Original Article

Sebacic acid, a royal jelly-containing fatty acid, decreases LPS-induced IL-6 mRNA expression in differentiated human THP-1 macrophage-like cells

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Macrophages produce many inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), in innate immune responses. However, excess production of these mediators by activated macrophages triggers deleterious effects, leading to disorders associated with inflammation. Royal jelly (RJ), a milky-white substance secreted by worker bees, contains unique fatty acids, including 10-hydroxy-2-decenoic acid (10H2DA) and sebacic acid (SA). 10H2DA has been reported to have various biological functions, such as anti-inflammation. However, the anti-inflammatory effect of SA is not fully understood. In this study, we investigated the effects of SA on lipopolysaccharide (LPS)-induced cytokine expression using differentiated human THP-1 macrophage-like cells. SA dose-dependently decreased LPS-induced mRNA expression of IL-6, but not TNF-α and IL-1β. SA suppressed the phosphorylation of signal transducers and activators of transcription 1 (STAT1) and STAT3, but hardly affected the activation of JNK, p38, or NF-κB. In addition, SA decreased LPS-induced interferon-β (IFN-β) expression, and the addition of IFN-β restored the inhibition by SA of LPS-induced STAT activation and IL-6 expression. Furthermore, SA suppressed LPS-induced nuclear translocation of interferon regulatory factor 3 (IRF3), a transcription factor responsible for IFN-β expression. Taken together, we conclude that SA selectively decreases LPS-induced expression of IL-6 mRNA through inhibition of the IRF3/IFN-β/STAT axis.

***Key Words*:** **macrophage, royal jelly, sebacic acid, inflammation, cytokine**

Inflammation is an essential immune response to protect the host from infection. However, chronic inflammation has been demonstrated to also be involved in the pathogenesis of several diseases. Macrophages play critical roles in innate immune responses. They produce inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and nitric oxide (NO), in response to pathogens. Meanwhile, excess production of these mediators by activated macrophages triggers deleterious effects, leading to disorders associated with inflammation, such as septic shock, atherosclerosis, and hepatic fibrosis and cirrhosis.[1] Blockade of inflammation in the liver was reported to ameliorate liver fibrosis.[2] Recently, macrophages were reported to play a critical role in the progression of non-alcoholic fatty liver disease (NAFLD).[3]

The expression of inflammatory cytokines has been reported to be regulated by toll-like receptor 4 (TLR4) in macrophages.[4] Lipopolysaccharide (LPS), an exogenous TLR4 ligand, activates several signaling pathways including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK), including JNK and p38. The activation of these pathways is required for LPS-induced expression of cytokines. Several studies have shown that TLR4 participates in the exacerbation of hepatic steatosis observed in NAFLD.[5] TLR4 knockout ameliorates high-fat diet (HFD)-induced accumulation of triglycerides and cytokine production in the liver.[6] Serum LPS levels are increased in the HFD-induced NAFLD mouse model, and the inhibition of TLR4 suppresses liver inflammation compared with normal mice.[7] A systematic review and meta-analysis showed that NAFLD is significantly correlated with inflammatory cytokines, such as TNF-α and IL-6.[8]

Royal jelly (RJ), a milky-white substance secreted by worker bees, contains proteins, carbohydrates, lipids, vitamins, and minerals. 10-Hydroxy-2-decenoic acid (10H2DA), 10-hydroxydecanoic acid (10HDA), and sebacic acid (SA) are known as the main fatty acids included in RJ. These fatty acids have been reported to exert various biological functions including anti-inflammatory, anti-oxidant, and anti-diabetic effects.[9–12] For example, 10H2DA inhibits LPS-induced NF-κB activation in murine macrophage cell line RAW264 cells and decreases production of cytokines and NO. These RJ-containing fatty acids induce the expression of extracellular superoxide dismutase via inhibiting histone deacetylase (HDAC).[13] Intake of SA improves hyperglycemia and insulin resistance in human diabetes patients and animal models.[10] Recently, we demonstrated that SA decreases the expression of angiopoietin-like protein 8, a risk factor for metabolic syndrome.[14] However, the inflammatory effects of SA are not fully understood. In this study, we investigated the effects of SA on LPS-induced cytokine expression using differentiated human THP-1 macrophage-like cells.

Materials and Methods

Materials. Sebacic acid, valproic acid, and *O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anti-mouse IgG peroxidase antibody were purchased from Sigma-Aldrich (St. Louis, MO). Brefeldin A was purchased from Tokyo Chemical Industry (Tokyo, Japan). Anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-NF-κB, anti-phospho-STAT1, anti-STAT1, anti-phospho-STAT3, and anti-STAT3

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antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-YY-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody and HRP-conjugated anti-mouse IgG antibody were purchased from Sigma-Aldrich. HDAC1 Inhibitor Screening Assay Kit was purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture. Human monocytic leukemia THP-1 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, 0.1 mg/ml streptomycin, and 100 U/ml penicillin G in a humidified 5% CO_2/95% air incubator at 37°C. THP-1 cells were differentiated into macrophage-like cells by treatment with 100 nM TPA for 24 h, and cultured in the growth medium for another 24 h. Differentiated THP-1 (dTHP-1) cells were used for experiments.

