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Genipin inhibits lipopolysaccharide-induced acute systemic inflammation in mice as evidenced by nuclear factor-κB bioluminescent imaging-guided transcriptomic analysis

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ABSTRACT

Genipin is a natural blue colorant in food industry. Inflammation is correlated with human disorders, and nuclear factor-κB (NF-κB) is the critical molecule involved in inflammation. In this study, the anti-inflammatory effect of genipin on the lipopolysaccharide (LPS)-induced acute systemic inflammation in mice was evaluated by NF-κB bioluminescence-guided transcriptomic analysis. Transgenic mice carrying the NF-κB-driven luciferase genes were administered intraperitoneally with LPS and various amounts of genipin. Bioluminescent imaging showed that genipin significantly suppressed LPS-induced NF-κB-dependent luminescence *in vivo*. The suppression of LPS-induced acute inflammation by genipin was further evidenced by the reductions of cytokine levels in sera and organs. Microarray analysis of these organs showed that the transcripts of 79 genes were differentially expressed in both LPS and LPS/genipin groups, and one third of these genes belonged to chemokine ligand, chemokine receptor, and interferon (IFN)-induced protein genes. Moreover, network analysis showed that NF-κB played a critical role in the regulation of genipin-affected gene expression. In conclusion, we newly identified that genipin exhibited anti-inflammatory effects in a model of LPS-induced acute systemic inflammation via downregulation of chemokine ligand, chemokine receptor, and IFN-induced protein productions.

1. Introduction

The fruit of *Gardenia jasminoides* Ellis is a medicinal herb that has been used for the treatment of inflammation, jaundice, and hepatic disorders in traditional Chinese medicine (Tseng et al., 1995). Genipin is the aglycon of geniposide found in gardenia fruit (He et al., 2006). Genipin has been used as a blue colorant in food industry (Fujikawa et al., 1987). It also has been used as the cross-linking agent for biological tissue fixation (Sung et al., 1998). Genipin possesses a variety of pharmacological activities, such as anti-microbial, hepatoprotective, and neurotrophic effects (Yamamoto et al., 2000; Yamazaki and Chiba, 2008). Anti-topical inflammatory potentials of genipin have also been reported (Koo et al., 2004, 2006); however, the therapeutic effect and mechanism of genipin on systemic inflammation *in vivo* remain to be clarified.

A wide range of human disorders, such as pneumonia, asthma, rheumatic arthritis, neurodegenerative diseases and obesity, correlates with inflammation (Amor et al., 2010; Thaler and Schwartz, 2010). Moreover, epidemiological studies have identified chronic infections and inflammation as major risk factors for various types of cancers (Karin, 2006). The development of inflammation is controlled by various cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Hanada and Yoshimura, 2002). The production of cytokine is further controlled by the transcription factor, nuclear factor-κB (NF-κB) (Baldwin, 1996). NF-κB is an inducible nuclear transcription factor that consists of heterodimers of RelA (p65), c-Rel, RelB, p50/NF-κB1, and

Abbreviations: BCR, B-cell receptor; CCL, chemokine (C–C motif) ligand; CXCL, chemokine (C–X–C motif) ligand; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldahyde-3-phosphate dehydrogenase; GO, gene ontology; Ifi202, IFN-activated gene 202B; IFN, interferon; IL-1β, interleukin-1β; LAPTM5, lysosomal-associated protein transmembrane 5; LPS, lipopolysaccharide; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NF-κB, nuclear factor-κB; Nrarp, Notch-regulated ankyrin repeat protein; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; RLU, relative luciferase unit; SNF, sucrose nonfermenting protein; TNF-α, tumor necrosis factor-α.

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p52/NF-κB2. NF-κB activity is induced by a large variety of signals, such as bacteria, viruses, necrotic cell products, and inflammatory cytokines. When stimulated, NF-κB binds to the NF-κB-responsive element present in the promoter of inflammatory genes, leading to the induction of gene expression. Accordingly, NF-κB is a critical molecule involved in the regulation of inflammatory cytokine production and inflammation (Bonizzi and Karin, 2004).

