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Original article

Immunomodulatory activities of phlorizin metabolites in lipopolysaccharide-stimulated RAW264.7 cells

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ABSTRACT

The immunomodulatory effects of two new phlorizin metabolites, phloretin 4-O-β-D-glucuronide (**1**), 6-methoxyl-phloretin-2-O-β-D-glucuronide (**2**), together with phloretin-2-O-β-D-glucuronide (**3**) on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were determined. **1**–**3** (1–5 μg/ml) significantly inhibited the production of NO ($p < 0.01$). At the concentration of 5 μg/ml, **2** and **3** further inhibited iNOS mRNA expression ($p < 0.01$ or 0.05), and **1**–**3** inhibited iNOS protein expression ($p < 0.01$). Conversely, they all promoted the proinflammatory cytokine TNF-α mRNA expression ($p < 0.01$). For IL-10 mRNA, **1** and **3**, which are main metabolism forms in rat plasma, obviously promoted its expression ($p < 0.01$), while metabolite **2**, which was only detected in rat urine, showed inhibitory activity, but **1**–**3** alone without LPS stimulation had no effect on the expression of both TNF-α and IL-10 mRNA expression. **1** further inhibited VEGF, CCL2 and CXCL1 mRNA expression at the concentration of 5–25 μg/ml ($p < 0.01$). These results indicated phloretin's metabolites with different structural forms showed different immunomodulatory activities.

1. Introduction

Phlorizin is a natural product belongs to the dihydrochalcone class of flavonoid. It is widely distributed in the bark, leaves, and fruit of apple trees [1], and also reported to be main constituent in *Lithocarpus polystachyus* extract [2,3]. Phlorizin has anti-diabetes, obesity, and stress hyperglycemia clinical application potential, but few corresponding mechanisms were reported [4]. In our previous study, phase II metabolites of phlorizin were reported to be main absorbed forms in rat plasma following oral administration of the *Lithocarpus polystachyus* extract, and two phase II metabolites were isolated and identified by Nuclear Magnetic Resonance (NMR) techniques [5]. The anti-inflammatory effects of phlorizin and its aglycone, phloretin had been studied and compared in LPS-stimulated RAW264.7 cells, phloretin significantly inhibited the levels of NO, PGE2, IL-6, TNF-a, iNOS and COX-2, but phlorizin did not show any anti-inflammatory response [6]. Combing our previous study and known report [5,7], phlorizin was not detected to be absorbed into rat plasma after oral administration, while its phase II metabolites were detected to be main existing forms. Phlorizin also has different pharmacological activities when compared to phloretin [4], but the mechanisms of phlorizin for treating metabolic syndrome-related diseases are still unknown. Since the absorbed metabolites should be their real acting forms *in vivo*, whether these metabolites have similar or different biological activities when compared to their parent forms needs to be further verified. Up to date, no biological activities about the phase II metabolites of phlorizin were reported, and here we firstly studied the immunomodulatory effects of three phlorizin metabolites.

2. Materials and methods

2.1. Compounds, reagents and cells

Three phlorizin metabolites, phloretin 4-O-β-D-glucuronide (**1**), 6-methoxyl-phloretin-2-O-β-D-glucuronide (**2**) and phloretin-2-O-β-D-glucuronide (**3**) were isolated from rat urine after orally administered of phlorizin-richfull extract.

RAW264.7 mouse macrophages were obtained from American Type Culture Collection (Rockville, MD, USA) and grown in High-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) and supplemented with 10% fetal bovine serum (FBS; Gibco BRL). Phosphate buffer saline (PBS) was purchased from Thermo Scienfic

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HyClone (Logan,UT). Lipopolysaccharide (LPS), dimethylsulfoxide (DMSO) and 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemicals Co. (MO, USA). Hypoderm Molecular Biology Grade Water was purchased from Thermo Scientific.

2.2. Instruments

NMR data were recorded in CD_3OD, using TMS as an internal reference on a Bruker-AM-600 instrument (^1H, 500 MHz; ^{13}C, 125 MHz). Mass spectra were recorded on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). UV spectra were obtained on Shimadzu-UV-2450 spectro photometer; λ_{max} (log ε) in nm. Semi-prep HPLC runs were carried on a Agilent-1200 apparatus equipped with a PDA detector (Agilent-1200 apparatus, Santa Clara, CA, USA), and a Phenomenex Luna C_{18} column (5 μm, 9.4 × 250 mm). MTT assay was performed on a Multi label Plate Reader (VICTOR^{TM}X5, PekinElmer, USA), and RT-PCR experiment was performed on a NanoDrop 2000C Spectrophotometer (Thermo Scientific) and ABI 7500 Real-Time PCR System.