Real-time RT-PCR. Cells were washed with phosphate-buffered saline (PBS), and total RNA was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). First-strand cDNA was synthesized according to the protocol of ReverTra Ace® qPCR RT Kit (TOYOBO, Otsu, Japan), and the real-time RT-PCR method was performed using Thunderbird® SYBR® qPCR Mix (TOYOBO). Target mRNAs were amplified using specific primers, as follows: TNF-α (forward primer, 5'-GGCGTGGAGCTGAGAGATAA-3'; reverse primer, 5'-TCG GCAAAGTCGAGATAGTC-3'), IL-6 (forward primer, 5'-AGA CAGCCACTCACCTCTTCA-3'; reverse primer, 5'-GATTTT CACCAGGCAAGTCTC-3'), interferon-β (IFN-β: forward primer, 5'-TTCAGTGTCAGAAGCTCCTGTG-3'; reverse primer, 5'-TAGTCTCATTCCAGCCAGTGCT-3'), IL-1β (forward primer, 5'-CACGATGCACCTGTACGATCA-3'; reverse primer, 5'-GTT GCTCCATATCCTGTCCCT-3'), and β-actin (forward primer, 5'-TGCGTGACATTAAGGAGAAGC-3'; reverse primer, 5'-GAG TTGAAGGTAGTTTCGTGG-3'). The respective mRNA expression levels were corrected for β-actin mRNA expression.

Western blot analysis. dTHP-1 cells were washed twice with ice-cold PBS, and then whole-cell and nuclear extracts were prepared as described in our previous report.[9] Whole-cell extracts (35 μg of total protein) or nuclear extracts (15 μg of total protein) were separated by SDS-PAGE using 10 or 12% polyacrylamide gel and then transferred onto a PVDF membrane. The membrane was incubated with specific primary antibodies (1:3,000) and sequentially with HRP-conjugated second antibody (1:5,000). Proteins were detected using SuperSignal West Pico (Thermo Fisher Scientific Inc., Waltham, MA) and imaged with ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA).

Cell viability. dTHP-1 cells were seeded in a 96-well plate at 2.5×10^4 cells/well. The next day, the cells were treated with varying concentrations of SA. Twenty-four hours later, cell viability was measured by MTT assay. This assay was carried out in quadruplicate. The results are expressed as percentages relative to untreated cells.

HDAC activity. HDAC activity was determined using HDAC1 Inhibitor Screening Assay Kit (Cayman Chemical). After adding 140 μl of assay buffer, 10 μl of recombinant HDAC1, and 10 μl of each sample to a 96-well plate, 10 μl of HDAC substrate was added and the plate was incubated at 37°C for 30 min. Then, 40 μl of HDAC developer was added, and the plate was incubated at room temperature for 15 min, followed by measuring the fluorescence (Ex 365 nm, Em 410–460 nm). Each HDAC activity is expressed as a percentage relative to the control.

Statistical analysis. Data were analyzed using ANOVA followed by the post-hoc Bonferroni test or Student's *t* test. A *p* value of less than 0.05 was considered significant.

Results

Effects of SA on LPS-induced gene expression of inflammatory cytokines. First, to examine the anti-inflammatory effects of SA, we investigated whether SA prevents LPS-induced inflammatory cytokine expression in dTHP-1 cells. dTHP-1 cells were pretreated with SA (0.5, 1, and 1.5 mM) for 24 h, and then were stimulated with LPS (100 ng/ml) for 2 h. The mRNA levels of inflammatory cytokines (TNF-α, IL-6, and IL-1β) were measured using real-time RT-PCR. LPS increased mRNA expression of these cytokines. SA dose-dependently suppressed LPS-induced IL-6 mRNA expression, while the induced expression of TNF-α and IL-1β mRNAs was not affected by SA treatment (Fig. 1A). SA also reduced LPS-induced expression of IL-6 at the protein level (Fig. 1B). Additionally, we confirmed that SA did not affect cell viability at the concentrations of SA used in this study (Fig. 1C).

Effects of SA on LPS-induced activation of signaling pathways. Activation of the MAPK and NF-κB pathways plays a key role in LPS-elicited inflammatory responses.[15] Therefore, we investigated the effects of SA on these pathways. dTHP-1 cells were pretreated with SA (0.5, 1, and 1.5 mM) for 24 h and then stimulated with LPS (100 ng/ml) for 2 h. LPS evoked phosphorylation of MAPK, namely JNK and p38, and increased the nuclear accumulation of NF-κB. However, SA did not inhibit the phosphorylation of JNK and p38 or the nuclear accumulation of NF-κB (Fig. 2A and B). These results indicate that the MAPK and NF-κB pathways were not involved in the decrease of IL-6 mRNA expression by SA.

