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8-Prenylkaempferol suppresses inducible nitric oxide synthase expression through interfering with JNK-mediated AP-1 pathway in murine macrophages

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ABSTRACT

8-Prenylkaempferol is a prenylflavonoid isolated from the roots of *Sophora flavescens*, a Chinese herb with anti-inflammatory properties. However whether 8-prenylkaempferol itself displayed an anti-inflammatory activity remained unclear. In this study, we evaluated the effect of 8-prenylkaempferol on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages. 8-Prenylkaempferol inhibited significantly LPS-induced NO production through suppressing inducible NO synthase (iNOS) expression at both protein and mRNA levels but failed to affect sodium nitroprusside-triggered NO production, iNOS enzyme activity, and cell viability. Further investigation of the mechanisms revealed that 8-prenylkaempferol inhibited LPS-induced c-Jun phosphorylation (a major component of activator protein-1, AP-1), but did not attenuate IkB-α degradation nor NF-κB nuclear translocation. Cellular signaling analysis using mitogen-activating protein kinase (MAPK) inhibitors including 2′-amino-3′-methoxyflavone (PD98059, MEK1/2 inhibitor), 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine (SB203580, p38 kinase inhibitor) and anthra[1-9-cd]pyrazol-6(2H)-one (SP600125, c-Jun N-terminal kinase inhibitor) demonstrated that extracellular signal-regulated kinase1/2 (ERK1/2), p38 and JNK all participated in LPS-stimulated iNOS expression and NO production, but 8-prenylkaempferol interfered selectively with JNK phosphorylation. On the other hand, LPS-induced c-Jun phosphorylation was attenuated in the presence of SP600125. We suggested that interfering with JNK-mediated c-Jun phosphorylation and thus blocking AP-1 activation might contribute to the suppression effects of 8-prenylkaempferol on iNOS. These findings provided the first molecular basis that 8-prenylkaempferol is an effective agent for attenuating pro-inflammatory NO induction.

1. Introduction

The root of *Sophora flavescens* Ait. (Leguminosae) is a natural product that has been used in traditional herbal preparations in China for centuries, and is prescribed as an anti-pyretic and anti-inflammatory agent (Chang and But, 1986). A variety of bioactive compounds with the ability to inhibit inflammatory responsiveness such as chemotaxis and cyclooxygenase-II induction and to treat cardiovascular disorders have recently been isolated from *S. flavescens* (Kim et al., 2002; Lee et al., 2005; Zheng et al., 1999). When screening for anti-inflammatory constituents from herbs, we found that a crude extract of the roots of *S. flavescens* exhibited anti-inflammatory activity against lipopolysaccharide (LPS)-induced nitric oxide (NO) production. Inflammation is a central feature of many pathological conditions and is mediated by a variety of soluble factors and cellular signaling events. For example, nuclear factor-κB (NF-κB)-dependent gene expression plays an important role in inflammatory responses and increases the expression of genes encoding cytokines and receptors involved in pro-inflammatory enzymes such as inducible NO synthase (iNOS) and cyclooxygenase-II (COX-II) (Giuliani et al., 2001). In addition, activator protein-1 (AP-1), another early transcriptional factor, is also involved in pro-inflammatory response either alone or by coupling with NF-κB (Adcock, 1997; Giuliani et al., 2001). Improper up-regulation of iNOS has been associated with pathophysiology of certain types of cancers as well as inflammatory disorders (Cross and Wilson, 2003; Trifan and Hla, 2003; Ristimaki, 2004). Furthermore, AP1, NF-κB, iNOS, and mitogen-activated protein kinases (MAPKs) have been exploited as molecular targets in drug discovery and development for inflammatory-related diseases. In the current study, we successfully purified and identified a prenylflavonoid 8-prenylkaempferol (Fig. 1) from *S. flavescens*, and found that this compound displayed potent anti-inflammatory property against LPS-stimulated NO production, suggesting that it is one of the active

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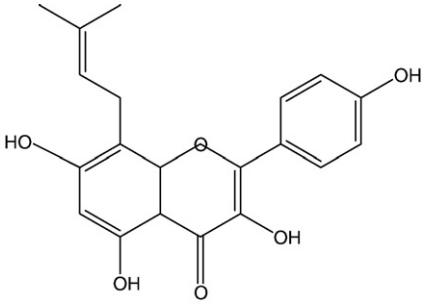

Fig. 1. Chemical structure of 8-prenylkaempferol.

components in *S. flavescens*. Herein, we investigated its possible anti-inflammatory mechanisms and our results demonstrated that 8-prenylkaempferol suppressed iNOS expression through the inhibition of JNK-mediated c-Jun phosphorylation to disturb AP-1 activation.

