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Synthetic Wogonin Derivatives Suppress Lipopolysaccharide-Induced Nitric Oxide Production and Hydrogen Peroxide-Induced Cytotoxicity

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Wogonin (5,7-dihydroxy-8-methoxyflavone) has been reported to exhibit a variety of biological properties including anti-inflammatory and neuroprotective functions. In this study, biological activities of diverse synthetic wogonin derivatives have been evaluated in two experimental cell culture models. Inhibitory activities of wogonin derivatives on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV2 microglial cells and on hydrogen peroxide (H_2O_2)-induced neuronal cell death in SH-SY5Y human neuroblastoma were examined. Wogonin derivatives such as WS2 and WS3 showed more potent suppressive activities on LPS-induced NO production and H_2O_2-induced cytotoxicity than wogonin itself. In addition, thiol substitution played a minor role in enhancing the activities of the derivatives. These findings may contribute to the development of novel anti-inflammatory and neuroprotective agents derived from wogonin.

Key words: Wogonin, Thiowogonin, Wogonin derivatives, Nitric oxide (NO), Neuronal death, BV2 microglia, SH-SY5Y neuroblastoma

INTRODUCTION

Flavonoids are a group of low molecular weight polyphenolic compounds found in plants, which exhibit a variety of biological activities including anti-inflammatory, anti-oxidant, anti-viral, anti-tumor, and neuroprotective actions (Kang *et al.*, 2004; Lee *et al.*, 2003; Middleton *et al.*, 2000). Wogonin (5,7-dihydroxyl-8-methoxyflavone), a flavonoid isolated from *Scutellaria baicalensis* Georgi, has been reported to be anti-inflammatory through the inhibition of NO and prostaglandin E_2 production (Kim *et al.*, 1999; Wakabayashi and Yasui, 2000) and antioxidant activities through the scavenging of hydroxyl radicals (Gao *et al.*, 1999). In addition, neuroprotective activities of wogonin have been reported in various models (Cho and Lee, 2004; Gao *et al.*, 2001; Lee *et al.*, 2003).

Reactive oxygen species (ROS) have been implicated in diverse neurodegenerative disorders including Alzheimers disease, Parkinsons disease, and many inflammatory conditions (Liu *et al.*, 2002; Murphy, 1999). During the pathogenesis of various neurological disorders excess levels of NO, released from activated microglia, often aggravate disease (Liu *et al.*, 2002). In addition, excessive NO results in the formation of ROS such as peroxynitrite anion from the reaction of NO with superoxide anion (Murphy, 1999). Drugs that inhibit ROS in both glia and neurons may have beneficial therapeutic effects in the treatment of diseases.

In the present study, various wogonin derivatives, which have neuroprotective and anti-inflammatory properties, were examined as potential candidates for more effective treatment against neuronal and inflammatory disease.

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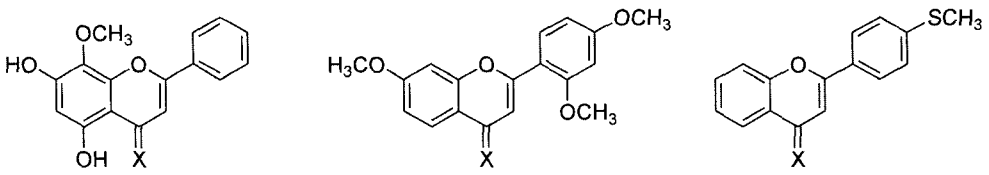
MATERIALS AND METHODS

Preparation of synthetic wogonin and wogonin derivatives

Wogonin (WS1) was synthesized from commercially available chrysin and was thiol-substituted to yield thiowogonin (TWS1) through a reaction with Lawessons reagent. Other derivatives were prepared from WS1 and TWS1. The structures of wogonin and its derivatives are illustrated in Fig. 1.



X=O: WS1 (wogonin)
X=S: TWS1 (thiowogonin)

X=O: WS2
X=S: TWS2

X=O: WS3
X=S: TWS3

Fig. 1. Chemical structures of wogonin and wogonin derivatives. WS1, 5,7-dihydroxy-8-methoxyflavone (wogonin); TWS1, 5,7-dihydroxy-8-methoxythiocarbonylflavone (thiowogonin); WS2, 2',4',7-trimethoxyflavone; TWS2, 2',4',7-trimethoxythiocarbonylflavone; WS3, 4'-methylsulfanylflavone; and TWS3, 4,-methylsulfanylthioflavone

Cell culture

An immortalized murine BV2 cell line that exhibits phenotypic and functional properties of reactive microglial cells (Blasi *et al.,* 1990) was obtained from M. McKinney (Mayo Clinic, Jacksonville, FL). Human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection (Rockville, USA). The cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/mL streptomycin and 10 U/mL penicillin at 37°C in a humidified incubator with 5% CO_2. All experiments were carried out on sub-confluent cultures.

