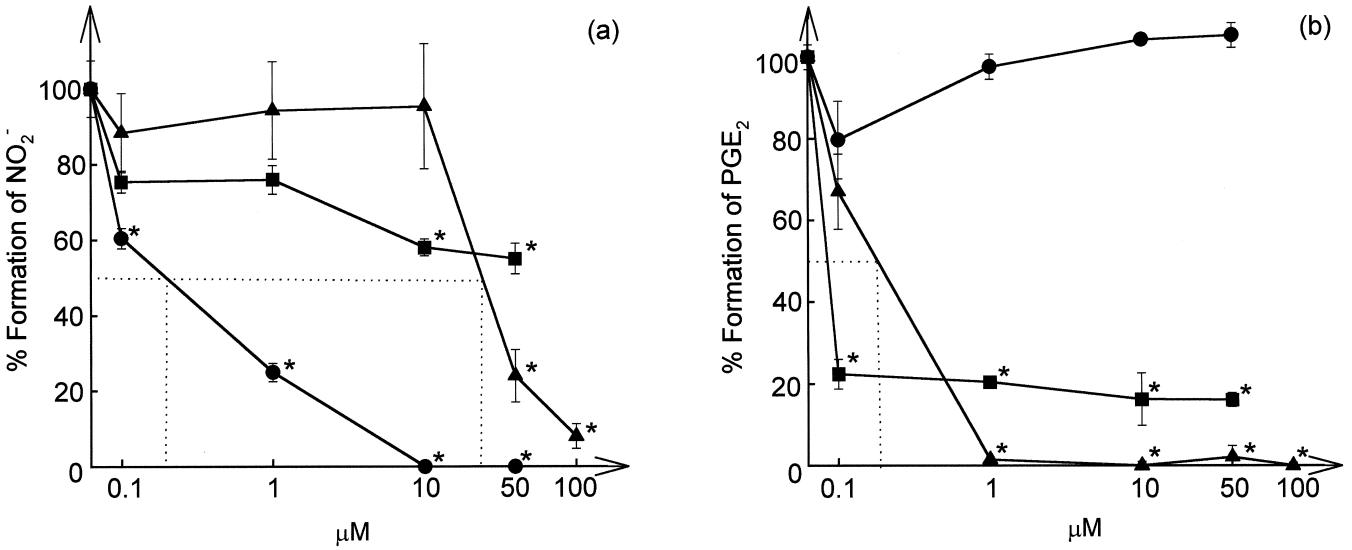

Fig. 2. Effects of wogonin on NO (a) and PGE_2 (b) production from LPS-induced RAW 264.7 cells. RAW 264.7 cells were incubated with LPS (1 μg/mL) and test compounds [AMT (●), prednisolone (■), and wogonin (▲)] for 24 hr, and NO and PGE_2 concentrations in the medium were measured as described in "Materials and methods." Basal levels of NO and PGE_2 without LPS were 1.0 ± 0.1 μM and 3.1 ± 0.1 nM, respectively. LPS treatment increased NO and PGE_2 production to 52.9 ± 4.9 μM and 34.9 ± 1.3 nM, respectively. Percent formation was calculated after subtracting basal level concentrations of NO and PGE_2. The data points and bars represent arithmetic means ± SD (N = 3). Key: (*) $P < 0.001$, significantly different from the LPS-treated control group.

15-min incubation, wogonin still inhibited PGE_2 formation (IC_{50} = 29 μM) but not NO production (Fig. 4).

3.2. Effects of wogonin on iNOS and COX-2 enzyme activities

The effect of wogonin on the enzyme activities of iNOS and COX-2 were examined using cell homogenates. As shown in panels a and b of Fig. 5, wogonin inhibited COX-2 activity directly in a concentration-dependent manner (IC_{50} = 46 μM), while it did not inhibit iNOS activity at these same concentrations. The IC_{50} values of AMT and NS-398 (a preferential COX-2 inhibitor) for iNOS and COX-2 activity were 0.5 and 4.9 μM, respectively. Using autoradiography (Fig. 5c) and HPLC separation (Fig. 5d) of PG production, it was also shown

