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N-feruloylserotonin inhibits lipopolysaccharide-induced inflammation *via* SIRT1 -stimulated FOXO1 and NF-κB signaling pathways in RAW 264.7 cells

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ABSTRACT

Macrophages become activated by a variety of stimuli such as lipopolysaccharide (LPS) and participate in the process of immune responses. Activated macrophages produce various inflammatory mediators. In the present study, we investigated the anti-inflammatory mechanism of a serotonin derivative, *N*-feruloylserotonin, isolated from safflower seeds in RAW 264.7 macrophages. *N*-Feruloylserotonin treatment significantly attenuated these effects on LPS-induced reactive oxygen species, nitric oxide, and prostaglandin E_2 production in RAW 264.7 macrophages. Furthermore, *N*-feruloylserotonin significantly decreased the abnormal expression of mitogen-activated protein kinase, such as phosphor (p)-c-Jun N-terminal kinase and p-extracellular-signal regulated kinase activation. Further research revealed that *N*-feruloylserotonin could stimulate sirtuin1 (SIRT1), then promote the forkhead box protein O1 (FOXO1), and suppress nuclear factor-kappa B (NF-κB) signaling pathways. The present study suggests that *N*-feruloylserotonin may be a new anti-inflammatory component and a promising candidate for anti-inflammatory therapeutic agents through the regulation of SIRT1-stimulated FOXO1 and NF-κB signaling pathways.

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Introduction

Rheumatoid arthritis is well-defined as a systemic autoimmune disorder associated with a chronic inflammatory process, which gradually leads to joint destruction, deformity, disability, chronic pain, and even premature death (1). Rheumatoid arthritis is also a serious disorder that significantly impairs people's quality of life and affects 1% of the world's population (2). Inflammatory responses complicate the development and progression of rheumatoid arthritis (3). Therefore, the development of effective anti–inflammatory medication to treat rheumatism is urgently required.

Safflower (*Carthamus tinctorius* L., a member of the chrysanthemum family), which is distributed widely throughout the world, including China, India, Southern Europe, and North America, is widely used for the treatment of bone formation, osteoporosis blood stasis, and prevention of rheumatism in Korea (4). Moreover, a large proportion of phenolic compounds, such as serotonin (5-hydroxytryptamine) derivatives, serotonin glycosides, lignin, and flavonoids, have been isolated from safflower seeds (5). Our previous study showed that safflower seeds exerted a pleiotropic effect on several parameters related to oxidative stress and inflammation. In addition, they had a renoprotective effect in cisplatin-treated mice (6), and serotonin and two of its derivatives, *N*-feruloylserotonin and *N*-(*p*-coumaroyl) serotonin, were identified as biologically active substances in the seeds (7). Furthermore, our previous study showed that serotonin and its major derivatives [*N*-feruloylserotonin and *N*-(*p*-coumaroyl) serotonin] suppressed inflammation- and apoptosis-related protein expressions by blocking mitogen-activated protein kinase (MAPK)-dependent nuclear factor-kappa B (NF-κB) activation pathway in mice (7). Overall, the anti-inflammatory properties of hydroxycinnamic acid (such as ferulic and *p*-coumaric acids) amides of serotonin were found to be superior to those of serotonin.

N-Feruloylserotonin (Figure 1), an alkaloid and polyphenol, is an amide formed between serotonin and ferulic acid (feruloyl) widely distributed in many plants (8-12), especially in safflower seeds; they contain abundant *N*-feruloylserotonin (37.06 mg/g) (6, 13). It was first identified as an anti-oxidant compound in safflower (14) and

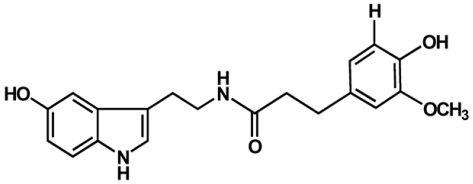

Figure 1. Chemical structure of *N*-feruloylserotonin.

