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Evaluation of the anti-inflammatory effects of phloretin and phlorizin in lipopolysaccharide-stimulated mouse macrophages

Wei-Tien Chang [a,1], Wen-Chung Huang [b,1], Chian-Jiun Liou [b,*]

[a] Department of Nutrition and Health Sciences, Chang Gung University of Science and Technology, 261 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan, ROC
[b] Department of Nursing, Chang Gung University of Science and Technology, 261 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan, ROC

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ABSTRACT

Many reports suggest that phloretin and phlorizin have antioxidant properties and can inhibit glucose transportation, the anti-inflammatory effects and mechanism of phloretin and phlorizin remain unclear. This study aims to evaluate the anti-inflammatory effects of phloretin and phlorizin in LPS-stimulated murine RAW264.7 macrophages. RAW264.7 cells were pretreated with various concentrations of phloretin or phlorizin (3–100 μM) and cell inflammatory responses were induced with LPS. Pretreated with 10 μM phloretin significantly inhibited the levels of NO, PGE_2, IL-6, TNF-α, iNOS and COX-2. Furthermore, it was demonstrated that phloretin suppressed the nuclear translocation of NF-κB subunit p65 proteins, and decreased phosphorylation in MAPK pathways. Surprisingly, phlorizin did not suppress the inflammatory response in LPS-stimulated RAW264.7 cells. These results suggest that phloretin has an anti-inflammatory effect that reduces levels of proinflammatory cytokines and mediators in RAW264.7 cells.

1. Introduction

Acute and chronic inflammation are multiple and complex processes enacted by activated immune-associated cells (McGettrick & O'Neill, 2010). Macrophages play a central role in various inflammatory responses (Olefsky & Glass, 2010). Gram-negative bacteria release lipopolysaccharide (LPS), which is recognised by macrophages and activated macrophages, and the expression of proinflammatory cytokines and mediators (McGettrick & O'Neill, 2010).

There are several reports that LPS-stimulated macrophages induce the expression of inducible nitric oxide synthase (iNOS) to convert L-arginine into L-citrulline and NO, a free radical (Kobayashi, 2010). Additional NO release by iNOS in macrophages, hepatocytes, or smooth muscles cells may enhance the inflammatory response. Cyclooxygenase-2 (COX-2) is also induced by activated macrophages to synthesise prostaglandin E_2 (PGE_2), which causes tissue inflammation and increases pain (Hu, Bradshaw, Chen, Tan, & Walker, 2008). In addition, activated macrophages also release proinflammatory cytokines, including interleukin (IL)-6, tumour necrosis factor alpha (TNF-α), and IL-1β, to enhance the inflammatory response and injure cells or tissues (Chong & Sriskandan, 2011; Olefsky & Glass, 2010). Therefore, inhibiting the release of these inflammatory cytokines and mediators by activated macrophages may suppress or decrease tissue injury during the inflammatory process.

Nuclear transcription factor kappa-B (NF-κB) is a transcription factor that is activated by the inflammatory response to involve the expression of proinflammatory cytokines and mediators (Ruland, 2011). In addition, recently studies have found that mitogen-activated protein kinase (MAPK) pathway activity may also modulate the expression of iNOS and COX-2 in LPS-induced macrophages (Cargnello & Roux, 2011).

Dietary compounds have recently been studied that may affect and improve acute and chronic disease (Gregory, Headley, & Wood, 2011; Kisely et al., 2011). Animal and clinical studies have indicated that some plant-based flavonoids may attenuate conditions mediated by the inflammatory response, including asthma, rheumatoid arthritis, and cardiovascular disease (Gonzalez-Gallego, Garcia-Mediavilla, Sanchez-Campos, & Tunon, 2010; Morinobu et al., 2008; Shen et al., 2011; Teixeira Damasceno, Apolinario, Dias Flauzino, Fernandes, & Abdalla, 2007).

Phloretin and its glycosylated product phloretin-2-β-D-glucose (phlorizin; also called phloridzin) belong to the chalcone class of flavonoids (Ehrenkranz, Lewis, Kahn, & Roth, 2005). They are widely distributed in the bark, leaves, and fruit of apple trees (Gonzalez-Gallego et al., 2010). Phloretin and phlorizin have many biological functions, including antioxidase activity, regulation of glucose transporters, and the ability to induce apoptosis in tumour cells (Duge de Bernonville et al., 2010; Ehrenkranz et al., 2005; Vasantha Rupasinghe & Yasmin, 2010).

* Corresponding author. Tel.: +886 3 2118999x5421; fax: +886 3 2118293.
E-mail address: ccliu@gw.cgust.edu.tw (C.-J. Liou).
[1] These authors contributed equally to this work.

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In addition, treatment with phloretin can also inhibit the expression of IL-8, CXCL10, and TNF-α mRNAs in LPS-stimulated human acute monocytic leukemia-derived cell line (MonoMac 6) (Jung, Triebel, Anke, Richling, & Erkel, 2009). However, the anti-inflammatory effects of phloretin and phlorizin have not been clearly studied. Therefore, the levels of proinflammatory cytokines and mediators were investigated and the expression of NF-κB and MAPK pathway components were analysed to evaluate the possible anti-inflammatory effects of phloretin and phlorizin in LPS-induced RAW264.7 macrophage cells.

