

The plant flavonoid wogonin suppresses death of activated C6 rat glial cells by inhibiting nitric oxide production

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Abstract

Flavonoids are a group of low molecular weight polyphenolic compounds derived from plants. 5,7-dihydroxy-8-methoxyflavone (Wogonin), a flavonoid originated from the root of *Scutellaria baicalensis* Georgi, has been shown to exert various anti-inflammatory effects such as inhibition of nitric oxide (NO) and prostaglandin E₂ production in macrophages. Because glial cells have been previously shown to undergo NO-dependent apoptosis upon inflammatory activation and this auto-regulatory process may be negatively affected by exogenous factors possessing anti-inflammatory activities, we examined the effects of wogonin on NO production and activation-induced cell death of C6 rat glial cells. Activation of C6 glial cells with lipopolysaccharide (LPS), interferon- γ , and tumor necrosis factor- α induced NO production followed by cell death. Pretreatment of C6 cells with wogonin before LPS and cytokine treatment dose-dependently inhibited NO production as well as death of activated C6 cells. Wogonin-mediated inhibition of NO production was accompanied by suppression of inducible nitric oxide synthase (iNOS) protein induction and nuclear factor kappa B (NF- κ B) reporter activity. Wogonin, however, did not affect a NO donor-induced cytotoxicity. Taken together, our results indicate that wogonin inhibits activation-induced death of C6 glial cells by suppressing NO production, and these inhibitory effects of wogonin on NO production are exerted through inhibition of NF- κ B-mediated iNOS induction. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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Flavonoids are a group of low molecular weight polyphenolic compounds of plant origin. They exhibit a variety of biological activities such as anti-inflammatory, antioxidant, antiviral, and antitumor actions [13]. A 5,7-dihydroxy-8-methoxyflavone (wogonin) is a flavonoid derived from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Oriental medicine. This flavonoid has been shown to exert anti-inflammatory effects. It inhibited lipopolysaccharide (LPS)-induced production of nitric oxide (NO) [9,20] and prostaglandin E₂ [21] in macrophages. It also showed free radical scavenging and antioxidant activities [7,17]. However, little information is available about its effects on the activation of astrocytes and their NO production.

Astrocytes play an essential role in maintaining function of neurons by producing and responding to a variety of

growth factors and cytokines [2]. Stimulated astrocytes produce diverse inflammatory mediators such as NO and tumor necrosis factor- α (TNF α) [6,16,18]. There is also growing evidence that toxic mediators produced by activated microglial cells and astrocytes might be involved in the pathogenesis of various neurodegenerative diseases [1,3,12]. Thus, production of toxic inflammatory mediators by activated glial cells needs to be tightly regulated. Potential mechanisms for down-regulation of activated astrocytes are the deactivation or elimination of activated cells. We have previously shown that activated microglial cells [11] and astrocytes [19] undergo apoptosis for the regulation of their own activation states. Because the process of auto-regulatory apoptosis found in glial cells heavily depends on autocrine NO production [11], this type of programmed cell death may be either positively or negatively influenced by exogenous factors that stimulate or inhibit NO production. Based on the known anti-inflammatory activities of wogonin, we hypothesized that wogonin may modulate

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NO production in glial cells, which in turn may regulate apoptosis of activated glial cells. In order to test this hypothesis we utilized C6 rat glial cells, which were activated with LPS and inflammatory cytokines in the presence or absence of wogonin, and determined the effects of the flavonoid on NO production as well as the cell viability. We also investigated the effects of wogonin on the inflammatory induction of inducible nitric oxide synthase (iNOS) protein and nuclear factor kappa B (NF- κ B) activity as the underlying mechanism of induced NO production.

LPS, *N*-monomethyl *L*-arginine (NMMA), and sodium nitroprusside (SNP) were obtained from Sigma (St. Louis, MO). Wogonin was purchased from Waco Pure Chemicals (Japan) and dissolved in 0.2 M NaOH to make 50 mM stock solution, which was then appropriately diluted in culture medium for assays. Recombinant mouse TNF α , which has been shown to be active on rat C6 cells [15], was purchased from R&D Systems (Minneapolis, MN). Recombinant rat interferon- γ (IFN γ) was generously provided by Dr van der Meide, TNO Primate Center (The Netherlands). A caspase inhibitor (*z*-VAD-fmk) was purchased from Enzyme Systems (Livermore, CA). C6 rat glial cells were obtained from American Type Culture Collection. The cell line was maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, and penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD).

