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Genipin attenuates lipopolysaccharide-induced persistent changes of emotional behaviors and neural activation in the hypothalamic paraventricular nucleus and the central amygdala nucleus

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ABSTRACT

Sickness behavior is a series of behavioral and psychological changes that develop in inflammatory disease, including infections and cancers. Administration of the bacterial endotoxin lipopolysaccharide (LPS) induces sickness behavior in rodents. Genipin, an aglycon derived from an iridoid glycoside geniposide extracted from the fruit of *Gardenia jasminoides*, has anti-inflammatory and antidepressant activities. However, the effects of genipin on inflammation-induced changes in emotional behaviors are unknown. In this study, we examined the effects of genipin on LPS-induced inflammation in BV-2 cells and sickness behavior in mice. Pretreatment with genipin inhibited LPS-induced increases in NO production and reduced the mRNA levels of inflammation-related genes (iNOS, COX-2, IL-1β and IL-6) in BV-2 cells. Oral administration of genipin ameliorated LPS-induced depressive-like behavior in the forced swim test and social behavior deficits 24 h after LPS administration in mice. LPS-induced expression of mRNAs for inflammation-related genes and the number of c-fos immunopositive cells decreased in the paraventricular nucleus (PVN) of the hypothalamus and the central nucleus of the amygdala (CeA), suggesting that genipin attenuates LPS-induced changes of emotional behaviors through inhibition of neural activation and inflammatory responses in the PVN and CeA. These novel pharmacological effects of genipin may be useful for treatment of patients with sickness behavior.

1. Introduction

Sickness behavior refers to a series of behavioral and psychological changes, such as fatigue, anhedonia, depression, anxiety, apathy and loss of appetite, that develop in inflammatory disease, including infections and cancers (Dantzer et al., 2008; DellaGioia and Hannestad, 2010; Kronfol and Remick, 2000). Systemic administration of LPS, the major component of the outer membrane of Gram-negative bacteria, triggers innate immune cells to secret inflammatory cytokines such as interleukin (IL)-1β and IL-6 (Kent et al., 1992). These peripheral inflammatory signals directly and rapidly activate the brain and induce delayed activation of immune cells in the central nervous system (CNS), including microglia. The inflammatory cytokines secreted from the activated immune cells may cause sickness behavior (Nguyen et al., 2002).

LPS induces decreased spontaneous locomotor activity, food intake, sucrose preference and social interaction, and increases the duration of immobility in the forced swim test and tail suspension test in rodents (Bluthé et al., 1992, 1999; Bluthé et al., 2000a, 2000b; de Paiva et al., 2010; DellaGioia and Hannestad, 2010; Dunn and Swiergiel, 2005; Frenois et al., 2007; Henry et al., 2008; Swiergiel et al., 1997; Yirmiya et al., 1996). In several of these studies, LPS-induced decreases in spontaneous locomotor activity and food intake occurred within 6 h after administration, but not 24 h after administration. In contrast, the LPS-induced decrease in social interaction and increase in duration of immobility in the forced swim test continued for more than 24 h after administration (Frenois et al., 2007). These studies suggest that LPS-induced changes of emotional behaviors occur independently of decreases in spontaneous locomotor activity and food intake, and that the persistent changes in emotional behaviors may be mediated by delayed activation of CNS immune cells.

Genipin is an aglycon derived from geniposide, an iridoid glycoside extracted from the fruit of *Gardenia jasminoides*. Genipin has pharmacological properties that include anti-inflammatory and antidepressant-like effects. Genipin inhibits LPS- and interferon-γ-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells (Koo et al., 2004), and LPS-induced production of tumor necrosis

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factor-α, IL-1β, prostaglandin E2, reactive oxygen species, nuclear factor-κB activation and NO release in rat microglial cultures (Nam et al., 2010). Moreover, in a behavioral study, genipin reduced the duration of immobility in the forced swim test and tail suspension test in mice (Tian et al., 2010). However, the effect of genipin on LPS-induced changes of emotional behaviors has yet to be analyzed.

In this study, to explore natural compounds that have ameliorative effects on LPS-induced changes of emotional behaviors, we screened 109 medicinal herb-derived compounds using an in vitro assay, and we investigated the effect of genipin that has relatively strong anti-inflammatory activity among the tested compounds, on LPS-induced sickness behavior in mice.

2. Material and methods

2.1. Drug treatments

LPS (from *Escherichia coli* O127:B8, Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) for in vitro analyses or in saline (0.9% w/v solution of NaCl) for in vivo analyses. Genipin (Wako, Osaka, Japan) was dissolved in 100% methanol for in vitro analyses or suspended in 0.5% w/v carboxymethylcellulose (CMC) for in vivo analyses. LPS was injected intraperitoneally 2 h or 24 h before behavioral tests or decapitation for mRNA measurement or immunohistochemistry. Genipin was injected orally 1 h before LPS administration. All drugs were injected at a fixed volume of 10 ml/kg body weight.