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inhibited proinflammatory cytokine production (15). In addition, serotonin (a simple indole alkaloid) is a physiologically active amine and a well-known neurotransmitter that regulates mood, sleep, and anxiety in mammals (16). Its hallucinogenic effect is well-known based on biochemical, electrophysiological, and behavioral studies. Serotonin also plays a role as an anti-oxidant by scavenging reactive oxygen species (ROS). Moreover, it has been reported to show strongly *in vitro* anti-oxidant activity (17). It was also reported that serotonin reduced lipopolysaccharide (LPS)-induced up-regulation of pro-inflammatory mediators and cytokines (18). The serotonin derivative *N*-feruloylserotonin, isolated from safflower seeds, was reported to have anti-inflammatory activities compared with those of serotonin (4). However, to the best of our knowledge, the mechanisms have not yet been studied in LPS-stimulated RAW 264.7 macrophages.

Inflammation is a defence mechanism against harmful pathogens, such as bacteria, viruses, and fungi, and macrophages are key mediators of immune responses (19). During inflammation, activated macrophages secrete pro-inflammatory cytokines and mediators, including nitric oxide (NO). NO plays a critical role in maintaining physiological homeostasis in the body. However, excessive NO can react with the superoxide anion (O_2^-) to form toxic peroxynitrite ($ONOO^-$), which has been implicated in the progression of degenerative and inflammatory diseases, such as cancer, diabetes, cardiovascular diseases, and Alzheimer's disease (20). LPS, a potent NO donor, is widely used to elucidate the possible mechanisms of NO-mediated oxidative stress and cell death. It has been reported that LPS induces phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) in macrophages. The activation of signalling pathways involving these MAPK leads to the production of pro-inflammatory cytokines and mediators, such as NO and prostaglandin E_2 (PGE_2) (21). In addition, a high level of LPS causes the production of ROS in a variety of cell types (22). Moreover, sirtuin1 (SIRT1), a member of the sirtuin family, has been reported to be closely related to inflammatory pathways (23). Practically, the regulation of SIRT1-related pathways can help inhibit the progression of inflammation-related disorders. Therefore, the current study aimed to investigate the anti-inflammatory effect of *N*-feruloylserotonin and its mechanisms through the regulation of SIRT1 using LPS-stimulated RAW 264.7 macrophages.

Materials and Methods

Materials

RAW 264.7 macrophages were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). LPS (*Escherichia coli*, serotype 0111:B4) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The enzyme immunoassay kit for PGE_2 was obtained from R&D Systems (Minneapolis, MN, USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR, USA). SIRT1, phosphor (p)-forkhead box protein O1 (FOXO1), superoxide dismutase (SOD), catalase, NF-κB, inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), ERK, p-ERK, JNK, p-JNK, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

The RAW 264.7 cells were cultured at 37°C in a CO_2 (5%) incubator in DMEM containing penicillin/streptomycin (1%) and FBS (10%) and sub-cultured weekly using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline.

Cell viability

After the cells had reached confluence, they were seeded at 5 x 10^4 cells per well into 24-well plates and incubated for 2 h, and then treated with *N*-feruloylserotonin for 24 h. RAW 264.7 cells were then stimulated with LPS (1 μg/mL) for 24 h. The cells were incubated with 1 mL of MTT solution (5 mg/mL) for 4 h at 37°C, and the medium containing MTT was removed. Then, the formazan crystals were dissolved in 1 mL of DMSO, and viable cells were quantified by measuring absorbance at 540 nm (24).

ROS measurement

The ROS scavenging activity was measured using DCFH-DA (25). RAW 264.7 cells were incubated with *N*-feruloylserotonin for 24 h at 37°C, followed by treatment with LPS (1 μg/mL) for another 24 h. Fluorescence was read for 60 min, at wavelengths of 480 nm for excitation and 535 nm for emission, using a fluorescence plate reader (BMG LAB-TECH, Ortenberg, Germany).

NO measurement

The nitrite concentration in the medium was measured as an indicator of NO production. RAW 264.7 macrophages were cultured in a 60-mm cell culture dish, preincubated for 1 h with different concentrations of *N*-feruloylserotonin, and then stimulated for 16 h with LPS. One-hundred microliter of each supernatant was mixed with the same volume of Griess reagent; absorbance of the mixture at 540 nm was determined with an ELISA plate reader (26).

PGE_2 measurement

RAW 264.7 macrophages were cultured in a 60-mm cell culture dish, pre-incubated for 1 h with different concentrations of *N*-feruloylserotonin, and then stimulated for 16 h with LPS. One-hundred microliter of supernatant of the culture medium was collected for the determination of PGE_2 concentrations using an ELISA kit.