In addition to the above-mentioned pathways, STAT signaling has been reported to be responsible for LPS-induced IL-6 expression.[16] We examined the effects of SA on LPS-induced STAT activation. dTHP-1 cells were cultured with SA (0.5, 1, and 1.5 mM) for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. LPS induced tyrosine-phosphorylation of STAT1 and STAT3, and SA significantly suppressed their increased phosphorylation (Fig. 2C).

Effects of SA on LSP-induced activation of IFN-β signaling. LPS has been reported to regulate expression of cytokines via autocrine/paracrine factors.[17] We investigated the effects of the protein synthesis inhibitor cycloheximide (CHX) on LPS-induced gene expression of IL-6. dTHP-1 cells were pretreated with CHX (5 μg/ml) for 30 min, and then stimulated with LPS (100 ng/ml) for 2 h. LPS-induced IL-6 mRNA expression was suppressed in the presence of CHX (Fig. 3A). Meanwhile, the treatment with CHX increased the levels of TNF-α mRNA, and did not affect those of IL-1β mRNA (Supplemental Fig. 1*). These results suggest that proteinaceous factors produced by LPS are important for LPS-induced IL-6 expression. IFN-β signaling through the type I IFN receptor has been shown to participate in LPS-induced IL-6 expression.[18,19] Therefore, we hypothesized that IFN-β may be responsible for the inhibitory effects of SA on LPS-induced IL-6 mRNA expression. We examined the effects of SA on LPS-induced IFN-β mRNA expression. dTHP-1 cells were pretreated with SA (0.5, 1, and 1.5 mM) for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. As expected, SA dose-dependently decreased LPS-induced IFN-β mRNA expression (Fig. 3B). In addition, we conformed that when dTHP-1 cells were treated with LPS, the expression peak of IFN-β preceded that of IL-6 under our experimental conditions (Supplemental Fig. 2*).

IFN-β addition abrogates the inhibitory effects of SA. Since IFN-β elicits phosphorylation of STAT via the type I IFN receptor, the inhibition of LPS-induced IFN-β expression by SA may be involved in the inhibition of STAT activation and IL-6 mRNA expression. We investigated whether the addition of IFN-β abrogates its inhibitory effects. dTHP-1 cells were pretreated with SA (1 mM) for 24 h, and then stimulated with LPS

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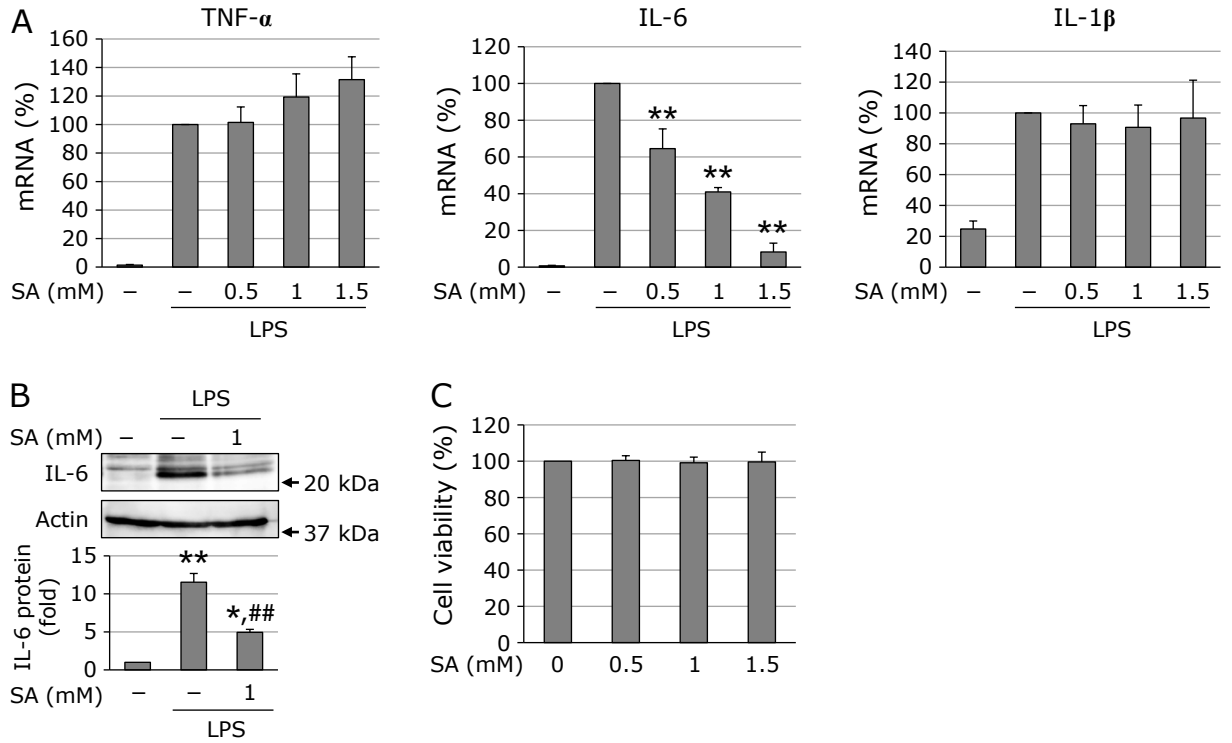