Cytotoxicity was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. In brief, cells (3×10^4 cells in 200 μ l of medium per well) were seeded in 96-well microtiter plates and treated with various reagents for the indicated time periods. In some experiments, cells were pretreated for 1 h with NMMA, wogonin, or *z*-VAD-fmk. After various treatments, medium was removed and MTT (0.5 mg/ml) was added before incubating at 37°C for 2 h in CO₂ incubator. After a brief centrifugation, supernatants were carefully removed and dimethylsulfoxide was added. After insoluble crystals were completely dissolved, absorbance at 540 nm was measured using a Thermomax microplate reader (Molecular Devices).

NO₂⁻ in culture supernatants was measured to assess NO production. Fifty μ l of sample aliquots was mixed with 50 μ l of Griess reagent (1% sulphanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader. NaNO₂ was used as standard to calculate NO₂⁻ concentrations.

For DNA ploidy analysis, C6 cells were suspended in PBS-5 mM EDTA, and fixed by adding 100% ethanol dropwise. RNase A (40 μ g/ml) was added to resuspended cells, and incubation was carried out at room temperature for 30 min. Propidium iodide (50 μ g/ml) was then added for flow cytometric analyses (FACS Vantage; Becton Dickinson).

Western blot analysis was carried out as follows. Cells were lysed in triple-detergent lysis buffer (50 mM Tris-HCl,

(pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF). Protein concentration in cell lysates was determined using Bio-Rad protein assay kit. An equal amount of protein for each sample was separated by 10% SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham). The membrane was blocked with 5% skim milk and sequentially incubated with polyclonal rabbit anti-mouse/rat iNOS antibody (Transduction Laboratories) or polyclonal goat anti-mouse/rat cyclooxygenase-2 (COX-2) antibody (Santa Cruz Biotechnology) and HRP-conjugated secondary antibodies (anti-rabbit or -goat IgG, Amersham) followed by ECL detection (Amersham).

NF- κ B reporter assays were performed as follows. C6 cells in six well plate were co-transfected with 1 μ g of plasmid carrying two copies of kB sequences linked to luciferase gene (pNF- κ B-luc, generously provided by Dr T. H. Lee at Yonsei University, Seoul, Korea) [8] together with 0.2 μ g of *lacZ* gene under SV40 early promoter (pCH110, Pharmacia) using lipofectAMINE reagent (GibcoBRL). Transfection was performed according to supplier's instructions. At 48 h after transfection, cells were treated with LPS, IFN γ , TNF α , and wogonin for 6 h, and then luciferase and β -galactosidase activity in transfected cells was measured using luciferase assay system (Promega, Madison, WI) and β -galactosidase enzyme assay system (Promega), respectively, according to supplier's protocols.

All data were presented as means \pm SD from three or more independent experiments. Statistical comparison between different treatments was done by Student's *t*-test. Differences with *P* value less than 0.05 were considered statistically significant.

Treatment of C6 glial cells with inflammatory stimuli, LPS/IFN γ /TNF α , induced NO production as well as a decrease in the cell viability (Fig. 1A). Neither a significant NO production nor the cell death was induced by LPS or cytokines alone, and combination of LPS/IFN γ /TNF α induced the strongest response (data not shown). C6 cell death caused by treatment with LPS/IFN γ /TNF α was due to apoptosis as determined by DNA ploidy analysis (Fig. 1D). Pretreatment of C6 cells with broad-spectrum caspase inhibitor, *z*-VAD-fmk (50 μ M), before LPS/IFN γ /TNF α treatment significantly inhibited the cell death (47.5% inhibition, *P* < 0.05), further indicating that the death of C6 cells was due to caspase-dependent apoptosis. Next, the effects of wogonin on NO production and activation-induced death of C6 cells were determined. Wogonin dose-dependently inhibited LPS/IFN γ /TNF α -induced NO production (Fig. 1B) as well as the death of activated C6 glial cells (Fig. 1C; 48.1% inhibition of the cell death). Inhibitory effects of wogonin on C6 cell death were comparable to NMMA, a NOS inhibitor. Concentrations of wogonin higher than 50 μ M was not used because of cytotoxicity. Treatment of C6 cells with wogonin of either 70 or 100 μ M alone resulted in 12.4 or 31.7% cytotoxicity, respectively. Fifty μ M of wogonin was the highest concentration without

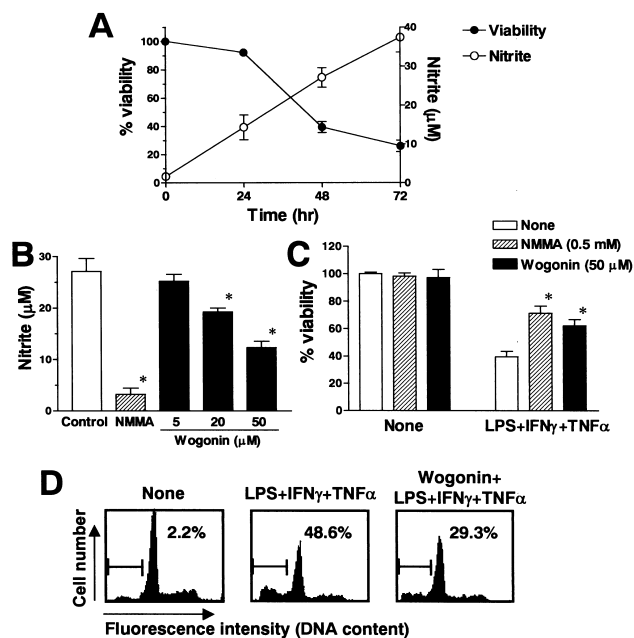


Fig. 1. Wogonin inhibits death of activated C6 glial cells and their NO production. (A) C6 cells were treated for the indicated time periods with combination of LPS (100 ng/ml), IFN γ (100 units/ml), and TNF α (10 ng/ml), and then cell viability or NO production was evaluated. (B,C) C6 cells were treated with LPS/IFN γ /TNF α for 48 h in the presence of NMMA (0.5 mM) or wogonin (5, 20, 50 μ M), and then NO production (B) or cell viability (C) was assessed. NMMA (NOS inhibitor) was used for comparison. Viability of untreated cells was set to 100%. The results are mean \pm SD of three independent experiments. Asterisks indicate significant differences from treatment with LPS/IFN γ /TNF α alone ($P < 0.05$) (B,C). 'Control' in panel (B) indicates treatment with LPS/IFN γ /TNF α without NMMA or wogonin. (D) C6 cells treated with LPS/IFN γ /TNF α in the presence or absence of wogonin (50 μ M) for 48 h were subjected to DNA ploidy analysis. Numbers in histograms indicate the percentage of sub-diploidy cells. The following concentrations of activating agents were used throughout all experiments: LPS, 100 ng/ml; IFN γ , 100 units/ml; TNF α , 10 ng/ml.

apparent cytotoxicity. Appearance of sub-diploidy cells caused by LPS/IFN γ /TNF α treatment was also inhibited by wogonin pretreatment (Fig. 1D), further supporting that wogonin inhibited apoptotic death of activated C6 cells.

LPS and cytokines are known to induce iNOS expression in astrocytes, and this is believed to be the underlying mechanism for the increased NO production [5]. Thus, we next investigated whether wogonin affects iNOS expression induced by LPS plus cytokines. Western blot analysis revealed that treatment of C6 cells with LPS/IFN γ /TNF α induced iNOS, and this was inhibited by 50 μ M of wogonin (Fig. 2A). In contrast, the expression of COX-2 that is closely associated with NO action in astrocytes [14] was not affected by LPS/IFN γ /TNF α or wogonin. These results indicate that inhibition of apoptosis of activated C6 glial cells and their NO production by wogonin is due to its specific inhibitory effects on iNOS expression. As the

inflammatory induction of iNOS in C6 cells is known to be mediated through NF- κ B activation [15], we speculated that wogonin may inhibit iNOS induction by down-regulating NF- κ B activation in C6 cells. NF- κ B reporter assays indicated that inflammatory activation of C6 cells by treatment with LPS/IFN γ /TNF α strongly induced NF- κ B activity, and this was inhibited by wogonin treatment (Fig. 2B), indicating that inhibition of NF- κ B is the underlying mechanism of wogonin action on iNOS induction. Wogonin, however, did not affect an exogenous NO donor-induced death of C6 cells (Fig. 2C). In contrast, z-VAD-fmk significantly inhibited NO donor-induced cytotoxicity. These results indicate that wogonin does not block cytotoxic action of NO, and the inhibitory effects of wogonin on the apoptosis of activated C6 glial cells appear to be mediated through the suppression of NO production. We also evaluated the effects of wogonin on C6 cell death induced by sublethal dose of SNP, because the effects of wogonin on LPS/IFN γ /TNF α -induced cell death was assessed under the condition of around 60% cytotoxicity. SNP of 0.25 mM induced 63.4% cytotoxicity, which was not influenced by pretreatment with wogonin (Fig. 2D,E), further supporting that the cytoprotective effect of wogonin was not due to inhibition of cytotoxic action of NO.

In the current work, we have demonstrated that wogonin, an anti-inflammatory flavonoid, suppressed auto-regulatory apoptosis of activated C6 glial cells. Together with our previous report on activation-induced cell death (AICD) of microglial cells [11], present work suggests that the elimination of activated glial cells by apoptosis could be an important mechanism whereby undesirable effects of long-term activation of glial cells can be minimized. Inflammatory mediators that are produced by activated glial cells in central nervous system may have harmful effects on neurons. Thus, in various neurodegenerative diseases involving chronic activation of glial cells, these cells appear to play a significant role in the pathogenic mechanisms [1,3,12]. Anti-inflammatory action of wogonin could be considered beneficial for the treatment of such neurodegenerative diseases that involve chronic inflammatory responses. However, it should be kept in mind that NO-inhibiting action of wogonin might also block auto-regulatory apoptosis of activated glial cells. Our preliminary work showed that wogonin blocks LPS-induced NO production and iNOS induction in BV-2 mouse microglial cells as well (unpublished data, Suk et al.). Thus, the AICD-inhibiting action of wogonin may not be limited to C6 glial cells. Other types of glial cells in vivo may also be affected by wogonin in such a way that the delicately regulated balance between inflammatory activation and suicide of activated glial cells may be perturbed leading to the exacerbated pathological inflammatory responses. Although NO may be the major cytotoxic mediator in AICD of glial cells, involvement of other inflammatory mediators could not be excluded. This possibility was indicated by our results that NOS inhibitor, NMMA, did not completely inhibit AICD of