Western blot analysis

Cellular proteins were extracted from control and *N*-feruloylserotonin-treated RAW 264.7 cells. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, 0.5 mM DTT, 5 mM Na fluoride, and 0.5 mM

Na orthovanadate) containing 5 μg/mL each of leupeptin and aprotinin and incubated for 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation by 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with a blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a primary antibody. Blots were washed three times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) (n=5). Data were compared using one-way ANOVA. *P*-values < 0.05 were considered significant. All analyses were performed using SPSS for Windows, version 23 (SPSS Inc., Chicago, IL, USA).

Results

Cell viability

Cells were treated with different concentrations of *N*-feruloylserotonin, and cell viability was determined after incubation by MTT. As shown in Figure 2, the survival rate was significantly reduced at 50 and 100 μM of *N*-feruloylserotonin. In subsequent experiments, therefore, the maximum concentration was limited to 25 μM of *N*-feruloylserotonin.

ROS levels

Activated macrophages have been reported to increase oxidative stress and reduce antioxidant enzymes that prevent cell or tissue damage (25). Therefore, we measured whether *N*-feruloylserotonin prevents LPS-induced ROS production using the DCFH-DA assay. Figure 3 indicates that LPS-stimulated RAW 264.7 cells exhibited a higher ROS level, while ROS levels were markedly decreased by *N*-feruloylserotonin to almost the level in the non-LPS-treated group.

NO and PGE_2 levels

Since NO and PGE_2 are products of iNOS and COX-2 enzymes, respectively, we analyzed the effect of *N*-feruloylserotonin treatment on the production of NO and PGE_2 in LPS-activated RAW 264.7 cells. Significant concentration-dependent suppression by *N*-feruloylserotonin of NO generation was observed in RAW 264.7 cells (Figure 4A). Also, *N*-feruloylserotonin concentration dependently diminished the production of PGE_2 in cells treated with LPS (Figure 4B).

SIRT1 expression

SIRT1 protects cells from FOXO1 and NF-κBp65-mediated apoptosis in response to oxidative stress (23). As presented in Figure 5, the expression levels of SIRT1 were markedly reduced in LPS-treated RAW 264.7 cells, but *N*-feruloylserotonin-treated RAW 264.7 cells showed higher expression levels.

p-FOXO1, MnSOD, and catalase expressions

As reported by Haigis and Sinclair (23), FOXO activa-

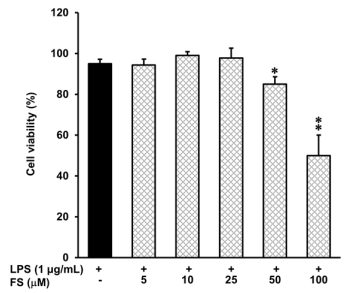

Figure 2. Effects of *N*-feruloylserotonin on cell viability in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Significance: **p*<0.05, ***p*<0.001 *vs*. LPS-treated cell values.

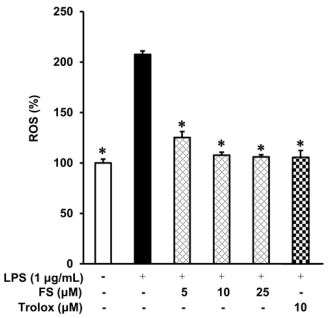

Figure 3. Effects of *N*-feruloylserotonin on ROS levels in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Trolox is used as a positive control. Significance: **p*<0.001 *vs*. LPS-treated cell values.

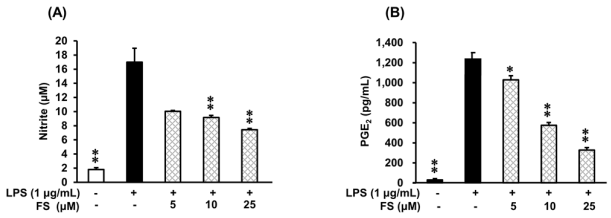

Figure 4. Effects of *N*-feruloylserotonin on NO (A) and PGE_2 (B) levels in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Significance: **p*<0.05, ***p*<0.001 *vs*. LPS-treated cell values.