Fig. 1. Effects of SA on LPS-induced gene expression of inflammatory cytokines. (A) Real-time PCR analysis of cytokine mRNAs. dTHP-1 cells were pretreated with or without the indicated concentrations of SA for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. The levels of TNF-α, IL-6, and IL-1β mRNAs were determined by real-time RT-PCR. Values (mean ± SEM, $n = 4$) are expressed as percentages relative to LPS alone. $^{**}p<0.01$ (vs LPS alone). (B) Effect of SA on LPS-induced production of IL-6 protein. dTHP-1 cells were pretreated with SA (1 mM) for 24 h, and then stimulated with LPS (100 ng/ml) and brefeldin A (300 ng/ml, last 3 h) for 6 h. After that, whole cell extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 3$) are expressed as fold-changes relative to untreated cells. $^{*}p<0.05$, $^{**}p<0.01$ (vs untreated cells); $^{\#\#}p<0.01$ (vs LPS alone). (C) Cell viability. dTHP-1 cells were treated with the indicated concentrations of SA for 24 h. Cell viability was measured using the MTT assay. The results are shown as means ± SD ($n = 4$).

(100 ng/ml) for 2 h in combination with IFN-β (20 ng/ml). The addition of IFN-β restored the decreased levels of IL-6 mRNA expression and STAT1/3 phosphorylation (Fig. 4A and B).

HDAC-inhibitory activity of SA. It has been reported that 10H2DA, another RJ-containing fatty acid, inhibits HDAC.[20] HDAC inhibitors, such as valproic acid (VPA), have anti-inflammatory effects.[21,22] Therefore, we investigated the possibility that the inhibitory effects of SA on LPS-induced IL-6 expression are attributed to HDAC inhibition by SA. First, we examined the effects of SA on histone acetylation. After dTHP-1 cells were treated with SA (0.5, 1, and 1.5 mM) for 24 h, histone H3 and H4 acetylation was determined by Western blotting. SA dose-dependently increased the levels of histone H4 acetylation, but hardly affected histone H3 acetylation (Fig. 5A). In addition, we determined whether SA directly inhibits HDAC using the recombinant HDAC1. SA reduced HDAC activity similarly to VPA (Fig. 5B).

We further ascertained the anti-inflammatory effects of VPA under our experimental conditions. dTHP-1 cells were pretreated with VPA (0.5, 1, and 1.5 mM) for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. VPA decreased the expression of both IL-6 and IFN-β mRNAs in a dose-dependent manner (Fig. 5C).

Effects of SA on LPS-induced activation of interferon regulatory factor 3. The transcription factor interferon regulatory factor 3 (IRF3) plays an important role in the regulation of IFN-β gene expression.[23] HDAC4 has been shown to suppress virus-induced nuclear translocation of IRF3.[24] We examined the effects of SA on its nuclear translocation. dTHP-1 cells were pretreated with SA (1.5 mM) for 24 h, and then stimulated with LPS (100 ng/ml) for 1 h. LPS increased the amount of IRF3 in the nucleus, and SA suppressed the increased nuclear accumulation of IRF3 (Fig. 6A). In addition, VPA also suppressed its nuclear accumulation (Fig. 6B).

Discussion

It has been reported that the main RJ-containing fatty acid, 10H2DA, exhibits pleiotropic biological functions such as antioxidation and anti-inflammation.[11,25] 10H2DA suppresses LPS-induced expression inducible NO synthase and IL-6, but not TNF-α, in RAW264 murine macrophages. Meanwhile, Chen *et al.*[26] demonstrated that the RJ-fatty acids 10H2DA and SA suppress LPS-induced expression of IL-6 and TNF-α at high concentrations. Therefore, the inhibitory effects of RJ-containing fatty acids on cytokine induction have remained controversial. Whether SA has anti-inflammatory effects is not fully understood. In the present study, we examined the effects of SA on LPS-induced expression of proinflammatory cytokines using dTHP-1 cells, which were differentiated into macrophage-like cells. SA specifically decreased LPS-induced IL-6 expression, whereas the increased TNF-α and IL-1β mRNA expressions were not affected by SA treatment.

The MAPK and NF-κB pathways play important roles in LPS-induced expression of inflammatory cytokines. Indeed, several natural products with anti-inflammatory activity, such as flavonoids, have been reported to inhibit these pathways.[27,28] However, SA hardly affected LPS-induced activation of JNK and p38, or nuclear translocation of NF-κB, suggesting that SA exerts its anti-inflammatory effects through mechanisms other than inhibition of these pathways. Meanwhile, since SA and 10H2DA