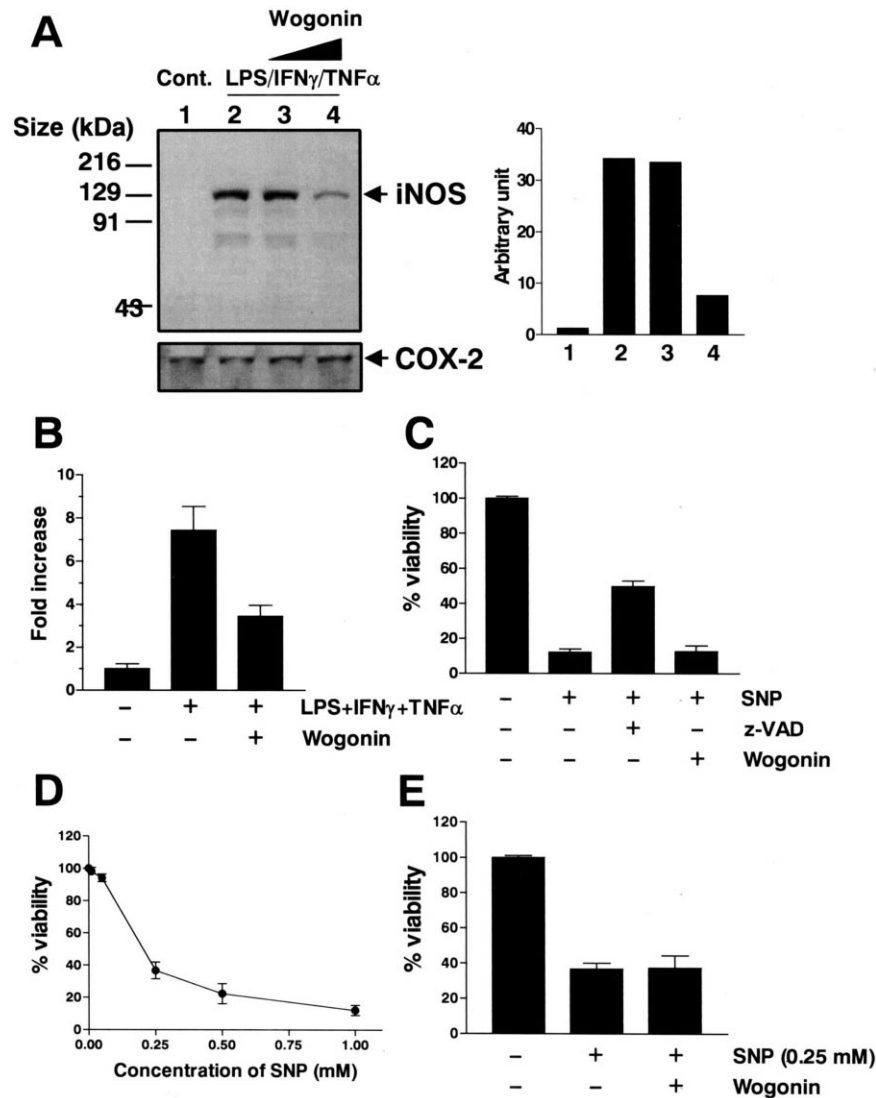


Fig. 2. Wogonin inhibits inflammatory induction of iNOS and NF- κ B. (A) After treatment of C6 cells with LPS/IFN γ /TNF α for 16 h in the absence or presence of wogonin (5, 50 μ M), iNOS or COX-2 expression was evaluated by Western blot analysis using antibodies specific for iNOS or COX-2 (left). Lane 1, untreated; lane 2, LPS/IFN γ /TNF α ; lane 3, LPS/IFN γ /TNF α and wogonin (5 μ M); lane 4, LPS/IFN γ /TNF α and wogonin (50 μ M). Fifty μ M of wogonin was not toxic to C6 cells as demonstrated by MTT assays shown in Fig. 1C. The result of iNOS Western blot was subjected to densitometric analysis (right). (B) C6 cells treated with LPS/IFN γ /TNF α with or without wogonin (50 μ M) for 6 h were subjected to NF- κ B reporter assays. Values are fold increases in luciferase activity normalized to β -galactosidase activity. Wogonin treatment showed 61.8% inhibition of NF- κ B reporter activity. (C) C6 cells were also treated with SNP (1 mM), a NO donor, for 24 h with or without wogonin (50 μ M) or z-VAD (50 μ M), and then cell viability was evaluated. The z-VAD, but not wogonin, inhibited SNP-induced cell death (52.2% inhibition for z-VAD). (D,E) C6 cells were treated with increasing concentrations of SNP (0.01–1 mM) (D) or 0.25 mM of SNP (E) with or without wogonin (50 μ M) for 24 h, then MTT assays were carried out. The results are mean \pm SD of three independent experiments.

C6 cells (Fig. 1C). This also accounts for the lack of the absolute correlation between NO production and cell death (Fig. 1A). It has been recently reported that water extract of *Scutellaria baicalensis* enhanced IFN γ -induced NO production in mouse peritoneal macrophages [10]. Conflicting results may be due to discrepancies in the types of stimuli (IFN γ versus LPS/IFN γ /TNF α) and cell types used (mouse peritoneal macrophages versus rat glioma cell line). More importantly, the effects of total extract of *Scutellaria baica-*

lensis and a single compound isolated from it may not be necessarily the same. Although baicalin, baicalein, and wogonin are three major constituents of the root of *Scutellaria baicalensis*, wogonin is present only in small amounts in the root (0.1–0.4%).

We have shown that AICD-inhibiting action of wogonin is mediated through its inhibitory effects on the production of NO, an autocrine toxic mediator. And, this NO-inhibiting effect of wogonin was explained by the specific inhibition of

iNOS induction. Down-regulation of NF- κ B activity by wogonin provided a molecular basis of its action on iNOS, activation of whose gene transcription has been shown to be under control of NF- κ B in C6 cells [15]. However, COX-2 gene expression at the protein level was not affected by wogonin in C6 cells (Fig. 2A), although transcriptional regulation of many inflammatory genes including COX-2 is also under control of NF- κ B [4]. In RAW 264.7 mouse macrophage-like cell line, wogonin inhibited LPS-induced COX-2 protein induction [21]. Thus, the regulation of inflammatory gene expression by wogonin appears to depend on the individual genes and cell types studied. Regarding the wogonin-mediated down-regulation of NF- κ B activity, the current study only addressed changes in the final activity of NF- κ B by reporter assays. In order to better understand NF- κ B-inhibitory action of wogonin in C6 cells, more detailed analyses are required on the mechanism of NF- κ B regulation by wogonin including I κ B degradation, nuclear translocation of NF- κ B, and possible co-activator competition with other transcriptional activators.

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