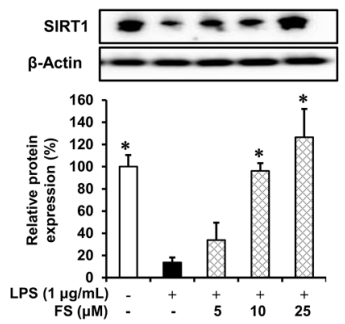

Figure 5. Effects of *N*-feruloylserotonin on SIRT1 expressions in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Significance: **p*<0.001 *vs*. LPS-treated cell values.

tion leads to increased levels of FOXO target genes like antioxidant proteins such as SOD and catalase. Our experimental results also showed that the expressions of FOXO and its regulated Mn-SOD and catalase were significantly lower in the LPS-treated group, as compared with the non-treated group. However, the reduced p-FOXO1 and its regulated antioxidant protein expressions were increased significantly in RAW 264.7 cells treated with *N*-feruloylserotonin (Figure 6).

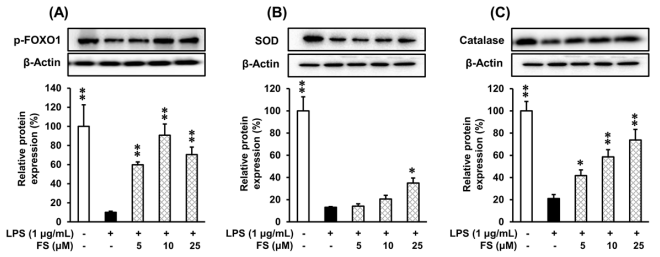

Figure 6. Effects of *N*-feruloylserotonin on p-FOXO1 (A), MnSOD (B), and catalase (C) expressions in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Significance: **p*<0.05, **p*<0.001 *vs*. LPS-treated cell values.

p-ERK and p-JNK expressions

The MAPK pathway is known to play an important role in the transcriptional regulation of LPS-induced iNOS and COX-2 expressions (27). To investigate the involvement of the MAPK pathway in the inhibitory effects of *N*-feruloylserotonin on NO and PGE_2 productions, the expression of MAPK (p-ERK and p-JNK) induced by LPS was evaluated in RAW 264.7 cells. As shown in Figure 7, *N*-feruloylserotonin (5, 10, and 25 μM) strongly inhibited LPS-induced activations of p-ERK and p-JNK in LPS-induced RAW 264.7 cells.

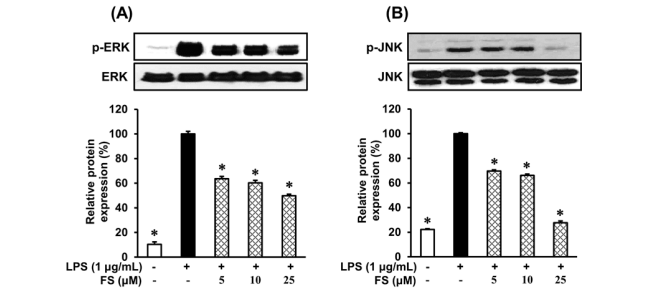

Figure 7. Effects of *N*-feruloylserotonin on p-ERK (A) and p-JNK (B) expressions in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Significance: **p*<0.001 *vs*. LPS-treated cell values.

p-NF-κBp65, iNOS, and COX-2 expressions

As ROS are involved in LPS-induced expression of proinflammatory genes such as p-NF-κBp65, iNOS, and COX-2 by macrophages (28), we tested whether *N*-feruloylserotonin treatment reduces expressions of these genes in LPS-activated RAW 264.7 cells. In our results, the expressions of inflammation-related proteins (p-NF-κBp65, iNOS, and COX-2) were significantly higher on stimulation with LPS. The increased protein expression of NF-κBp65 was decreased significantly in RAW 264.7 cells treated with *N*-feruloylserotonin. In addition, treatment with *N*-feruloylserotonin resulted in concentration-dependent down-regulation of protein expressions involved in the inflammatory response. The reduced expression of iNOS was stronger than that of COX-2, indicating that the iNOS enzyme plays a key role in promoting the anti-inflammatory actions of *N*-feruloylserotonin (Figure 8).