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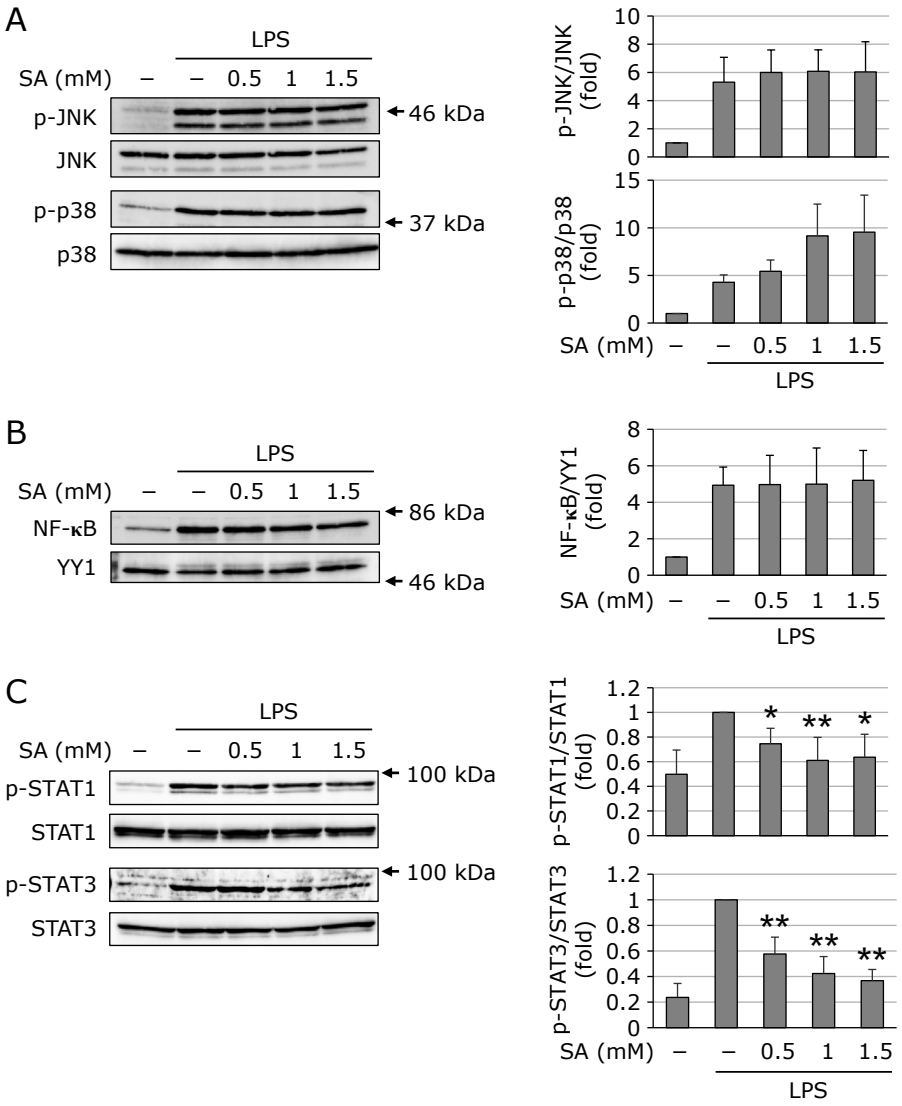

Fig. 2. Effects of SA on LPS-induced activation of signaling pathways. (A) Effects of SA on LPS-induced JNK and p38 phosphorylation. dTHP-1 cells were pretreated with or without indicated concentrations of SA for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. After the treatment, whole cell extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 3$) are expressed as fold-changes relative to untreated cells. (B) Effects of SA on LPS-induced nuclear accumulation of NF-κB. dTHP-1 cells were pretreated with or without indicated concentrations of SA for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. After the treatment, nuclear extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 3$) are expressed as fold-changes relative to untreated cells. (C) Effects of SA on LPS-induced STAT phosphorylation. dTHP-1 cells were pretreated with or without indicated concentrations of SA for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. After the treatment, whole cell extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 5$) are expressed as fold-changes relative to LPS alone. *$p<0.05$, **$p<0.01$ (vs LPS alone).

were reported to suppress JNK activation by LPS at higher concentrations of SA (2.5 and 5 mM) in RAW264.7 cells,[26] high concentrations of SA are likely to cause the inhibition of JNK. In addition, 10H2DA inhibits LPS-induced activation of NF-κB and subsequent expression of IκB-ζ.[29] These findings suggest that each RJ-fatty acid has different inhibitory properties for signaling pathways.

It is known that an autocrine mechanism is involved in the induction of IL-6 expression by LPS.[18,19] In fact, we found that LPS-induced IL-6 mRNA expression was decreased in the presence of CHX in dTHP-1 cells. This result strongly supports the suggestion that the production of proteinaceous factors contribute to this phenomenon. Since type I IFN receptor 1 deficient suppresses IL-6 production by synthetic double-stranded RNA,[30] IFN-β may function as an autocrine factor involved in IL-6 induction. IFN-β promotes tyrosine-phosphorylation of STAT1/3 via type I IFN receptors to form homo- or heterodimers. The STAT dimers regulate gene expression via binding to STAT-binding elements in promoter regions of target genes such as IL-6, leading to inflammatory responses. Therefore, the STAT pathway has been reported to be important for IL-6 gene expression.[16] In the present study, we found that SA decreased LPS-induced IFN-β expression and subsequent STAT1/3 phosphorylation. Furthermore, the addition of IFN-β restored the decreased STAT1/3 phosphorylation and IL-6 expression by SA. 10H2DA does not inhibit LPS-induced IFN-β expression or STAT activation.[29] These results suggest that the inhibitory effect of SA on LPS-induced IL-6 expression is in part due to inhibition of IFN-β autocrine signaling. IFN-β alone was shown to increase IL-6 expression in astrocytes.[31] However, since the treatment with IFN-β alone slightly induced IL-6 mRNA expression in dTHP-1 cells, IFN-β may not be sufficient on its own to induce IL-6

