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Anti-Inflammatory Effect of Aqueous Extract from Kawachi-Bankan Peel on Lipopolysaccharide-Induced Inflammatory Responses in RAW264.7 Cells

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Abstract: Kawachi-bankan (*Citrus maxima*) is one of the citruses produced in Ehime, Japan. Bioactive substances in citrus peel such as flavonoids and carotenoids have been studied very well. However, health functions of water-soluble substances in citrus peel have not been focused. We herein indicated the anti-inflammatory effect of Kawachi-bankan peel aqueous extract (KPE) on mouse macrophage-like RAW264.7 cells. RAW264.7 cells were treated with lipopolysaccharide (LPS) to induce inflammation, and the effect of KPE on LPS-induced hyperinflammatory response in the cells was examined. KPE significantly inhibited the production of inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α by LPS-stimulated RAW264.7 cells without cytotoxicity. KPE also significantly inhibited the mRNA expression levels of IL-6 and TNF-α in the cells, suggesting that KPE inhibits the production of inflammatory cytokines by suppressing the gene expression levels. Immunoblot analysis revealed that KPE suppressed the activation level of p38 MAPK and translocation of NF-κB into nucleus in the LPS-stimulated cells, suggesting that KPE shows an anti-inflammatory effect on macrophages through downregulation of MAPK and NF-κB cascades. Taken together, our findings indicated that KPE contributes to alleviating of a hyperinflammatory response in macrophages.

Keywords: Kawachi-bankan; anti-inflammatory effect; macrophages

1. Introduction

An inflammatory response is a local defense reaction to maintain homeostasis in a living body. The inflammatory response begins with recognition of the infection or injury by immune cells such as macrophages and dendritic cells. Recognition of them by macrophages causes the activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) cascades. Eventually, the activated macrophages produce proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α [1,2]. These actions lead to activation of other immune cells and contribute to the elimination of non-self substances. However, continued inflammatory state caused by excessive production of inflammatory substances leads to autoimmune diseases and chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease. Thus, the inhibition of excessive or chronic macrophage activation is great importance for the prevention and alleviation of these diseases.

Citrus peel contains abundant flavonoids and carotenoids such as hesperidin, naringin, and β-cryptoxanthin, and various biological functions based on these components have been reported. Kawachi-bankan (*Citrus maxima*) is one of the citruses produced in Ehime, Japan. The peel of Kawachi-bankan contains abundant auraptene, which is a kind of coumarin, as compared with other citrus peels [3]. It has been reported that auraptene has an immunomodulatory effect on lymphocytes [4] and anti-inflammatory effect on macrophages [5]. Although health functions of lipid-soluble substances in citrus peel such as auraptene have been studied very well, those of water-soluble substances in the peel have

not been focused. Therefore, we examined the anti-inflammatory effect of Kawachi-bankan peel aqueous extract (KPE) on mouse macrophage-like RAW264.7 cells.

2. Materials and Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, bovine serum albumin (BSA), fetal bovine serum (FBS), and lipopolysaccharides (LPS) from *Escherichia coli* 026/B6 were products of Sigma-Aldrich (St. Louis, MO, USA). Goat anti-actin antibody and horseradish peroxidase-labeled (HRP-labeled) anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled anti-rabbit IgG antibody, HRP-labeled anti-mouse IgG antibody, mouse anti-IκBα antibody, and rabbit antibodies against histone H3, NF-κB p65, extracellular signal-regulated protein kinases (ERK)1/2, phosphorylated ERK1/2, c-Jun N-terminal kinase (JNK), phosphorylated JNK, p38 MAPK, and phosphorylated p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

2.2. Sample preparation

Fruits of Kawachi-bankan were harvested in Ehime, Japan. The peels were manually separated from the fresh fruit and freeze-dried. The dried peels were then powdered with a mill mixer (Iwatani, Tokyo, Japan) and used as Kawachi-bankan peel powder. The powder was suspended in 10 mM sodium phosphate buffer (NaPB; pH 7.4) at 0.1 g/mL at 12°C for 20 h. After centrifugation at 15,000 × *g* at 4°C for 20 min, the supernatant was collected and centrifuged at 270,000 × *g* at 4°C for 20 min. After centrifugation, the supernatant was collected and adjusted to pH 7.4. The supernatant was then filtrated through a 0.22 μm membrane and used as Kawachi-bankan peel aqueous extract (KPE). Dry weight of KPE was measured by weighing a portion of freeze-dried samples.

2.3. Cells and cell culture

Mouse macrophage-like cell line RAW264.7 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). RAW264.7 cells were cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS at 37°C under humidified 5% CO_2. RAW264.7 cells were detached using phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% ethylenediamine-*N,N,N′,N′*-tetraacetic acid (Dojindo Laboratories, Kumamoto, Japan) for the subsequent experiments.

2.4. Cytokine production assay

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 96-well culture plate (Corning, Corning, NY, USA) at 3.0×10^4 cells/well, and cultured for 12 h at 37°C under humidified 5% CO_2. After washing with PBS, the cells were treated with 200 μL of 10% FBS-DMEM containing 20 ng of LPS and various concentrations of KPE or 10 mM NaPB as control and incubated for 12 h at 37°C. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. After incubation, the concentrations of TNF-α and IL-6 in the culture media were measured by enzyme-linked immunosorbent assay (ELISA) using Mouse TNF-α ELISA Ready-SET-GO! (eBioscience, San Diego, CA, USA) and Mouse IL-6 ELISA MAX Standard Set (BioLegend, San Diego, CA, USA), respectively, according to the manufacturer's instructions. Cell viability was measured using Cell Count Reagent SF (Nacalai Tesque) after collecting the culture media from each well for ELISA.

2.5. Real-time RT-PCR

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 24-well culture plate (BD Falcon, Franklin Lakes, NJ, USA) at 1.5×10^5 cells/well and cultured for 12 h at 37°C under humidified

5% CO_2. After washing with PBS, the cells were treated with 1 mL of 10% FBS-DMEM containing 100 ng of LPS and various concentrations of KPE or 10 mM NaPB as control, and incubated for 12 h at 37°C. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. Total RNA was then isolated from the cells using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instructions and used as a template for cDNA synthesis with MMLV-reverse transcriptase (Promega, Madison, WI, USA) and an oligo-$(dT)_{20}$ primer (Toyobo, Osaka, Japan). A real-time PCR mixture, with a final volume of 20 μL, consisted of Thunderbird SYBR qPCR Mix (Toyobo), 10 pmol of a forward primer, 10 pmol of a reverse primer, and 0.1 μg of a cDNA sample. Thermal cycling conditions were 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. PCR products were measured on a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated based on the comparative CT method using StepOne Software v2.1 (Applied Biosystems). Expression of the β-actin gene was used as an endogenous control. Specific oligonucleotide sequences for each gene are as follows. Mouse β-actin: sense, 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and antisense, 5'-ATGGAGCCACCGATCCACA-3'; mouse TNF-α: sense, 5'-CTACTCCCAGGTTCTCTTCAA-3' and antisense, 5'-GCAGAGAGGAGGTTGACTTTC-3'; mouse IL-6: sense, 5'-AAGCCAGAGTCCTTCAGAGAGAT-3' and antisense, 5'-TTGGATGGTCTTGGTCCTTAGC-3'.

2.6. Immunoblot analysis

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 35 mm dish (BD Falcon) at 1.0×10^6 cells/dish and cultured for 12 h at 37°C under humidified 5% CO_2. After washing with PBS, the cells were treated with 2.0 mL of 10% FBS-DMEM containing 200 ng of LPS and 20 mg/mL of KPE or 10 mM NaPB as control and incubated for 15 min. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. Cytosolic and nuclear proteins were prepared using a CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. Denatured proteins were then separated using SDS-PAGE and transferred onto a PVDF membrane (Hybond-P; GE Healthcare, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described [6].

2.7. Statistical analysis

Data obtained were expressed as mean ± standard deviation. One way ANOVA followed by Tukey-Kramer test as used to assess the statistical significance of the difference. Values with $^{*}p < 0.05$ or $^{**}p < 0.01$ were considered statistically significant.

3. Results

3.1. Effect of KPE on inflammatory cytokine production by LPS-stimulated RAW 264.7 cells

We first examined the effect of KPE on inflammatory cytokine production by LPS-stimulated RAW264.7 cells. As shown in Figure 1a, KPE significantly inhibited the production of IL-6 and TNF-α by LPS-stimulated RAW264.7 cells in a dose-dependent manner. The highest concentration of KPE showed the inhibition rate of approximately 90% for the production of both IL-6 and TNF-α compared to control. Cell viability test showed that KPE was not cytotoxic within the tested concentrations (Figure 1b). Therefore, we performed further experiments at 20 mg/mL or lower concentrations of KPE.

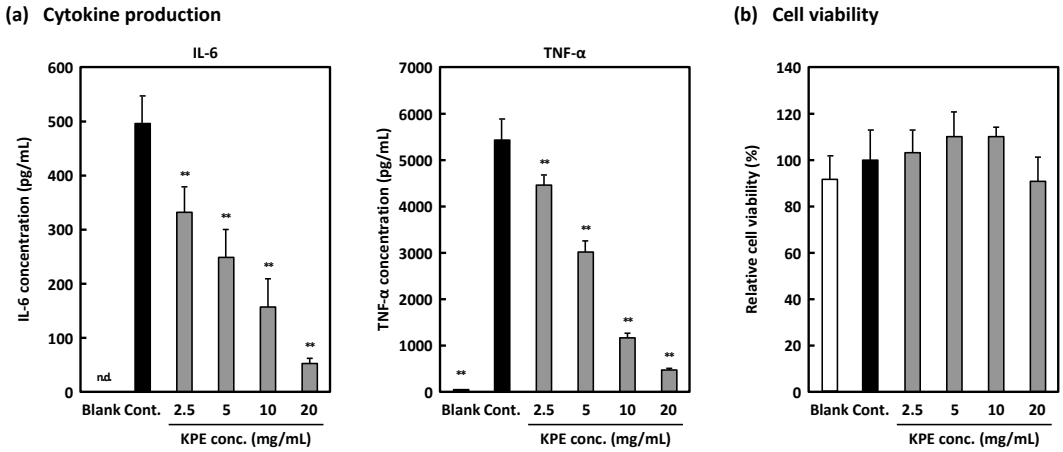

Figure 1. Effect of KPE on cytokine production and cell viability of RAW264.7 cells. (**a**) RAW264.7 cells were treated with 10% FBS-DMEM containing 100 ng/mL of LPS and various concentrations of KPE or 10 mM NaPB as control and incubated for 12 h. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. After incubation, the concentrations of TNF-α and IL-6 in the culture media were measured by ELISA. (**b**) Cell viability was measured after collecting the culture media from each well for ELISA. Experiments were performed in triplicate, and error bars indicate as the mean ± standard deviation. $^{**}p < 0.01$ against control by Tukey-Kramer test. n.d. indicates not determined.

3.2. Effect of KPE on expression levels of IL-6 and TNF-α genes in RAW264.7 cells

Next, the effect of KPE on gene expression of inflammatory cytokines in LPS-stimulated RAW264.7 cells was examined. The result showed that KPE significantly inhibited the mRNA expression levels of IL-6 in the cells (data not shown). On the other hand, a suppression tendency was observed in the mRNA expression levels of TNF-α, although there was no significant difference. These results suggested that KPE inhibits the production of inflammatory cytokines by suppressing the gene expression levels.

3.3. Effect of KPE on the signaling pathways involved in macrophage activation

LPS, a major outer membrane component of gram-negative bacteria, binds to TLR4 on the cell surface of macrophages and leads to macrophage activation through MAPK and NF-κB cascades. Therefore, we examined the effect of KPE on the signal molecules in MAPK and NF-κB cascades. The result showed that the phosphorylation level of p38 MAPK was inhibited by KPE, whereas that of ERK or JNK was not affected (data not shown). In addition, KPE inhibited translocation of NF-κB into the nucleus in the LPS-stimulated cells, suggesting that KPE shows an anti-inflammatory effect on macrophages through downregulation of MAPK and NF-κB cascades.

4. Discussion

In this study, we found that KPE inhibits the production of IL-6 and TNF-α by LPS-stimulated RAW264.7 cells without cytotoxicity. KPE also inhibited the mRNA expression levels of IL-6 and TNF-α in the cells. These results suggested that KPE inhibits production of inflammatory cytokines by suppressing the gene expression of cytokines in LPS-stimulated RAW264.7 cells.

MAPK and NF-κB cascades play essential roles in macrophage activation. There are mainly three families of MAPKs: ERK, JNK, and p38 kinases. MAPKs are activated by specific MAPK kinases (MAPKKs) and eventually lead to the synthesis of transcription factors such as c-Jun and c-Fos. These transcription factors form the activator protein (AP)-1 dimers, which bind on DNA after translocating

into the nucleus, and promote the transcriptional activity of target genes. In addition, NF-κB is translocated into the nucleus after activation, and regulates the transcriptional activity as a transcription factor. KPE suppressed the phosphorylation level of p38 MAPK and translocation of NF-κB from cytosol to nucleus in the LPS-stimulated cells. Our data indicated here suggest that KPE suppresses the expression levels of cytokine genes through inhibition of AP-1 activity and NF-κB translocation.

5. Conclusions

Although bioactive substances in citrus peel such as flavonoids and carotenoids have been studied very well, health functions of water-soluble substances in citrus peel have not been focused. In this study, we found that KPE inhibits the excessive production of inflammatory cytokines by LPS-stimulated RAW264.7 cells through downregulation of MAPK and NF-κB cascades. Taken together, our findings indicated that KPE contributes to alleviating a hyperinflammatory response in macrophages.

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