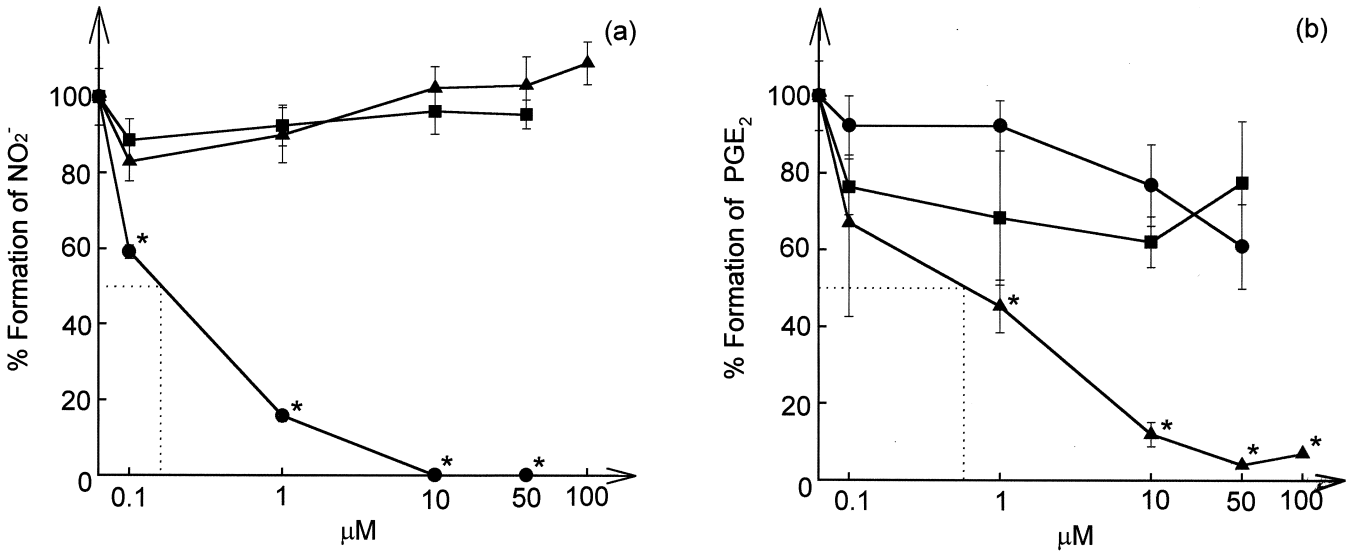

Fig. 3. Effects of wogonin on NO (a) and PGE_2 (b) production from RAW 264.7 cells pre- induced by LPS. RAW 264.7 cells were incubated with LPS for 24 hr, and test compounds [AMT (●), prednisolone (■), and wogonin (▲)] were added. After incubation for another 24 hr, NO and PGE_2 concentrations in the medium were measured. One hundred percent formation represented 42.3 ± 4.2 μM NO and 26.4 ± 3.3 nM PGE_2. The data points and bars represent arithmetic means ± SD (N = 3). Key: (*) $P < 0.001$, significantly different from the LPS-treated control group.