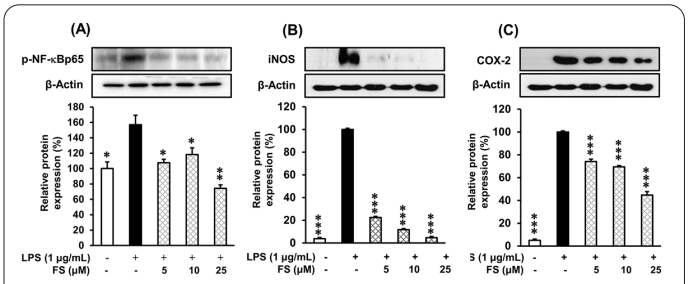

Figure 8. Effects of *N*-feruloylserotonin on NF-κBp65 (A), iNOS (B), and COX-2 (C) expressions in RAW 264.7 macrophages treated with LPS. FS, *N*-feruloylserotonin. Data are the mean ± SD. Significance: **p*<0.05, ***p*<0.01, ****p*<0.001 *vs*. LPS-treated cell values.

Discussion

Alkaloids refer to a broad class of compounds, and alkaloids that contain a ring system, called indole, have been further classified as indole alkaloids. Many kinds of plant-based indole alkaloids have numerous biological activities, which are relevant to the field of medicine, such as anti-bacterial, anti-malarial, anti-cancer, anti-diabetic anti-cholinesterase, and anti-inflammatory activities (29). Most plant-derived indole alkaloid-type compounds have an indole moiety linked to serotonin (30). *N*-Feruloylserotonin is a conjugated serotonin and unique polyphenol identified as the anti-oxidant constituent of safflower seeds. Chemically, it is an indole hydroxycinnamic acid amide formed between tryptamine (serotonin) and phenylpropanoid acid (feruloyl acid) (31). Moreover, its functional parent ferulic acid is a polyphenolic compound that is also well-known for its strong anti-oxidant properties. In addition, these serotonin derivatives have been reported to exhibit health-beneficial effects including anti-inflammatory activities (8). Nevertheless, the mechanism of cellular biology is not well-established. To our knowledge, the anti-inflammatory role of *N*-feruloylserotonin in SIRT1-stimulated FOXO1 and NF-κB signaling pathways is still unknown. Therefore, we first performed a cell viability assessment of LPS-treated RAW 264.7 macrophages to examine the anti-inflammatory activity and mechanism involving feruloylserotonin. The data indicated that no toxic sign was observed up to 25 μM of *N*-feruloylserotonin, and thereby we investigated the effect on NO and ROS productions through regulation of the MAPK pathway in RAW 264.7 macrophages against LPS. In addition, our data suggested that *N*-feruloylserotonin significantly reduces LPS-induced activation of NO and PGE_2.

Macrophages are versatile cells, but it is as sentinels of the immune system that they show their full functional repertoire. They detect pathogenic substances through pattern-recognition receptors and subsequently initiate and regulate inflammatory responses using a wide range of soluble pro-inflammatory mediators (32). LPS is one of the most powerful activators of macrophages, and macrophages induced by LPS are known to be activated through the production of inflammatory mediators, such as NO and other free radicals. During inflammatory processes, large amounts of pro-inflammatory mediators, NO and

PGE_2, are generated by iNOS and COX-2, respectively (33). Under inflammatory progression, iNOS and COX-2 are significantly up-regulated, which promotes NO generation. Abnormalities in NO production by iNOS lead to cytotoxicity, which is implicated in the pathogenesis of various inflammatory disorders such as rheumatoid arthritis. The present study showed that *N*-feruloylserotonin could improve inflammation by effectively reversing these inflammatory mediators in LPS-treated RAW 264.7 cells.

Next, we focused on SIRT1 and its downstream signaling pathways to investigate the molecular mechanism of *N*-feruloylserotonin. SIRT1 has been recognized for its anti-inflammatory activity through the regulation of FOXO1 and NF-κB (23). FOXO1 and NF-κB transcription factors play key roles in the onset and progression of chronic inflammatory disorders, such as rheumatoid arthritis (34, 35). Therefore, they are considered to be a good target for anti-inflammatory medications. The activation of SIRT1 not only increases the expression of FOXO1 but also promotes the transcription of FOXO1 from the cytoplasm to the nucleus (36). Moreover, MnSOD and catalase, major ROS scavengers, were upregulated by FOXO1 to relieve oxidative stress (37). Our results demonstrated that *N*-feruloylserotonin increased the activation of SIRT1, promoting the expression and transcription of FOXO1, upregulating MnSOD and catalase and reducing ROS in LPS-induced RAW 264.7 cells.