2. Materials and methods

2.1. Isolation of 8-prenylkaempferol

The dried, chipped roots of *S. flavescens* (8 kg), collected from Hualien, Taiwan, were extracted with MeOH (80 L×3). The combined extracts were evaporated in vacuum to give a black residue, which was suspended in water (10 L) and centrifuged (9000 ×*g*, 30 min) to give water-soluble and water-insoluble portions. The water-insoluble portion was chromatographed repeatedly using silica gel, Sephadex LH-20, preparative TLC, and HPLC to afford 8-prenylkaempferol and other twenty-eight compounds (Shen et al., 2006). The structure of 8-prenylkaempferol was elucidated with mass and NMR spectroscopy in comparison to published data (Hillerns and Wink, 2005). The purity of 8-prenylkaempferol was >98% as judged by HPLC and ^{1}H NMR (Shen et al., 2006).

2.2. Cell culture condition

RAW264.7 is a mouse macrophage cell line, and was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) Gibco BRL, Grand Island, NY 14072, USA) supplemented with antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% heat inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek 25115, Israel) and maintained at 37 °C in 5% CO2 humidified air. Peritoneal macrophages were collected from ICR mice anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Peritoneal macrophages were collected by washing the cavity with 4 ml sterile phosphate- buffered saline (PBS) containing heparin (50 U/ml). Peritoneal fluid was centrifuged and the cell pellet was washed twice with PBS. Cells were then plated onto 35-mm culture dishes ($4–5\times10^6$ cells per dish) containing DMEM. After 3 h at 37 °C, non-adherent cells were removed by washing with sterile PBS. Macrophages were removed from the culture dishes by vigorous pipetting and then centrifuged (300 ×*g* for 10 min) and resuspended in DMEM at a concentration of 2×10^7/ml. Macrophage viability (95–98%) was determined by exclusion of Trypan blue. The committee for animal experiments of the National Research Institute of Chinese Medicine gave its approval for the project.

2.3. Measurement of cell viability

Cell viability was monitored by Alamar Blue Assay kit (Serotec Ltd, 22 Bankside, Kidlington, Oxford, UK) as described previously (Don et al., 2007). In brief, cells were plated at a density of 10^5 cells/well into 96-well plates for 12 h, followed by treatment with different concentrations of 8-prenylkaempferol for a further 18 h. Thereafter, Alamar Blue growth indicator dye (10%, v/v) was added for another 4 h-incubation at 37 °C. The change in color could be monitored with an ELISA reader at 620 nm. Cell viability correlates with optical density. Wells containing medium and Alamar Blue dye without cells were used as blanks. In each case, the experiments were performed in duplicate. All experiments were repeated at least twice with similar results. The mean absorbance for the duplicate cultures of each drug was calculated and the mean blank value was subtracted from these. Cell viability in control medium without any treatment was represented as 100%.

2.4. Nitrite assay

Cells were plated at a density of 10^5 cells/ml in 96-well plates for 12 h, followed by treatment with LPS and different concentrations of the indicated compounds for a further 18 h. Nitrite (NO_2^-) accumulation was used as an indicator of NO production in the cell culture medium by the Griess reaction. One hundred microliters of each supernatant were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylendiamine dihydrochloride in water). The absorbance of each sample after Griess reaction was determined by ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories) at 550 nm (Chiou et al., 2000). The nitrite concentration is evaluated by means of a calibration curve (3.125 to 100 μM), using sodium nitrite as a standard.

2.5. Western blot analysis

Total cellular extracts were prepared according to our previous paper (Tsai et al., 2004), separated on 8% SDS-polyacrylamide minigels, and transferred to nitrocellulose membranes (Amersham, Buckinghamshire, U.K.). The membrane was blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with first antibodies overnight at 4 °C. Expression of the protein was detected by ECL (Amersham). In some experiments, we transfected RAW 264.7 cells with 100 nM siRNA targeting murine c-Jun (a pool of four siRNAs, Dharmacon) or a non-targeting control siRNA (siControl 1, Dharmacon). Cells were transfected at 50–70% confluence with a final concentration of 100 nM SMARTpool siRNA or nonspecific control pool using DharmaFECT siRNA transfection reagents (Dharmacon) according to the manufacturer's instructions. After 24 h, the medium was replaced and cells were treated for an additional 8 h with LPS. We then harvested protein for Western blot analysis. In another set of experiment, culture medium was collected from LPS-stimulated cells for nitrite measurement after siRNA transfection.

2.6. Quantifying iNOS mRNA

After stimulated with LPS in the absence or presence of 8-prenylkaempferol for 4 h, total RNA was extracted by using TRIZOL reagent according to the manufacture's instructions. The concentration of mouse iNOS mRNA was determined with Quantikine mRNA colorimetric quantification kit (R&D Systems Inc, USA) as described in our previous study (Ko et al., 2005). In brief, 2 μg of total RNA samples in 150 μl diluent was hybridized with 50 μl of iNOS gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labelled detection probes in a 96-well microplate in duplicate in a 65 °C water bath for 1 h. About 150 μl of hybridization solution was then transferred to a streptavidin-coated microplate and incubated for 60 min at room temperature on a shaker. After washing, 200 μl of antidigoxigenin conjugate was added to each well and incubated for