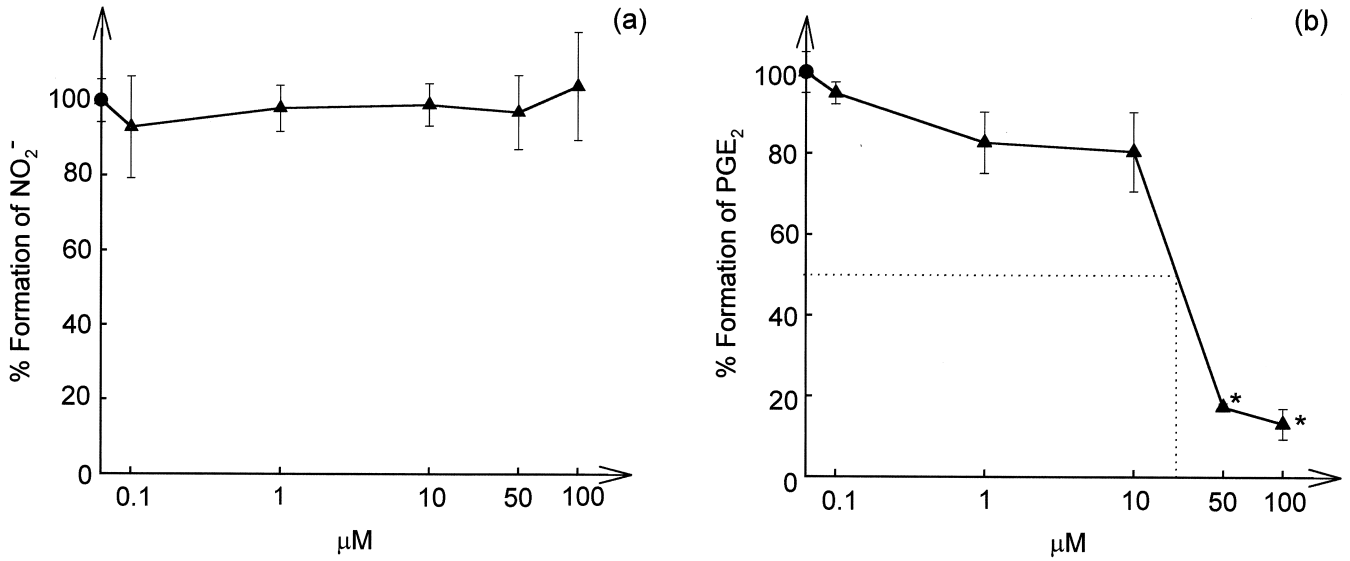

Fig. 4. Effects of wogonin on NO (a) and PGE_2 (b) production from RAW cells pre-induced with exogenously added AA. RAW 264.7 cells were incubated with LPS for 24 hr. Wogonin and AA (100 μM) were added. After incubation for another 15 min, NO and PGE_2 concentrations in the medium were measured. One hundred percent formation represented 1.8 ± 0.1 μM NO and 46.9 ± 2.1 nM PGE_2. The data points and bars represent arithmetic means ± SD (N = 3). Key: (*) $P < 0.001$, significantly different from the LPS-treated control group.

that wogonin (100 μM) inhibited both PGD_2 and PGE_2 production.

3.3. Effects of wogonin on iNOS and COX-2 induction

Enzyme levels were checked with a western blotting technique. Wogonin (100 μM) and prednisolone (1 and 10 μM) inhibited induction of both iNOS and COX-2 in LPS-induced RAW 264.7 cells (Fig. 6, a and b). When the effect of incubation time on the induction of iNOS and COX-2 was examined in the LPS-induced cells, it was seen that for both enzymes induction began at 5 hr and reached a maximum at about 10–20 hr after LPS addition. When added simultaneously with LPS, wogonin (100 μM) constantly inhibited the induction of both of the inducible enzymes throughout all the incubation periods (Fig. 6, c and d).

3.4. Effect of wogonin on PLA_2 activity from LPS-induced RAW 264.7 cells

When RAW 264.7 cells were labeled with [^{3}H]AA and then activated by LPS, they released approximately 2.3-fold more total radioactivity into the medium in the first hour than did cells not treated with LPS. After 1 hr of incubation the total radioactivity released into the medium plateaued and remained stable over the 22-hr time course. Wogonin (100 μM) slightly increased the release of total radioactivity ([^{3}H]AA released/metabolites) from the LPS-induced RAW 264.7 cells over the same period (Fig. 7).

3.5. Effect of RU-486 on inhibition of NO production by wogonin

In this system, prednisolone significantly inhibited NO production at 10 μM. When RU-486, a steroid receptor antagonist, was added, NO productivity was recovered in a concentration-dependent fashion (0.1 to 10 μM). In contrast, the addition of RU-486 did not increase NO production in the presence of wogonin (Fig. 8a). When iNOS levels were checked by western blotting, RU-486 (10 μM) was clearly shown to reverse the suppressive effects of prednisolone on iNOS induction. In contrast, no change was observed in RU-486/wogonin-treated cells (Fig. 8b).