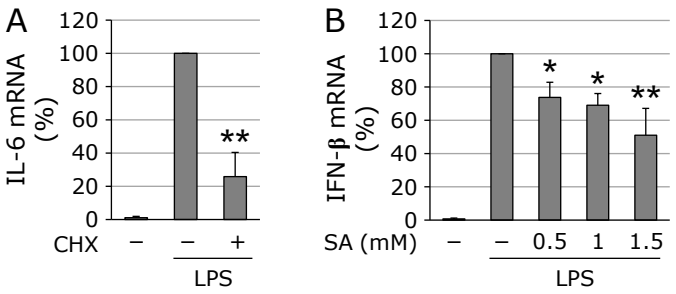

Fig. 3. Contribution of an autocrine factor to LPS-induced IL-6 expression. (A) Effects of cycloheximide on LPS-induced expression of IL-6 mRNA. dTHP-1 cells were pretreated with or without cycloheximide (CHX: 5 μg/ml) for 30 min, and then stimulated with LPS (100 ng/ml) for 2 h. The levels of IL-6 mRNA were determined by real-time RT-PCR. Values (mean ± SEM, $n = 3$) are expressed as percentages relative to LPS alone. $^{**}p<0.01$ (vs LPS alone). (B) Effects of SA on LPS-induced expression of IFN-β mRNA. dTHP-1 cells were pretreated with or without indicated concentrations of SA for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. The levels of IFN-β mRNA were determined by real-time RT-PCR. Values (mean ± SEM, $n = 4$) are expressed as percentages relative to LPS alone. $^{*}p<0.05$, $^{**}p<0.01$ (vs LPS alone).

expression in macrophage-like cells. Thus, the activation by LPS of other signaling pathways such as MAPK and NF-κB is likely to be required for LPS-induced IL-6 expression in macrophage-like cells.

The STAT pathway was reported to be involved in LPS-induced IL-1β expression in RAW 264.7 cells.[16] However, the contribution of STAT to expression of IL-1β mRNA in cells treated with LPS remains controversial. Since we found that CHX did not affect the increased IL-1β mRNA expression in dTHP-1, it is unlikely that the activation of STAT signaling via IFN-β participates in LPS-induced IL-1β expression under our experimental conditions.

HDAC inhibitors, such as trichostatin A and VPA, have been reported to exert potent anti-inflammatory effects.[21,22,32] For example, HDAC inhibitors suppress LPS-induced IFN-β expression and prevent subsequent IFN signaling.[33] Knockdown of HDAC3, an HDAC family member, suppresses LPS-induced STAT phosphorylation and IL-6 expression.[34] These findings indicate that HDACs are closely related to the regulation of inflammation. Here, we also showed that VPA inhibited LPS-induced expression of IFN-β and IL-6 mRNAs in dTHP-1 cells. Recent studies demonstrated that medium-chain fatty acids enhance gene expression via HDAC inhibition.[35] 10H2DA, a main RJ-containing fatty acid, functions as an HDAC inhibitor.[20] Therefore, we examined whether the anti-inflammatory effects of SA are attributed to the inhibitory activity of SA against HDAC. SA enhanced acetylation of histone H4 and inhibited enzymatic additivity of HDAC1. These results suggest that the inhibitory effects of SA on LPS-induced IL-6 expression may be partially due to HDAC inhibition by SA.

The transcription factor IRF3 is a master regulator of IFN-β gene expression. TLR4 activation in response to LPS promotes the nuclear translocation of IRF3 and its binding to the IFN-stimulated response elements in the *IFN-β* gene, leading to the increased expression of IFN-β.[23] LPS fails to activate the IRF3/IFN-β/STAT axis in IRF3-deficient cells.[36] We found that SA decreased LPS-induced nuclear accumulation of IRF-3, suggesting that the inhibition by SA of LPS-induced activation of IRF3 is likely to be involved in its anti-inflammatory effects. Unfortunately, at present, the mechanism for suppressing LPS-induced nuclear accumulation of IRF3 by SA remains unknown. However, VPA, an HDAC inhibitor, suppressed the increased nuclear levels of IRF3. This result suggests the possibility that the HDAC inhibitory activity of SA may contribute to the decreased expression of IFN-β mRNA through suppressing LPS-mediated IRF3 signaling. Further experiments are needed to fully understand the mechanisms.