Measurement of nitrite release

Accumulated nitrite was measured in the cell supernatant by the Griess reaction (Green *et al.,* 1982). The conditions of cell culture and treatment were the same as those used in ELISA. Briefly, 100 μL of Griess reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) was added to 100 μL of each sample in a 96-well microtiter plate and absorbance was read at 540 nm using a plate reader. Sodium nitrite, diluted in culture media at concentrations ranging from 10 to 100 μM, was used to prepare a standard curve.

Determination of cell viability

Cell viability was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. MTT dissolved in phosphate-buffered saline was added at the end of the incubation to a final concentration of 0.5 mg/mL, and then incubated at 37°C for 2 h, the resultant formazan product was extracted for 4 h with the extraction solution (20% SDS, 50% DMF and 2% acetic acid) and detected by a UV-VIS spectrometer (Perkin Elmer Co.) at 570 nm.

Statistics

The data are expressed as mean±SEM values. The significance of the protective effects of wogonin and its derivatives was assessed by analysis of variance (ANOVA). Significant differences between drug-treated groups and control (LPS- or H_2O_2-only) were analyzed with Dunnett's test.

RESULTS

Research into the development of more potent protective agents against neurodegenerative and inflammatory conditions has led to structural modifications of wogonin. As depicted in Fig. 1, six compounds, including wogonin were synthesized.

To determine the inhibitory effects of wogonin and its derivatives on LPS-induced NO production, BV2 microglial cells were challenged with LPS (200 ng/mL) for 16 h in the absence or presence of pretreatment with wogonin and its derivatives at various concentrations (Fig. 2). Cytotoxic effects of these compounds were not observed at the tested concentrations (data not shown). As previously reported (Chen *et al.,* 2001; Kim *et al.,* 1999), wogonin significantly suppressed LPS-induced NO production in a concentration-dependent manner (Fig. 2). However, the thiol-substituted derivative (TWS1) of wogonin failed to enhance the inhibitory activity of unmodified wogonin. Notably, two other wogonin derivatives, WS2 and WS3 exhibited more potent suppressive activity than wogonin itself. No significant improvement of the suppressive activities was observed in TWS2 and TWS3, thiol-substituted derivatives of WS2 and WS3, respectively, compared to the parent derivatives (Fig. 2).

The protective actions of wogonin derivatives against oxidative stress-induced neuronal damage were examined in SH-SY5Y human neuroblastoma cells challenged with 100 mM H_2O_2 for 2 h in the absence or presence of pretreatment with wogonin and its derivatives (Fig. 3). Consistent with previous reports (Gao *et al.,* 2001; Kang *et al.,* 2004), wogonin showed minor protective effects against H_2O_2-induced neuronal cell death in this model. Furthermore, higher concentrations exceeding 10 μM did not reduce oxidative stress-induced neuronal damage, although cytotoxic effects were not observed. WS2 and

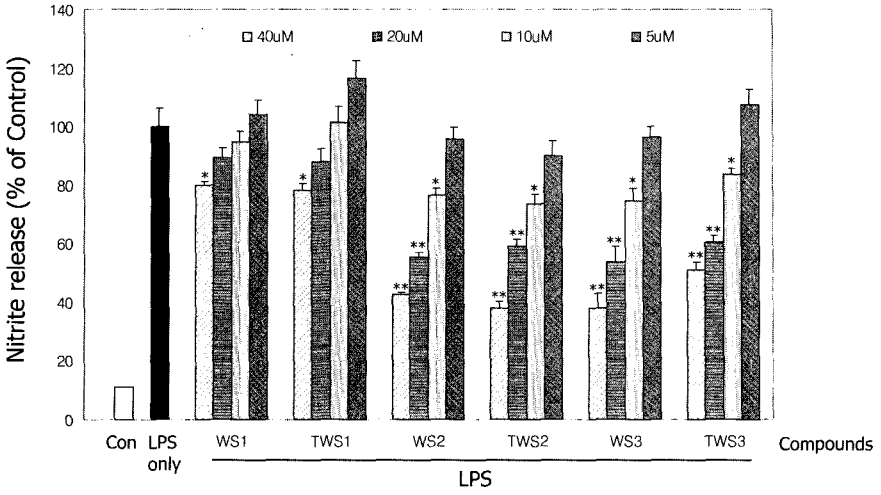


Fig. 2. Effects of wogonin and wogonin derivatives on the nitrite production of LPS-activated BV2 microglial cells. BV-2 microglial cells were challenged with LPS (200 ng/mL) in the presence or absence of pretreatment of each compound. Released nitrite in culture medium was measured. Data were presented as % of control value. Data represent three independent experiments and were expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the LPS alone group.