3.6. Effects of an NO donor or PGE_2 on the suppression of COX-2 or iNOS induction by wogonin

The suppressive activity of wogonin on COX-2 or iNOS induction by LPS treatment was challenged with an NO donor (SNAP) or PGE_2. However, the inhibition of COX-2 induction by wogonin was not changed by treatment with the NO donor (Fig. 9a), nor did the addition of PGE_2 (1–100 pg/mL) change the production level of NO (Fig. 9b).

4. Discussion

Previously, various flavonoids including wogonin were reported to be inhibitors of iNOS induction [3–7], and wogonin was found to be the most potent of the flavonoids tested thus far [3]. Several investigators have also demonstrated that some flavonoid derivatives, such as apigenin

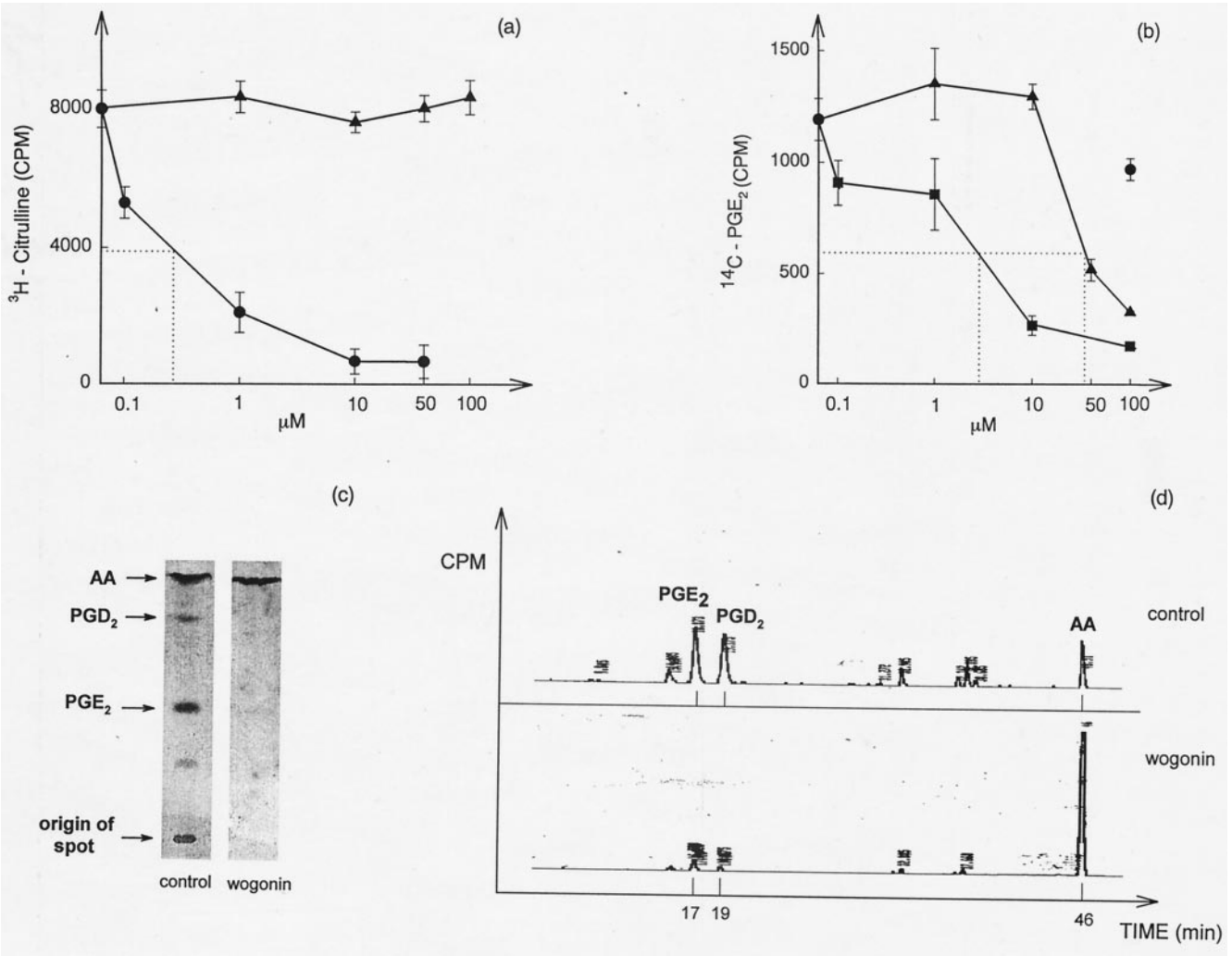

Fig. 5. Effects of wogonin on iNOS and COX-2 enzyme activities. (a) Effect on iNOS activity. [^{3}H]Citrulline formation from [^{3}H]arginine was measured in the presence of AMT (●) or wogonin (▲). (b) Effect on COX-2 activity. [^{14}C]PGE$_2$ formation from [^{14}C]AA was measured in the presence of aspirin (●), NS-398 (■), or wogonin (▲). (c) Autoradiogram of the effect of wogonin (100 μM) on PGD$_2$ and PGE$_2$ formation. (d) HPLC chromatographic profile of the effect of wogonin (100 μM) on PGD$_2$ and PGE$_2$ production.

and tectorigenin, inhibit COX-2 induction [8, 9]. This investigation was designed to elucidate the effects of wogonin on COX-2 in relation to the effects on iNOS, and to determine some mechanisms of action. The effects of wogonin on iNOS found in this study were in accord with results reported previously [3, 7].