2. Materials and methods

2.1. Materials

Fig. 1A illustrates the chemical structures of phloretin (from apple wood, ⩾99% by HPLC) and phlorizin (from apple wood, ⩾99% by HPLC), which were purchased from Sigma–Aldrich (Sigma, St. Louis, MO, USA). Phloretin and phlorizin were dissolved in dimethyl sulfoxide (DMSO), each at a stock concentration of 100 mM. The final culture concentration of DMSO was ⩽0.1%.

2.2. Cell line and culture medium

The RAW264.7 murine macrophage-derived cell line was purchased from the Bioresource Collection and Research Center (BCRC, Taiwan) and cultured in Dulbecco's modified Eagle's medium (Invitrogen-Gibco™, Paisley, Scotland) supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (Biological Industries, Haemek, Israel), and 100 U/ml each of penicillin and streptomycin. The cells were incubated at 37 °C in 5% CO_2 humidified air and were subcultured twice each week.

2.3. Measurement of cell viability

Cell viability was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (10^5 cells/well) were cultured in 96-well plates and treated with varying concentrations of phloretin or phlorizin for 24 h. Next, the supernatant was removed and the cells were incubated with MTT (50 mg/ml, Sigma) for 4 h at 37 °C. The plates were washed and isopropanol was added to dissolve formazone crystals, then the absorbance values were measured at 570 nm using a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

2.4. Nitrite assay

As an indicator of NO production, the nitrite level in the culture supernatant of RAW264.7 cells was determined. Cells (2×10^6 cells/ml) were pretreated with a range of concentrations of phloretin or phlorizin for 1 h, then incubated with LPS (1 μg/ml) for 24 h. The supernatant was mixed with an equal volume of Griess reagent

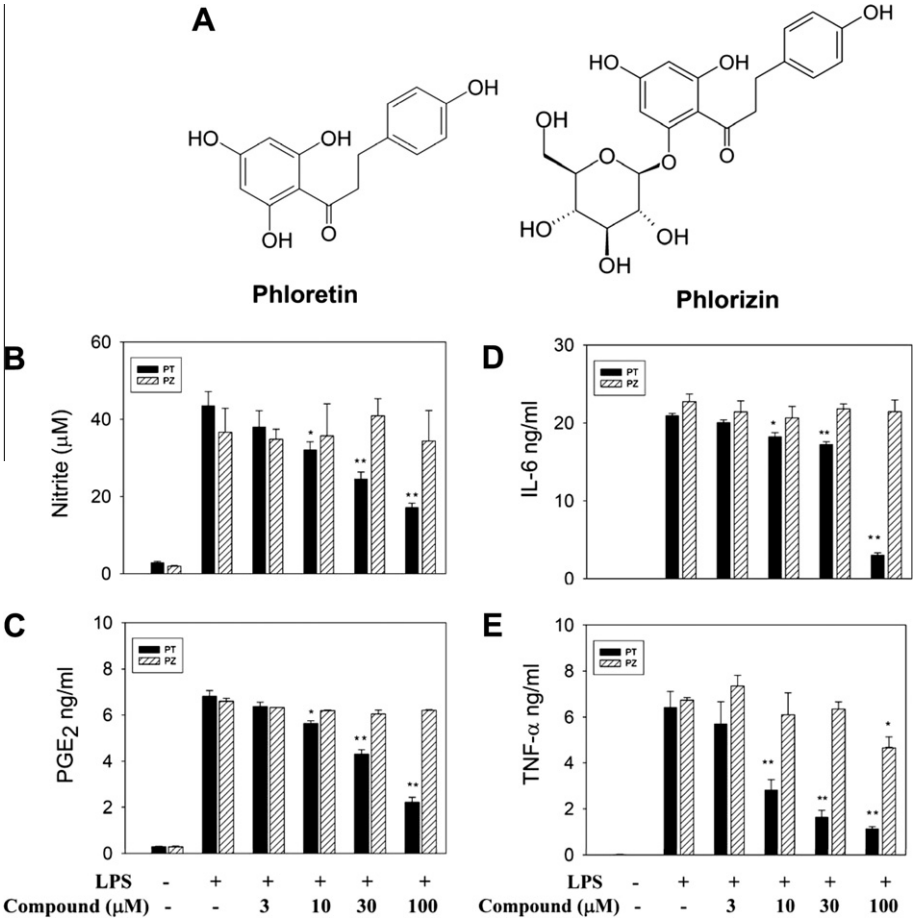

Fig. 1. Chemical structures of phloretin (PT) and phlorizin (PZ) (A), and effects of PT and PZ on LPS-induced production of (B) nitrite, (C) PGE_2, (D) IL-6 and (E) TNF-α. RAW264.7 cells (2×10^6 cells/ml) were pretreated with the indicated concentrations of phloretin or phlorizin for 1 h and then stimulated with LPS (1 μg/ml) for 8 h or 24 h. Data are presented as mean ± SD, $n = 12$. $^{*}p < 0.05$, $^{**}p < 0.01$; compared with the LPS-treated group.