MAPK is a group of signaling molecules that may also play important roles in inflammatory processes. At least three MAPK cascades: ERK, JNK, and p38, are well-described, and have been reported to differentially activate depending on the stimuli and cell types (27). Several studies demonstrated that activation of MAPK is significant in the regulation of inflammation *via* controlling the activation of ROS. In the present study, we investigated whether *N*-feruloylserotonin inhibits ROS *via* disrupting MAPK signals, and non-toxically inhibits the inflammatory response in RAW 264.7 macrophages.

LPS rapidly phosphorylates ERK and JNK, leading to NF-κB activation in macrophages (38). This activation leads to an increase in the production of pro-inflammatory mediators such as NO and PGE_2 (39, 40). The activation of ERK is considered to be involved in LPS-induced macrophage responses, such as the increased production of pro-inflammatory cytokines and iNOS (41, 42). Moreover, LPS stimulation of RAW 264.7 cells rapidly activates the JNK pathway (43). Therefore, the activations of ERK and JNK are used as hallmarks of LPS-induced signal transduction in RAW 264.7 cells. To further confirm the mechanism of pro-inflammatory mediator inhibition by *N*-feruloylserotonin, we investigated the effects of this compound on p-ERK and p-JNK in RAW 264.7 cells, and it was found that p-ERK and p-JNK were suppressed by *N*-feruloylserotonin in a concentration-dependent manner. Even though other signals (ERK and JNK) are also significantly decreased by *N*-feruloylserotonin, their expression was only slightly decreased by serotonin treatment. These findings indicate that *N*-feruloylserotonin can modulate MAPK pathways.

Under the influence of specific stimuli such as LPS, NF-κB is phosphorylated and transported to the nucleus, where it regulates the expression of various inflammatory mediators and cytokines such as iNOS, COX-2, IL-1β, IL-6, IL-10, and TNF-α (44). The inhibition of NF-κB phosphorylation, regulated by activation of SIRT1, ameliorates inflammation by suppressing the expression of downstream signaling pathways (45). Our results suggest that *N*-feruloylserotonin increased the activation of SIRT1, suppressing NF-κB expression and its transcription and reducing the inflammatory mediators.

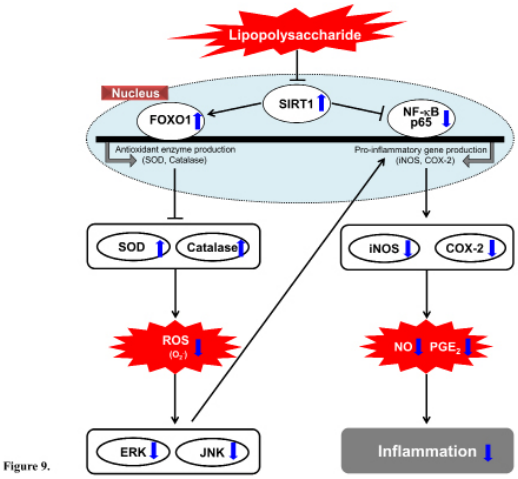

Figure 9. The possible anti-inflammation mechanism of *N*-feruloylserotonin in LPS-induced RAW264.7 cells

Conclusion

The present study revealed that *N*-feruloylserotonin, a major safflower seed anti-inflammatory agent, could ameliorate LPS-induced inflammation in RAW 264.7 cells through the activation of SIRT1 to modulate SIRT1/FOXO1 and SIRT1/NF-κB signaling pathways (Figure 9). Based on these results, *N*-feruloylserotonin may facilitate basic research using various cell lines and animal models of rheumatoid arthritis, supporting *N*-feruloylserotonin as a promising anti-inflammatory drug in pharmacologic theory. Moreover, it provided scientific evidence that *N*-feruloylserotonin may be a major anti-inflammatory component of safflower seeds.

Author contributions

Chan Hum Park: conceptualization; formal analysis; investigation; resources; writing – original draft; writing – review & editing; visualization; supervision. Su Hui Seong: formal analysis; validation; data curation. Jae Sue Choi: validation; data curation. Jin Pyeong Jeon: validation; data curation. Takako Yokozawa: conceptualization; writing – original draft, writing – review & editing; supervision.

Conflicts of interest

There is no conflict of interest regarding the authors of this manuscript.

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