The present study clearly demonstrated that wogonin at 1–100 μM inhibited NO as well as PGE$_2$ production via suppression of the induction of both iNOS and COX-2 in LPS-induced RAW 264.7 cells. But the potency with which wogonin reduced NO and PGE$_2$ production was somewhat different. Wogonin inhibited PGE$_2$ production more potently than NO production. The reason for the various sensitivities to inhibition by wogonin is not known at present. It may be explained in part by the fact that, in addition to the suppressive effects of wogonin on iNOS and COX-2 induction, it also inhibited COX-2 activity from the homogenate of LPS-induced RAW 264.7 cells, whereas the same flavonoid did not affect iNOS activity (Fig. 5). It was also shown that wogonin inhibited PGE$_2$ synthesis, but not NO production, when it was added after iNOS and COX-2 were induced (Figs. 3 and 4). These findings support the hypothesis that wogonin behaves as a direct COX-2 inhibitor. In contrast, wogonin did not reduce the basal level of PGE$_2$ production, suggesting that wogonin might not inhibit COX-1 activity (data not shown). This speculation is also supported by our previous study demonstrating that up to 100 μM wogonin did not significantly inhibit COX-1 activity from human platelet homogenates [12].

PGE$_2$ is formed from its precursor, AA. Thus, the reduction of PGE$_2$ production by wogonin might be caused by the reduction of free AA released from the membrane, which is mainly catalyzed by PLA$_2$. This possibility was checked in [^{3}H]AA-prelabeled cells, and it was found that wogonin did not inhibit PLA$_2$ activity (Fig. 7). A similar observation that tectorigenin inhibits COX-2 expression, but not PLA$_2$ ac-