We have previously applied NF-κB bioluminescent imaging to assess the host inflammatory responses to *Antrodia camphorata*, vanillin, and biomaterials *in vivo* (Ho et al., 2007; Hseu et al., 2010; Hsiang et al., 2009; Wu et al., 2009). Transgenic mice carrying the NF-κB-driven luciferase genes were used to monitor the host inflammatory responses, and the anti-inflammatory mechanism was further analyzed by transcriptomic tools. In this study, we applied such a platform to evaluate the anti-inflammatory potential of genipin in an *in vivo* model of lipopolysaccharide (LPS)-induced acute systemic inflammation. Our findings showed that genipin exhibited systemic anti-inflammatory effects *in vivo* via inhibiting the expressions of chemokine ligand, chemokine receptor, and IFN-induced protein genes.

2. Materials and methods

2.1. Materials

LPS was purchased from Sigma (St. Louis, MO) and dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2). Genipin was purchased from Wako (Osaka, Japan). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO) and dissolved in PBS at 5 mg/ml. D-Luciferin was purchased from Xenogen (Hopkinton, MA) and dissolved in PBS at 15 mg/ml. Mouse monoclonal antibodies against IL-1β and TNF-α were purchased from Santa Cruz (Santa Cruz, CA).

2.2. Cell culture and genipin treatment

Recombinant HepG2/NF-κB cells, which contained the luciferase genes driven by NF-κB-responsive elements, were constructed as described previously (Ho et al., 2007). HepG2/NF-κB cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For genipin treatment, cells were cultured in 96-well plates at 37 °C. Twenty-four hours later, cells were treated with 100 ng/ml LPS and various amounts of genipin for an additional 24 h.

2.3. Luciferase assay and cell viability assay

Luciferase assay was performed as described previously (Ho et al., 2007). Relative NF-κB activity was calculated by dividing the relative luciferase unit (RLU) of treated cells by the RLU of untreated cells. Cell viability was monitored by MTT colorimetric assay as described previously (Ho et al., 2007). Cell viability (%) was calculated by (OD of genipin-treated cells/ OD of solvent-treated cells).

2.4. Animal experiments

Mouse experiments were conducted under ethics approval from the China Medical University Animal Care and Use Committee (Ethics Approval Number 97–28N). Transgenic mice, carrying the luciferase genes driven by NF-κB-responsive elements, were constructed as described previously (Ho et al., 2007). All transgenic mice were crossed with wild-type F1 mice to yield NF-κB-*luc* heterozygous mice with the FVB genetic background.

A total of 25 transgenic mice (female, 6 to 8 weeks old) were randomly divided into five groups of five mice: (1) mock, no treatment; (2) LPS (4 mg/kg), (3) LPS plus genipin (1 mg/kg), (4) LPS plus genipin (10 mg/kg), and (5) LPS plus genipin (100 mg/kg). Mice were challenged intraperitoneally with LPS and then with genipin 10 min later. Four hours later, mice were imaged for the luciferase activity, and subsequently sacrificed for *ex vivo* imaging, RNA extraction, and immunohistochemical staining.

2.5. In vivo and ex vivo imaging of luciferase activity

For *in vivo* imaging, mice were anesthetized with isoflurane and injected intraperitoneally with 150 mg/kg luciferin. Ten minutes later, mice were placed face up in the chamber and imaged for 1 min with the camera set at the highest sensitivity by IVIS Imaging System® 200 Series (Xenogen, Hopkinton, MA). Photons emitted from tissues were quantified using Living Images® software (Xenogen, Hopkinton, MA). For *ex vivo* imaging, mice were anesthetized and injected with luciferase intraperitoneally. Ten minutes later, mice were sacrificed and tissues were rapidly removed. Tissues were placed in the IVIS system and imaged with the same setting used for *in vivo* studies. Signal intensity was quantified as the sum of all detected photon counts per second from tissues and presented as photon/s.

2.6. Cytokine enzyme-linked immunosorbent assay (ELISA)

IL-1β and TNF-α were quantified by ELISA with the OptEIA™ mouse IL-1β and TNF-α sets (Pharmingen, San Diego, CA). Sera were placed into wells that were coated with monoclonal antibody against IL-1β or TNF-α. After three washes with 0.05% Tween 20 in PBS, peroxidase-conjugated avidin, biotinylated antibody against IL-1β or TNF-α, and chromogenic substrate were added to each well in that order. The absorbance at 450 nm was measured in an ELISA plate reader.

2.7. Immunohistochemical staining

Parafilm-embedded organs were cut into 5-μm sections, deparaffinized in xylene, and then rehydrated in graded alcohol. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 15 min and the nonspecific binding was blocked with 1% bovine serum albumin at room temperature for 1 h. Sections were incubated with mouse monoclonal antibody against IL-1β or TNF-α at 1:50 dilution overnight at 4 °C and then incubated with biotinylated secondary antibody (Zymed Laboratories, South San Francisco, CA) at room temperature for 20 min. Finally, slides were incubated with avidin–biotin complex reagent and stained with 3,3′-diaminobenzidine according to manufacturer's protocol (Histostain®-Plus Kit, Zymed Laboratories, South San Francisco, CA). IL-1β- and TNF-α-positive areas were measured using Image-Pro Plus (Media Cybernetics, Bethesda, MD) to quantify the expression levels of IL-1β and TNF-α. The proportions of IL-1β- and TNF-α-positive areas (%) were calculated as areas occupied with brown color/area of whole tissue.

2.8. Microarray analysis

Total RNA was extracted from brain, heart, liver, or kidney using the RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA was quantified and evaluated as described previously (Cheng et al., 2010). Microarray analysis was performed as described previously (Cheng et al., 2010). Briefly, fluorescence-labeled RNA targets were prepared from 5 μg of total RNA using MessageAmp™ aRNA kit (Ambion, Austin, TX) and Cy5 dye (Amersham Pharmacia, Piscataway, NJ). Fluorescent targets were hybridized to the Mouse Whole Genome OneArray™ (Phalanx Biotech Group, Hsinchu, Taiwan), and the slides were scanned by an Axon 4000 scanner (Molecular Devices, Sunnyvale, CA). Number of replicates was three. The Cy5 fluorescent intensity of each spot was analyzed by genepix 4.1 software (Molecular Devices, Sunnyvale, CA). The signal intensity of each spot was corrected by subtracting background signals in the surrounding. We filtered out spots that signal-to-noise ratio was less than 0 or control probes. Spots that passed these criteria were normalized by the limma package of the R program. Normalized data were tested for differential expression using the Gene Expression Pattern Analysis Suite v3.1 (Montaner et al., 2006). Genes with fold changes >2.0 or <−2.0 were selected and analyzed by gene ontology (GO) on Gene Ontology Tree Machine (http://bioinfo.vanderbilt.edu/gotm/) (Zhang et al., 2004). We used the WebGestalt tool to test significant GO terms. The hierarchical clustering analysis of differential expressed genes was further performed and displayed using the TIGR Multiexperiment Viewer (http://www.tm4.org/index.html). Furthermore, we constructed the interaction networks of differential expressed genes using BiblioSphere Pathway Edition software (Genomatix Applications, http://www.genomatix.de/index.html). Microarray data are MIAME compliant and the raw data have been deposited in a MIAME compliant database (Gene Expression Omnibus, accession number GSE35934).

2.9. Quantitative real-time polymerase chain reaction (qPCR)

The expression levels of IFN-induced protein genes, including IFN-inducible GTPase (Iigp1) and IFN-activated gene 202B (Ifi202) genes, in the brains of three mice per group were validated by qPCR. RNA samples were reverse-transcribed for 2 h at 37 °C with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). qPCR was performed using 1 μl of cDNA, 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 200 nM of forward and reverse primers. The reaction condition was followed: 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, 1 min at 60 °C. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates. Primer sets used in this study were designed using Primer3 program (http://frodo.wi.mit.edu/primer3/). The specificities of primer sets were analyzed by nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Each primer set was able to amplify a target DNA fragment from the respective gene with specificity. The primer set for each gene is followed: Iigp1 forward, 5′-CTTGACATGGTGACTGAGGATG-3′; Iigp1 reverse, 5′-AGGTGGATAAAGCCCGAATAAC-3′; Ifi202 forward, 5′-AAGGCTGGTTGATGGAGAG-3′; Ifi202 reverse, 5′-GTCAATTCAAAGCAGACAAGTC-3′; glyceraldahyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-TCACCCACACTGTGCCCATCTATGA-3′; GAPDH reverse, 5′-GAGGAAGAGGATGCGGCAGTGG-3′.