2.3. Cell viability and nitric oxide assay

Cell viability was evaluated by MTT assay and nitric oxide was determined by measuring the amount of nitrite as described previously [8]. RAW264.7 cells (5×10^4 cells/well) were grown in 96-well plates for 24 h. After the addition of a range of concentrations of **1**–**3** for 2 h, then stimulation with LPS (1 μg/ml) for 24 h. The nitrite concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

2.4. Total RNA extraction and RT-PCR

RT-PCR was performed as described previously [8]. The total cellular RNA from RAW264.7 cells was extracted using a TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to instructions of the manufacturer. Total RNA (1 μg) was converted to cDNA using a Thermo RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The PCR primers of iNOS, TNF-α and GADPH used in this study were identical as previous report [9], the other PCR primers are listed below and were purchased from Life Technology: Mouse IL-10 (Forward TGGGAAGAGAAACC AGGGAGA and Reverse GTTTTCAGGGATGAAGCGGC; VEGF (Forward TATT CAGCGGACTCACCAGC, and Reverse AACCAACCTCCTCAAACCGT; CCL2 (Forward TTAAAAACCTGGATCGGAACCAA and Reverse GCATTA GCTTCA GATTTACGGGT; CXCL1 (Forward ACTGCACCCAAACCGAAGTC, and Reverse TGGGGACACCTTTTAGCATCTT. The gene expressions of TNF-α, iNOS, VEGF, CCL2 and CXCL1 were amplified from the synthesized cDNA. Real-time PCR was performed using Roche FastStart Universal SYBR Green Master. GADPH mRNA levels were used as internal controls. The PCR reactions were carried out as the following: 95 °C for 10 min; 40 cycles of 95 °C for 15 s. Final extension was performed at 60 °C for 1 min.

2.5. Western blot analysis

Western blot analysis was also performed as described previously [8]. The rabbit anti-iNOS was utilized as primary antibody, HRP-goat anti-rabbit IgG was used as a secondary antibody, and GAPDH was used as a loading control.

3. Results and discussion

3.1. Isolation and structural elucidation of compounds *1* and *2*

The metabolites-containing urine samples were collected as previous description [5]. The concentrated urine was subjected to ODS C_{18} chromatography (MeOH-0.05%CH_3COOH, 20:80, 30:70, 40:60 and 60:40). The subfractions containing target components were purified by preparative HPLC using the mobile phase of MeOH− 0.05%CH_3COOH (45:55) to obtain **1** (5.0 mg) and **2** (10.3 mg).

Compound **1** was obtained as a white amorphous powder. Its molecular formula was assigned as $C_{21}H_{22}O_{11}$ on the basis of the negative HR-ESI–MS (*m/z* 449.10712 $[M-H]^-$, calcd. for 449.10894). The structure was elucidated based on comparing their ^1H- and ^{13}C NMR data with those of our previous report (Table 1). The nucleus of 2, 4, 6, 4′-tetrahydroxy-dihydrochalcone conjugating a glucuronide was easily elucidated. The identical hydrogen and carbon chemical shifts indicate 3 and 5-H should be

Table 1
^1H- and ^{13}C NMR data of compounds **1**–**3** and phloretin-4′-*O*-β-D-glucuronide (CD_3OD, δ ppm, TMS)[a].

	phloretin-4-*O*-*β*-D-glucuronide (**1**)		6-methoxylphloretin-2-O-*β*-D-glucuronide (**2**)		phloretin-2-*O*-*β*-D-glucuronide (**3**)		phloretin-4′-*O*-*β*-D-glucuronide	
Position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
β-CH_2	2.87 (2H, t, 7.6 Hz)	31.0	2.87 (2H, t, 7.6 Hz)	30.9	2.84 (2H, t, 7.6 Hz)	31.2	2.90(2H, m)	31.6
α-CH_2	3.50 (2H, m)	47.0	3.50 (2H, m)	46.8	3.49 (2H, m)	47.4	3.28(2H, m)	47.2
1		107.2		106.8		106.8		105.5
2		162.3		161.9		165.3		166.0
3	6.15 (1H, s)	95.7	6.11 (1H, s)	95.4	6.08 (1H, s)	96.0	5.81(1H,s)	95.9
4		166.0		166.0				166.0
5	5.96 (1H, s)	98.8	5.97 (1H, s)	98.7	6.08 (1H, s)	96.0	5.81(1H,s)	95.9
6		167.7		169.2				166.3
1′		134.0		133.9		133.8		137.5
2′, 6′	7.08 (2H, d, 8.4)	130.6	7.07 (2H, d, 8.4)	130.2	7.03 (2H, d, 8.0)	130.2	7.16 (2H, d, 8.4)	130.5
4′		156.5		156.3		156.4		157.5
3′, 5′	6.68 (2H, d, 8.4)	116.2	6.69 (2H, d, 8.4)	116.1	6.68 (2H, d, 8.0)	116.1	7.02 (2H, d, 8.4)	118.3
CO		206.7		206.6		207.2		206.3
1”	5.08 (1H, d, 6.4)	102.4	5.12 (1H, d, 6.8)	102.2	4.98(1H, d, 6.0)	101.1	4.92 (1H)	103.0
2”	3.50(1H, m)	74.6		74.4		74.6	3.48	74.9
3”	3.50(1H, m)	78.0		77.4		78.0	3.49	77.7
4”	3.60(1H, m)	72.9		72.7		72.9	3.57	73.5
5”	3.60(1H, m)	77.1		76.8		77.1		76.8
6”		170.8		170.7		170.8		170.5
6-Me			3.78	53.0				

[a] Measured at 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR.