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Anti-inflammatory function of sudachitin and demethoxysudachitin from *Citrus sudachi*

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Abstract. It has been recognized that chronic inflammation, which differs in mechanism from acute inflammation triggered by normal immune responses, induces various diseases, including lifestyle-related diseases. Sudachitin (4',5,7-trihydroxy-3',6,8-trimethoxyflavone) and demethoxysudachitin (4',5,7-trihydroxy-6,8-dimethoxyflavone) are types of polymethoxyflavones found in the peel of sudachi (*Citrus sudachi*). This study focused on evaluation of the anti-inflammatory effects of these compounds, their mode of action, and their effect *in vivo*. Mouse macrophage cell line RAW264.7 cells and primary peritoneal macrophages (P-mac) were cultured with sudachitin or demethoxysudachitin, followed by stimulation with lipopolysaccharide (LPS) to induce an inflammatory response. Sudachitin and demethoxysudachitin suppressed IL-6, TNF-α, nitric oxide (NO) and MCP-1 production by RAW264.7 cells without cytotoxicity. Regarding their mode of action, they downregulated the activation of the MAPK and NF-κB pathways activated by LPS, thereby inhibiting the gene expression of inflammatory factors and suppressing the inflammatory response. Similarly, both compounds suppressed the production of inflammation mediators of in P-mac. Sudachitin exhibited a stronger inhibitory effect than demethoxysudachitin, suggesting that the methoxy group at the 3' position of sudachitin enhances its anti-inflammatory effect. To examine the anti-inflammatory effects *in vivo*, mice were orally administered samples for seven days and then subjected to LPS-induced systemic inflammation. Oral administration in the systemic inflammation model mice resulted in reduced blood levels of inflammatory cytokines. These findings confirm the efficacy of sudachitin and demethoxysudachitin in exerting anti-inflammatory effect.

1 Introduction

Macrophages and dendritic cells phagocytose and eliminate invading non-self substances and altered self cells, which in turn produce inflammatory factors and trigger an inflammatory response. This activates other immune cells and helps eliminate the non-self substances. However, excessive production of inflammatory substances or the persistence of an inflammatory state without the intrusion of non-self substances can lead to autoimmune diseases and chronic inflammatory diseases [1, 2]. Chronic inflammation differs in mechanism from acute inflammation triggered by normal immune responses. Chronic inflammation induces non-communicable diseases (NCDs). NCDs are responsible for 38 million deaths each year, approximately 28 million occur in low and middle-income countries. Cardiovascular disease, cancer, and diabetes are the NCDs that most death causes [3].

Meanwhile, citrus peels are rich in phytochemicals such as nobiletin and hesperidin, and these components have been reported to have various physiological activities, including anti-allergic, antioxidant, and fat accumulation inhibitory effects. [4]. Sudachitin (4',5,7-trihydroxy-3',6,8-trimethoxyflavone) and demethoxysudachitin (4',5,7-trihydroxy-6,8-dimethoxyflavone) are types of polymethoxyflavones found in the peel of sudachi (*Citrus sudachi*). This study aimed to elucidate the anti-inflammatory effects of these compounds, their mode of action, and their efficacy *in vivo*.

2 Materials and Methods

2.1 Reagents

Roswell Park Memorial Institute 1640 (RPMI 1640) medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and lipopolysaccharides (LPS) from *E. coli* 026/B6 were products of Sigma-Aldrich (St. Louis, MO, USA). HRP-labeled anti-rabbit IgG antibody, HRP-labeled anti-mouse IgG antibody and rabbit antibodies against histone H3, NF-κB p65, extracellular signal-regulated protein kinases

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(ERK)1/2, phosphorylated ERK1/2, p38 MAPK, and phosphorylated p38 MAPK and c-Jun N-terminal kinase (JNK), phosphorylated JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan) or Fujifilm Wako Pure Chemical (Osaka, Japan) unless otherwise noted.

2.2 Cells and cell culture

Mouse macrophage-like cell line RAW264.7 cells were distributed by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). RAW264.7 cells were cultured in 10% FBS-DMEM at 37°C under humidified 5% CO_2. Mouse peritoneal macrophages (P-mac) were prepared as previously described with some modifications [4]. In brief, female 8-week-old BALB/c mice were injected with 3.0% thioglycollate medium into the peritoneum. Four days after injection, the thioglycollate-elicited peritoneal macrophages were collected and cultured in a culture dish containing 10% FBS-RPMI 1640 medium. After cultivation for 1 h, to remove non-adherent cells, the culture dish was washed with PBS, and the adherent cells were collected and used as P-mac. The purity of P-mac prepared by this method was around 80%. P-mac were seeded into a culture plate and cultured at 37°C under humidified 5% CO_2 for use in the subsequent experiments.

2.3 Quantification of cytokines in culture medium

RAW264.7 cells and P-mac were cultured at 3.0×10^4 cells/well in a 96-well culture plate in 10% FBS-DMEM and 10% FBS-RPMI 1640, respectively. The cells were cultured for 12 h at 37°C under humidified 5% CO_2. After washing with PBS, the cells were treated with 200 µL of culture media containing 100 ng/mL of LPS and various concentrations of sudachitin (SUD) and demethoxysudachitin (DEM) or 10 mM sodium phosphate buffer (NaPB) as control and incubated for 12 h at 37°C. After incubation, the concentrations of TNF-α and IL-6 in the culture media were measured by enzyme-linked immunosorbent assay (ELISA) using ELISA MAX Standard Sets for mouse TNF-α and mouse IL-6 (BioLegend, San Diego, CA, USA), respectively.

2.4 Real-time RT-PCR

RAW264.7 cells suspended in 10% FBS-DMEM were inoculated at 1.5×10^5 cells/well in a 24-well culture plate, and cultured for 12 h at 37°C. After washing with PBS, the cells were treated with 1 mL of 10% FBS-DMEM containing 100 ng/mL of LPS and various concentrations of samples or 10 mM NaPB as control, and incubated for 12 h at 37°C. Blank cells were treated with 10% FBS-DMEM without LPS. Total RNA was then prepared from the cells by using Sepasol-RNA I Super G (Nacalai Tesque) for templates 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.

2.5 Immunoblot analysis

RAW264.7 cells were inoculated in a 35 mm dish at 1.0×10^6 cells/dish, and cultured for 12 h at 37°C. After washing with PBS, the cells were treated with 2.0 mL of 10% FBS-DMEM containing 100 ng/mL of LPS and samples or 10 mM NaPB as control and incubated for 15 min. Blank cells were treated with the medium without LPS. Cytosolic and nuclear proteins were prepared by using CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich). Denatured proteins were separated by using SDS-PAGE and transferred onto a PVDF membrane. Immunoblotting with various antibodies was performed as previously described [5].

2.6 Effect on a mouse model of SIRS

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice took standard food and water *ad libitum*. All animal experiments were approved by the Animal Experiment Committee of Ehime University and were performed in accordance with the Guidelines of Animal Experiments of Ehime University.

Systemic inflammatory response syndrome (SIRS) was induced in mice by injection of LPS as previously described with some modifications [6, 7]. Following adaptation period for 1 week, 9-week-old female BALB/c mice were randomly divided into 4 groups (6 mice/group). Intact group and control group were orally administered 20 μL of 10 mM NaPB for 7 consecutive days. SUD- and DEM-administered group mice were orally administered 20 μL of SUD and DEM at 5 mg/kg body weight/day for 7 days, respectively. One hour after the last oral administration, SIRS was induced in mice in control group and SUD group and DEM group by intraperitoneally injected of LPS at 10 mg/kg body weight. The mice in intact group were intraperitoneally injected with PBS alone. The blood was collected 2 h after LPS injection, and the serum levels of inflammatory cytokines were evaluated by ELISA.

2.7 Statistical analysis

Data obtained were expressed as mean ± tandard deviation. One-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey-Kramer test was used. Values with $*p < 0.05$ or $**p < 0.01$ were considered statistically significant.

3. Results and Discussion

3.1 Anti-inflammatory activity of sudachitin and demethoxysudachitin

Firstly, the effects of sudachitin (SUD) and demethoxysudachitin (DEM) on IL-6 and TNF-α production by LPS-stimulated RAW264.7 cells were evaluated 12 h after inoculation. The concentrations of IL-6 and TNF-α in the culture media were measured by ELISA. As indicated in Fig. 1A, both SUD and DEM significantly inhibited IL-6 and TNF-α production of LPS-stimulated RAW264.7 cells in a dose-dependent manner. The specific activities of SUD and DEM against LPS-treated RAW264.7 cells were almost same levels. On the other hand, the suppressive effect of SUD on IL-6 and TNF-α production of P-mac was significantly higher than that of DEM (Fig. 1B).

3.2 Effects of SUD and DEM on gene expression in LPS-stimulated RAW264.7 cells.

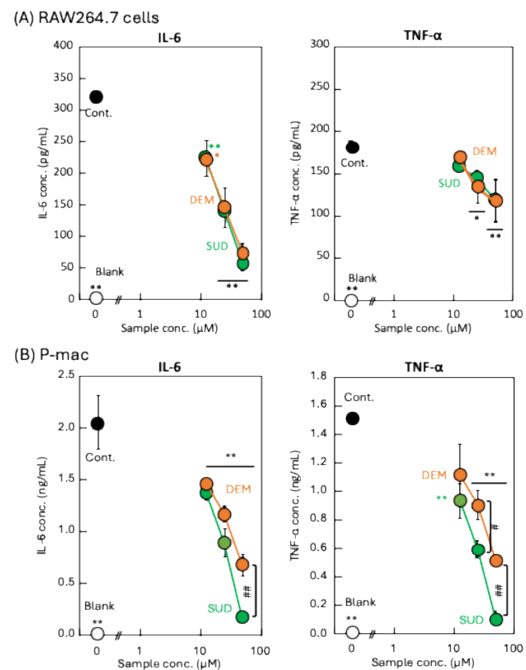

Fig. 1. Effects of SUD and DEM on IL-6 and TNF-α production of LPS-stimulated (A) RAW264.7 cells and (B) P-mac.

Effects of SUD and DEM on gene expression levels of inflammatory mediators in LPS-

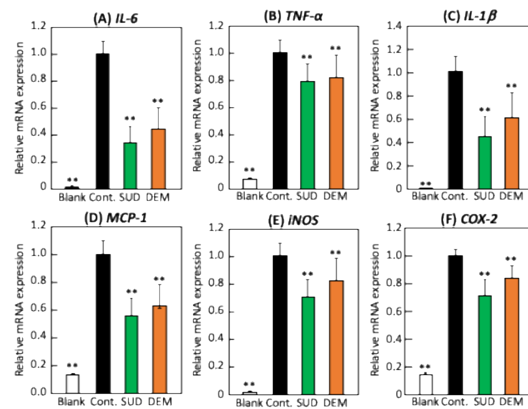

Fig. 2. Effects of SUD and DEM on gene expression in LPS-stimulated RAW264.7 cells. (A) *IL-6*, (B) *TNF-α*, (C) *IL-1β*, (D) *MCP-1*, (E) *iNOS* and (F) *COX-2* genes.

stimulated RAW264.7 cells were examined. SUD and DEM significantly inhibited the mRNA expression levels of inflammatory mediators such as IL-6 (Fig. 2A), TNF-α (Fig. 2B), IL-1β (Fig. 2C), MCP-1 (Fig. 2D), iNOS (Fig. 2E) and COX-2 (Fig. 2F). These results suggested that SUD and DEM inhibit the production of inflammatory cytokines by suppressing the gene expression levels.

3.3 Effects of SUD an DEM on signaling pathways in macrophage

To evaluate the mode of action of SUD and DEM on cytokine production by macrophages, the effects of SUD and DEM on MAPK pathway in

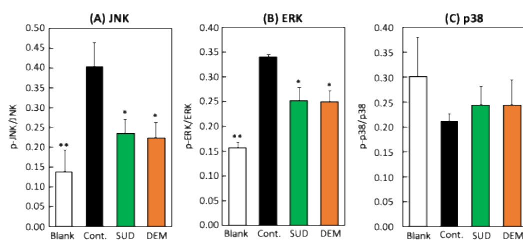

Fig. 3. Effects of SUD and DEM on phosphorylation of (A) JNK, (B) ERK and (C) p38 in MAPK pathway.

macrophage was examined. SUD and DEM significantly suppressed the phosphorylation of JNK (Fig. 3A) and ERK (Fig. 3B). The phosphorylation of p38 was also suppressed, but not significant (Fig. 3C).

Furthermore, the effects of SUD and DEM on NF-κB pathway was examined. As a result, SUD and DEM inhibited the translocation of NF-κB from the cytosol to the nucleus in LPS-stimulated RAW264.7 cells as indicated in Fig. 4.