In conclusion, we demonstrated that SA, like other RJ-

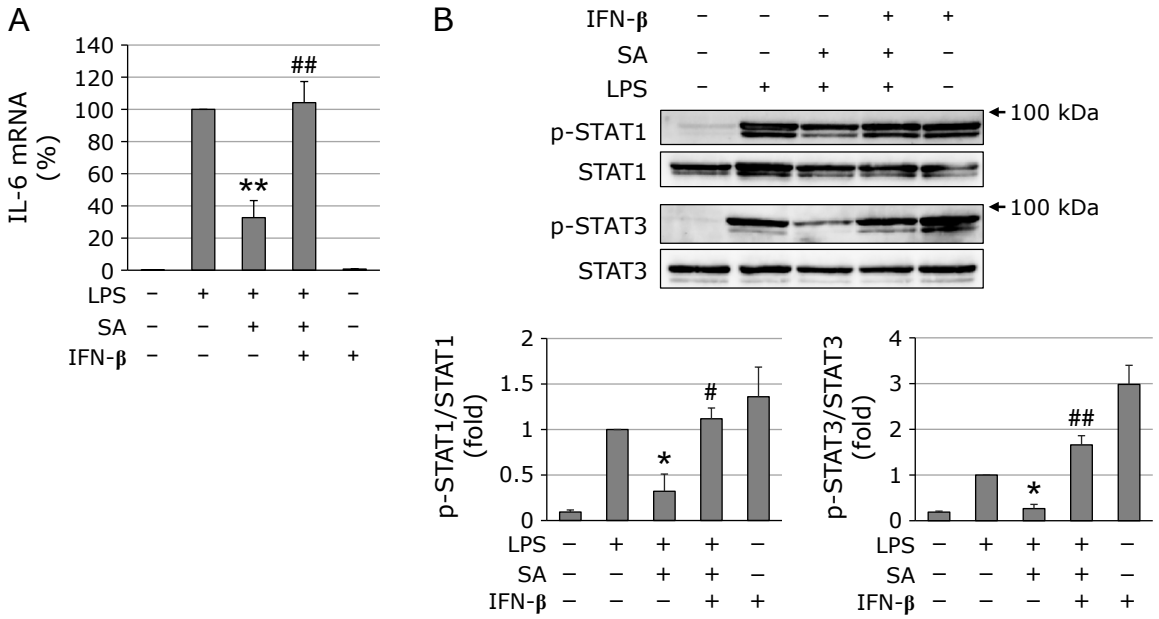

Fig. 4. IFN-β addition abrogates inhibitory effects of SA on LPS-induced IL-6 expression. (A) Effects of addition of IFN-β on suppression of IL-6 mRNA induction by SA. dTHP-1 cells were pretreated with or without SA (1 mM) for 24 h, and then stimulated with LPS (100 ng/ml) and IFN-β (20 ng/ml) for 2 h. The levels of IL-6 mRNA were determined by real-time RT-PCR. Values (mean ± SEM, $n = 4$) are expressed as percentages relative to LPS alone. $^{**}p<0.01$ (vs LPS alone); $^{\#\#}p<0.01$ (vs LPS + SA). (B) Effects of addition of IFN-β on suppression of LPS-induced STAT phosphorylation by SA. dTHP-1 cells were pretreated with or without SA (1 mM) for 24 h, and then stimulated with LPS (100 ng/ml) and IFN-β (20 ng/ml) for 2 h. After treatment, whole-cell extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 3$) are expressed as fold-changes relative to LPS alone. $^{*}p<0.05$ (vs LPS alone); $^{\#}p<0.05$, $^{\#\#}p<0.01$ (vs LPS + SA).

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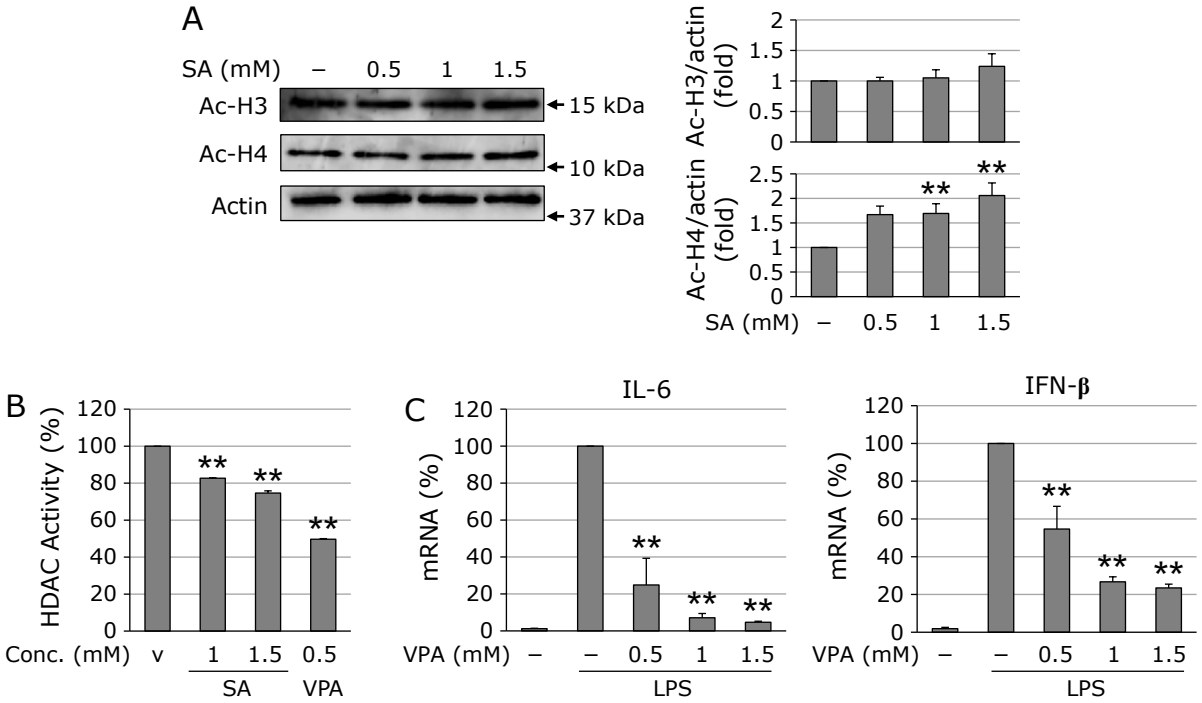

