**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 E2 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-extra-cellular-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.

**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 hydroxyccinnamic 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 feruloyl 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

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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 prostanoid 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, phenylmethylsulfonlyfluoride (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 confluency, 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 each 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 electrophoresed 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 activation was considered significant when p-FOXO1, MnSOD, and catalase expressions were induced by N-feruloylserotonin treatment.
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).

**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 PGE₂ 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.

**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).

**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 PGE₂.

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...
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.

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/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: conceptualization; formal analysis; investigation; resources; writing – review & editing; visualization; supervision. Jae Sue Choi: investigation; resources; writing – original draft; writing – review & editing; visualization; supervision. Takako Yokozawa: conceptualization; writing – original draft; writing – review & editing; supervision. Jin Pyeong Jeon: validation; data curation. Min Soon Kim: validation; data curation. Young Doo Park: validation; data curation.
N-feruloylserotonin and its derivatives, N-feruloylserotonin and N-(p-coumaroyl) serotonin, against cisplatin-induced renal damage in mice. 


10. Kawashima S, Hayashi M, Takii T et al. Serotonin derivative, 5-LOX.


15. Kawashima S, Hayashi M, Takii T et al. Serotonin derivative, 5-LOX.


25. Kawashima S, Hayashi M, Takii T et al. Serotonin derivative, 5-LOX.