3.4 Effects of oral administration of SUD and DEM on SIRS model mouse

The mice were orally administered with SUD and DEM at 5 mg/kg body weight/day for 7 days. One hour after the last oral administration of SUD and DEM, they were intraperitoneally injected with 10 mg/kg body weight of LPS/PBS to induce SIRS. Blood was collected 2 h after LPS injection, and IL-6 and TNF-α levels in serum were evaluated by ELISA. As a result, administration of SUD and DEM significantly inhibited IL-6 (Fig. 5A) and TNF-α (Fig. 5B) levels in serum from SIRS model mice. The concentrations of SUD and DEM in serum is still under investigation.

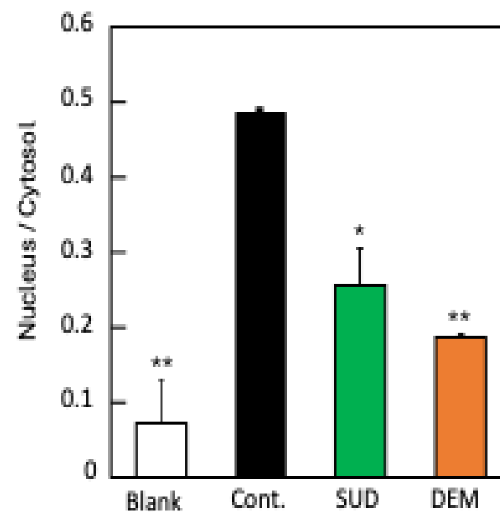

Fig. 4. Effects of SUD and DEM on translocation of NF-κB from cytosol to nucleus.

4 Conclusions

Citrus peel contains many kinds of flavonoids. SUD and DEM are types of polymethoxyflavones specifically found in the peel of sudachi (*Citrus sudachi*). In this study, it was revealed that both SUD and DEM suppressed IL-6 and TNF-α production by both LPS-stimulated RAW264.7 cells and P-mac. Specific activities of SUD and DEM on suppression of IL-6 and TNF-α production by LPS-stimulated RAW264.7 cells was almost same level, however, that of SUD on P-mac was significantly higher than that of DEM.

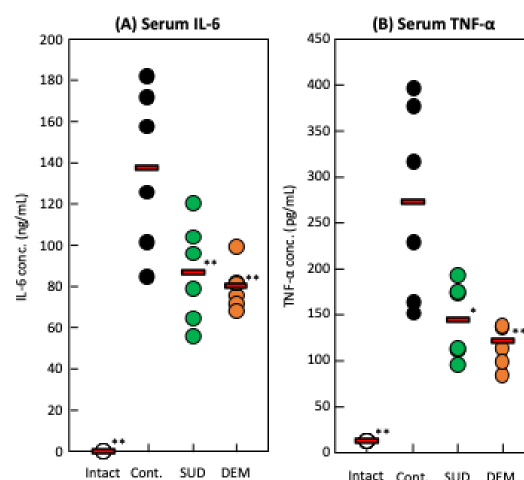

Fig. 5. Effect of oral administration of SUD and DEM on serum (A) IL-6 and (B) TNF-α levels in SIRS model mice.

This means that P-mac is more sensitive against SUD than DEM. This may come from the difference in the sensitivity of specific receptors against SUD and DEM.

SUD and DEM suppressed gene expression levels of IL-6 and TNF-α to reduce these inflammatory cytokine production by LPS-stimulated RAW264.7 cells. Not only suppression of gene expression levels of IL-6 and TNF-α, SUD and DEM suppressed iNOS gene expression, suggesting that nitric oxide (NO) production may also be suppressed.

Following binding of LPS with TLR4 on macrophages surface, activation of MAPK (ERK, JNK, and p38) pathway and NF-κB pathway were induced. ERK, JNK, and p38 form the activator protein (AP)-1 dimers, which bind with DNA after translocating into the nucleus, and promote the transcription of target genes. In NF-κB pathway, IKK is initially phosphorylated for activation [8]. The activated IKK phosphorylates IκBα, and the phosphorylated IκBα is rapidly degraded by the ubiquitin-proteasome system to translocate NF-κB from the cytosol to the nucleus, and promotes the transcription [5].

It is suggested from results that SUD and DEM suppress the activation of MAPKs, especially JUN and ERK, and translocation of NF-κB from cytosol to nucleus. Suppression of MAPK and NF-κB pathways by SUD and DEM resulted in inhibition of gene expression of inflammatory factors. In addition, oral administration of SUD and DEM against SIRS model mice clearly suppressed serum IL-6 and TNF-α levels, suggesting SUD and DEM suppressed inflammation response *in vivo.* Our findings suggested that SUD and DEM exhibit a strong anti-inflammatory activity *in vitro* and *in vivo*.

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