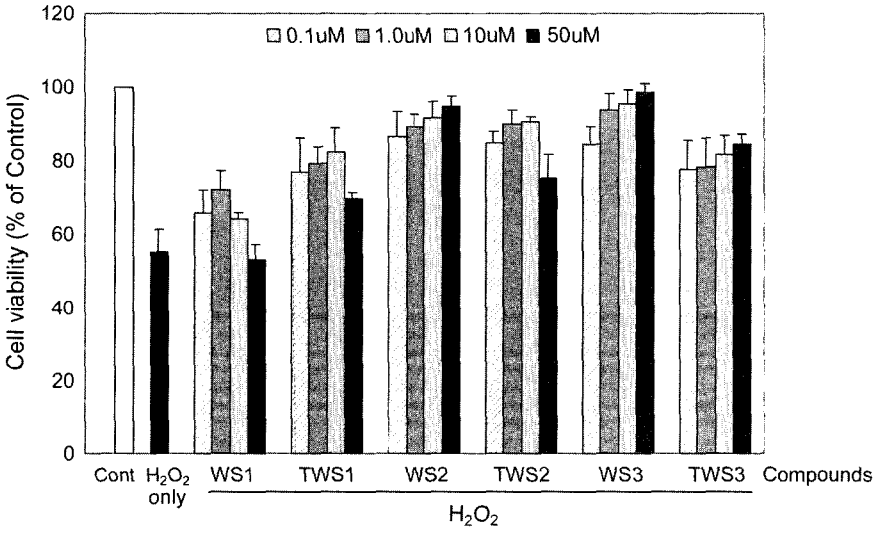


Fig. 3. Effects of wogonin and wogonin derivatives on the cell viability of H_2O_2-challenged SH-SY5Y human neuroblastoma cells. SH-SY5Y human neuroblastoma cells were challenged with 100 mM H_2O_2 for 2 hr in the presence or absence of pretreatment of each compound. Cell viability was determined using MTT assay. Data were presented as % of control value. Data represent three independent experiments and were expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the H_2O_2 alone group.

WS3 showed a greater protective effect compared to unmodified wogonin, and was in a concentration-dependent manner. However, the protective activity of WS2 and WS3 was attenuated by thiol substitutions as was found with TWS2 and TWS3, respectively. However, TWS2 and TW3 still exhibited significantly enhanced protective activity compared to wogonin itself (WS1) (Fig. 3).

DISCUSSION

The present study demonstrates that certain synthetic derivatives of wogonin exhibited more potent inhibitory activities on LPS-induced NO production and H_2O_2-induced cytotoxicity than wogonin itself. The data suggest that synthetic wogonin derivatives may be valuable therapeutic agents for the treatment of abnormal microglial activation and oxidative stress-induced neuronal cell death. Although NO has been recognized to be an important mediator of cellular communication, pathologically excessive levels of NO, released from activated microglia, have been reported to be implicated in the pathogenesis of neurodegenerative diseases (Liu *et al.*, 2002; Murphy, 1999). Previous studies report that LPS treatment (200 ng/mL) results in increased production of NO in BV2 microglial

cells through the induction of inducible nitric oxide synthase (iNOS) (Kim *et al.*, 2004). Oxidative stress has also been implicated in a number of neurodegenerative diseases (Simonian and Coyle, 1996). It has been reported that H_2O_2 induces apoptosis in neurons of the central nervous system (Chandra *et al.*, 2000; Kang *et al.*, 2004).

Among 12 synthesized compounds (data not shown), only WS1 and WS2 exhibited significantly greater protective effects than wogonin itself, whereas other derivatives, for example, carrying chloride substitutions failed to enhance activities (data not shown). In addition, the biological activities of derivatives were not enhanced by thiol substitutions. It has been suggested that some functional residues in wogonin play an important role in the modulation of its biological activities (Dao *et al.*, 2004a, 2004b; Kim *et al.*, 1999). However, further studies are necessary to determine the exact roles of diverse functional residues for the modulation of biological activities.

In conclusion, the data demonstrated that the introduction of certain functional residues into wogonin significantly enhanced the biological activities of the parent wogonin, providing insights into the design of wogonin derivatives with optimal protective activities. However, further studies are necessary to determine the mechanism by which the derivatives exert greater protective actions.

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