Fig. 5. Contribution of HDAC inhibition to inhibitory effects of SA on IL-6 expression. (A) Effects of SA on histone acetylation. dTHP-1 cells were pretreated with or without indicated concentrations of SA for 24 h. After treatment, whole cell extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 3$ or 5) are expressed as fold-changes relative to untreated cells. $^{**}p<0.01$ (vs untreated cells). (B) HDAC inhibitory activity of SA. HDAC inhibitory activity of SA and valproic acid (VPA) was determined using HDAC1 Inhibitor Screening Assay Kit. Values (mean ± SEM, $n = 3$) are expressed as percentages relative to control (v: vehicle). $^{**}p<0.01$ (vs vehicle). (C) Effects of VPA on LPS-induced expression of IL-6 and IFN-β. dTHP-1 cells were pretreated with or without indicated concentrations of VPA for 24 h, and then stimulated with LPS (100 ng/ml) for 2 h. The levels of IL-6 and IFN-β mRNA were determined by real-time RT-PCR. Values (mean ± SEM, $n = 3$) are expressed as percentages relative to LPS alone. $^{**}p<0.01$ (vs LPS alone).

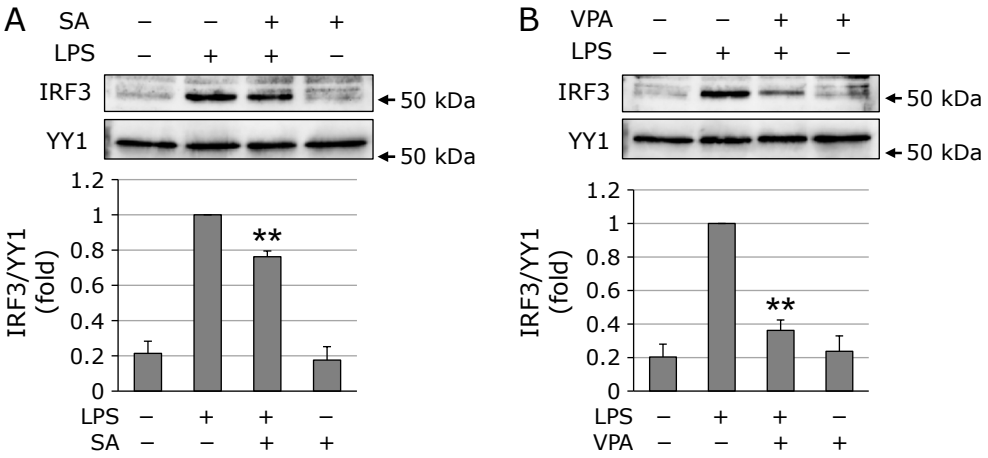

Fig. 6. Effects of SA on LPS-induced nuclear accumulation of IRF3. dTHP-1 cells were pretreated SA (1.5 mM: A) or VPA (0.5 mM: B) for 24 h, and then stimulated with LPS (100 ng/ml) for 1 h. After treatment, nuclear extracts were prepared from the cells and subjected to Western blot analysis. Values (mean ± SEM, $n = 3$ or 4) are expressed as fold-changes relative to LPS alone. $^{**}p<0.01$ (vs LPS alone).

containing fatty acids, has anti-inflammatory effects. SA selectively decreases LPS-induced expression of IL-6 mRNA through inhibition of the IRF3/IFN-β/STAT axis. These findings suggest that RJ-containing fatty acids play important roles to exert anti-inflammatory effects of RJ.

Author Contributions

HH designed the study; EO, NS, and HH performed experiments and acquired data; EO, TK, and HH analyzed and interpreted data; HH wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

HH received a research grant from API Co., Ltd. However, the company was not involved in the study design, collection, analysis and interpretation of the data, or preparation of the manuscript. All other authors declare no conflict of interest.

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