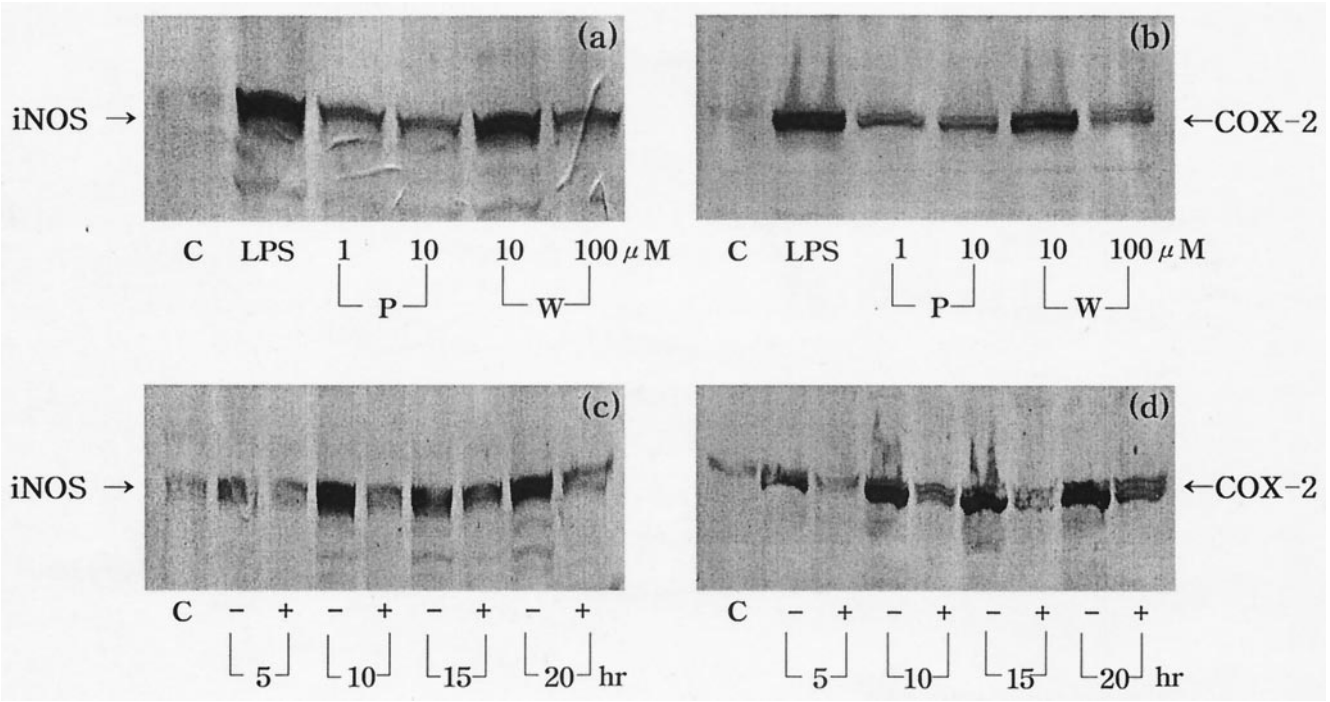

Fig. 6. Effects of wogonin on iNOS and COX-2 expression (western blot). (a,b) Effects on iNOS and COX-2 expression in the presence of prednisolone (P) or wogonin (W). (c,d) Time-dependent effects of wogonin on iNOS and COX-2 expression. Key: (+) presence or (−) absence of wogonin (100 μM). Numbers indicate the incubation time (hr) after LPS treatment.

tivity in mouse macrophages was reported previously [8]. From our study, it is evident that wogonin inhibits the induction of COX-2 expression and activity, thereby reducing PG production. There have been only a few reports describing the direct inhibition of COX-2 by flavonoids. Flavan-3-ols such as (+)-catechin, 4′-methyl-ent-gallocatechin, and mearnsitrin weakly inhibited COX-2 at very high concentrations (>100 μM), and they were not selective for COX-2 over COX-1 [15,16]. To the best of our knowledge, this is the first paper to find a flavonoid compound having COX-2 inhibitory activity at reasonable concentrations.

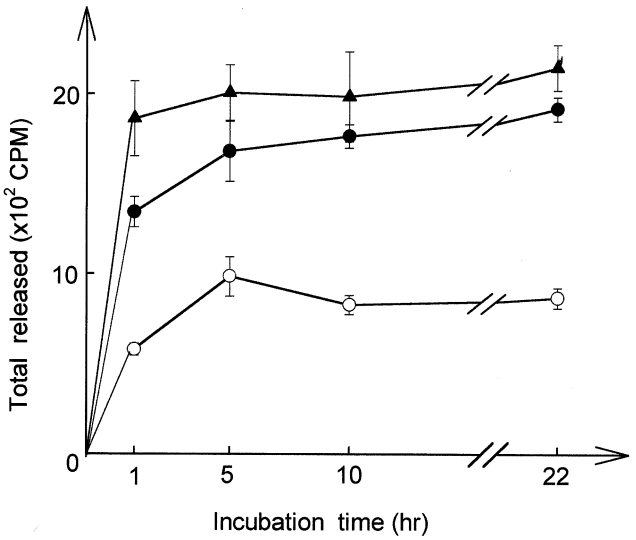

Fig. 7. Effect of wogonin on PLA_2 activity in RAW 264.7 cells prelabeled with [^{3}H]AA. RAW 264.7 cells were labeled with [^{3}H]AA for 24 hr. Wogonin (100 μM) and LPS were added, and the radioactivity released into the medium was counted at appropriate time intervals. Key: vehicle (○), LPS (●), and LPS and wogonin (▲). The data points and bars represent arithmetic means ± SD (N = 3). There was no statistically significant difference ($P < 0.001$) between the LPS-treated and the LPS plus wogonin-treated groups.