2.2. Cell culture

The murine microglial cell line BV-2 was generously donated by Dr. E. Blasi. BV-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD, USA) containing 100 μg/ml streptomycin and 100 IU/ml penicillin in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. The cells were plated at a density of 7.5×10^4 cells/well in 96-well tissue culture plates for Griess and MTS assays, or at 1.0×10^6 cells/well in a 60-mm tissue culture dish for real-time PCR. Genipin was added 1 h before exposure to 0.1 μg/ml LPS.

2.3. Animals

Experimental procedures concerning the use of animals were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. Every effort was made to minimize animal suffering and to reduce the number of animals used. Seven-week-old male ddY mice were obtained from Shimizu Laboratory Supplies Co., Ltd., (Kyoto, Japan) and housed in cages ($24 \times 17 \times 12$ cm^3) in groups of 5 animals under controlled environmental conditions (23 ± 1 °C; 12:12-h light-dark cycle, humidity of 55%, food and water ad libitum) for 1 week before use in experiments. We used 163 mice in total and in single use for each purpose.

2.4. Griess assay

At 24 h after LPS treatment, cell-free supernatants were collected and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA) and nitrite concentrations were determined with reference to a calibration curve prepared with sodium nitrite standards.

2.5. MTS assay

Cell toxicity was measured with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) using cells in 96-well plates. BV-2 cell cultures were incubated in a CO_2 incubator for 2 h with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and phenazine methosulfate (final concentrations: 333 μg/ml MTS and 25 μM phenazine methosulfate). Absorbance was measured at 490 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Total RNA isolation and reverse transcription

Total RNA was isolated from BV-2 cells or the mouse hypothalamus and amygdala with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were directly lysed in the culture dish with 1 ml TRIzol reagent or tissue were sonicated in 1 ml TRIzol reagent by a handy sonicator (TOMY SEIKO, Tokyo, Japan), and the samples were incubated for 5 min at room temperature. Then 0.2 ml chloroform was added and the sample was shaked by hand for 15 min. After an incubation for 3 min at room temperature and a centrifugation at 12,000*g* for 15 min at 4 °C, the aqueous phase of the sample was placed into a tube and isopropanol was added to the aqueous phase. The mixture was incubated for 10 min at room temperature and centrifuged at 12,000*g* for 10 min at 4 °C. Then the supernatant was removed and wash the pellet with 75% ethanol. After a centrifugation at 7500*g* for 5 min at 4 °C, the pellet was air dried for 10 min and dissolved in 20 μl nuclease free water.

Total RNA (1 μg) was used to perform reverse transcription with ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Briefly, the 20 μl reactions were incubated for 40 min at 42 °C, 5 min at 99 °C, and then preserved at 4 °C.

2.7. Quantitative real-time PCR

Quantitative real-time PCR was performed using Thunderbird qPCR Mix (Toyobo, Osaka, Japan) and the following primers for iNOS: 5′-AGA CCT CAA CAG AGC CCT CA-3′ (forward) and 5′-GGC TGG ACT TTT CAC TCT GC-3′ (reverse), COX-2: 5′-GGC CAT GGA GTG GAC TTA AA-3′ (forward) and 5′-GGG ATA CAC CTC TCC ACC AA-3′ (reverse), IL-1β: 5′-TGT GAA ATG CCA CCT TTT GA-3′ (forward) and 5′-CAG GTC AAA GGT TTG GAA GC-3′ (reverse), IL-6: 5′-GTT CTC TGG GAA ATC GTG GA-3′ (forward) and 5′-TTC TGC AAG TGC ATC ATC GT-3′ (reverse), β-actin: 5′-ACC CAC ACT GTG CCC ATC TA-3′ (forward) and 5′-GCC ACA GGA TTC CAT ACC CA-3′ (reverse), according to the manufacturer's protocol of 20 μl reaction. Quantitative real-time PCR was conducted using a Thermal Cycler Dice Real Time System Single (Takara Bio Inc., Shiga, Japan). Changes in gene expression were calculated relative to the endogenous β-actin standard.

2.8. Forced swim test

For the forced swim test, mice were individually placed in a polymethylpentene beaker (height. 27 cm, diameter. 18 cm) containing 25 ± 1 °C water of depth 13 cm, so that the mice could not support themselves by touching the bottom with their paws. The performance of the mice for 6 min in the swimming session was videotaped using a digital video camera for subsequent analysis. After the session, mice were removed from the beakers, dried with paper towels and returned to their home cages. The total duration of immobility was measured in the final 4 min of a 6-min test session by an observer blinded to the treatment conditions.