The suppressive effects of wogonin on iNOS and COX-2 expression were similar to those of an anti-inflammatory steroid (prednisolone). Glucocorticoids are well known as potent anti-inflammatory agents, down-regulating iNOS and COX-2 expression via a receptor-mediated process [17]. The mechanism of action of steroidal anti-inflammatory drugs for the suppression of these inducible enzymes is not understood completely. They suppress gene expression through glucocorticoid receptor complex formation, possibly mediated by direct interaction between the steroid receptor and nuclear transcription factors like NF-κB [18] or by binding to glucocorticoid responsive elements that regulate the transcription of various known genes. Figure 6 demonstrated that prednisolone down-regulated iNOS and COX-2 expression. As expected, RU-486 (a steroid receptor antagonist) blocked the suppressive activity of prednisolone on NO production as well as enzyme expression (Fig. 8). However, the same compound did not block the suppressive effects of wogonin. These results indicate that the cellular mechanism of action of wogonin is quite different from the steroidal action mechanism, and the effect on enzyme expression by wogonin is not mediated via complex formation

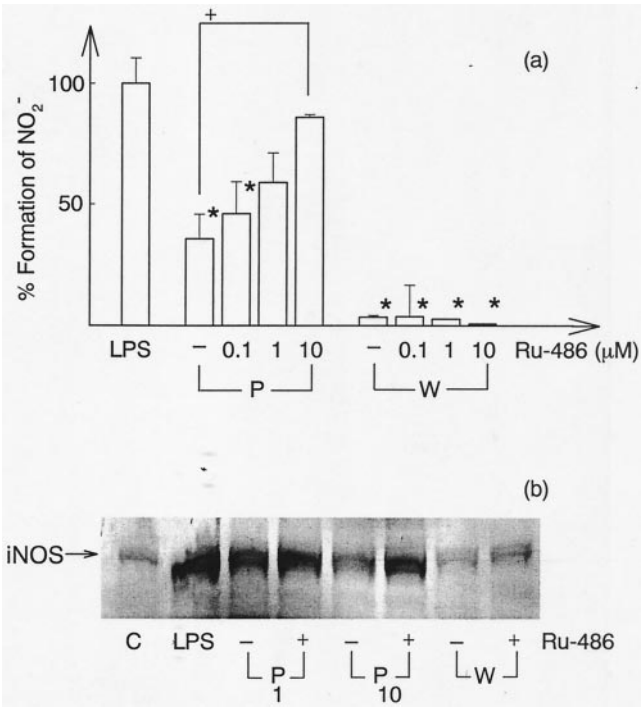

Fig. 8. Effect of RU-486 on the suppressive activity of NO production by wogonin. (a) Effect on NO production. LPS and prednisolone (P, 10 μM) or wogonin (W, 100 μM) in the presence (0.1, 1, 10 μM) or absence (−) of RU-486 were added to RAW 264.7 cells, and NO concentration was measured after 24 hr of incubation. The LPS-treated control produced 55.0 ± 5.2 μM NO (100%). The data points and bars represent arithmetic means ± SD. Key: (*) $P < 0.001$, significantly different from the LPS-treated control group, and (+) $P < 0.001$, significantly different from the group indicated. N = 3. (b) Effect on iNOS expression in RAW 264.7 cells (western blot). LPS and prednisolone (P, 1 and 10 μM) or wogonin (W, 100 μM) in the presence (+) or absence (−) of RU-486 (10 μM) were added to the cells. After 20 hr, electrophoresis was carried out on the cell homogenate.

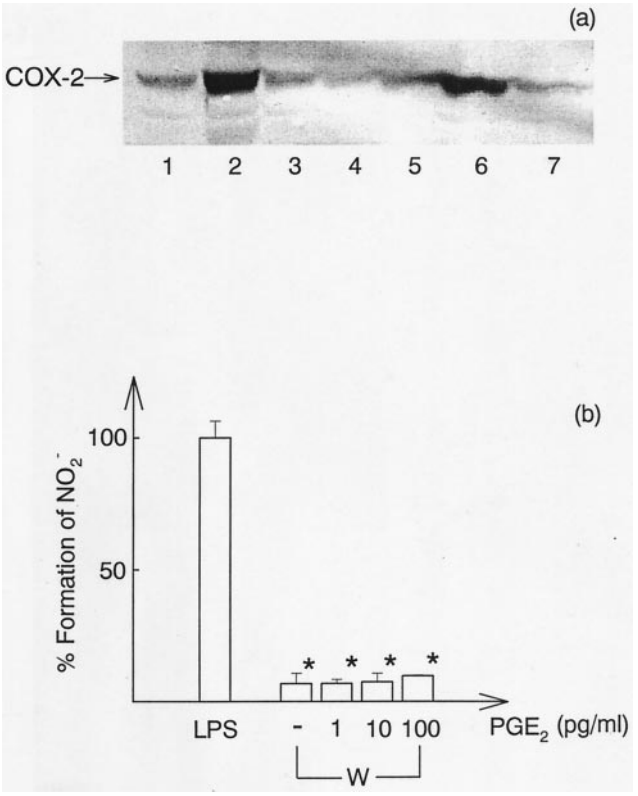

Fig. 9. Effects of an NO donor (SNAP) and PGE_2 on COX-2 and NO production. (a) Effect of SNAP on COX-2 expression (western blot). Lane 1: vehicle; 2: LPS; 3: LPS and wogonin (100 μM); 4: SNAP (100 μM); 5: SNAP (100 μM) and wogonin (100 μM); 6: LPS and SNAP (100 μM); and 7: LPS and SNAP (100 μM) plus wogonin (100 μM). (b) Effect of PGE_2 on NO production in RAW 264.7 cells. LPS and wogonin (W, 100 μM) in the presence (1, 10, 100 pg/mL) or absence (−) of PGE_2 were added to the cells. NO concentration was measured after 24 hr of incubation. The LPS-treated control produced 54.9 ± 4.0 μM NO (100%). The data points and bars represent arithmetic means ± SD. Key: (*) $P < 0.001$, significantly different from the LPS-treated control group. N = 3.

with a glucocorticoid receptor. There have been some previous reports showing the inhibition of NF-κB activation by several flavonoid derivatives [5,9]. However, whether wogonin inhibits *de novo* protein synthesis by modification of nuclear transcription factors or by another mechanism(s) remains to be elucidated.

Several investigators have claimed that NO affects the level of COX-2 and that PGE_2 affects NO production. For instance, elevated levels of NO induce COX-2 expression [10]. Thus, the wagonin-induced reduction in NO may suppress COX-2 expression indirectly. In checking this possibility, we found that when SNAP (an NO donor) was added to wogonin-treated cells, it did not restore COX-2 levels, and PGE_2 added exogenously did not change the suppressive action of wogonin on NO production (Fig. 9). These results show that the inhibition of iNOS and of COX-2 expression by wogonin are independent processes, and that the decreased levels of NO and PGE_2 did not affect the levels of COX-2 and iNOS, at least in our experimental system.

Wogonin is a major component of members of the Scutellaria species. It has been used as an anti-inflammatory agent in Chinese medicine in East Asia since ancient times. The methanol extract of *Scutellaria baicalensis* and its major constituent (wogonin) were reported to possess anti-inflammatory activity *in vivo* in an animal model for chronic arthritis [19]. The present investigation gives a scientific basis for using the Scutellaria species as a source for anti-inflammatory agents. Our study also opens the possibility for using plant flavonoids, including wogonin, in cancer chemoprevention or anticancer therapy since elevated COX-2 levels have been found in certain types of cancer.

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