Loganin Inhibits Lipopolysaccharide-Induced Inflammation and Oxidative Response through the Activation of the Nrf2/HO-1 Signaling Pathway in RAW264.7 Macrophages

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Inflammation caused by the excessive secretion of inflammatory mediators in abnormally activated macrophages promotes many diseases along with oxidative stress. Loganin, a major iridoid glycoside isolated from Cornus officinalis, has recently been reported to exhibit anti-inflammatory and antioxidant effects, whereas the underlying mechanism has not yet been fully clarified. Therefore, the aim of the present study is to investigate the effect of loganin on inflammation and oxidative stress in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Our results indicated that loganin treatment markedly attenuated the LPS-mediated phagocytic activity and release of nitric oxide (NO) and prostaglandin E₂, which was associated with decreased the expression of inducible NO synthase and cyclooxygenase-2. In addition, loganin suppressed the expression and their extracellular secretion of LPS-induced pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β. Furthermore, loganin abolished reactive oxygen species (ROS) generation, and promoted the activation of nuclear factor-E2-related factor 2 (Nrf2) and the expression of heme oxygenase-1 (HO-1) in LPS-stimulated macrophages. However, zinc protoporphyrin, a selective HO-1 inhibitor, reversed the loganin-mediated suppression of pro-inflammatory cytokines in LPS-treated macrophages. In conclusion, our findings suggest that the upregulation of the Nrf2/HO-1 signaling pathway is concerned at least in the protective effect of loganin against LPS-mediated inflammatory and oxidative stress, and that loganin can be a potential functional agent to prevent inflammatory and oxidative damage.

Key words loganin; inflammation; oxidative stress; reactive oxygen species (ROS); nuclear factor-E2-related factor 2 (Nrf2); heme oxygenase-1 (HO-1)

INTRODUCTION

A properly regulated inflammatory response helps the body resist the insults, but abnormal or excessive inflammation causes hyperactivity in the body and is harmful. Excessive inflammatory reactions have been reported as a leading cause of various diseases including immune system disorders.1–3 Among the cells involved in immune regulation, macrophages play a critical role in the innate immune response. Lipopolysaccharide (LPS), a major component of Gram-negative bacterial cell walls, is widely used in various assays to study interference in the inflammatory pathway. In LPS-stimulated macrophages, the generation of pro-inflammatory mediators is increased by activation of the toll-like receptor (TLR) 4-mediated nuclear factor-kappaB (NF-κB) signaling pathway.4,5 Nitric oxide (NO) and prostaglandin E₂ (PGE₂) are typical pro-inflammatory mediators, and inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, and facilitate inflammation.6,7 Moreover, the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), which are involved in the production of NO and PGE₂, respectively, is positively correlated with the production of inflammatory cytokines.8,9 LPS also can induce and accelerate oxidative stress along with the inflammatory cascade. Upon the LPS stimulation of macrophages, the generation of reactive oxygen species (ROS) is increased, contributing to the manifestation of inflammation, and overproduced inflammatory factors may promote excessive ROS production.2,10 Currently, non-steroidal anti-inflammatory drugs are widely used to suppress inflammatory symptoms and relieve oxidative stress, but various side effects have been reported from long-term use.11–13 Therefore, research on reliable and effective alternative agents to overcome inflammatory-mediated various diseases is urgently required.

Loganin is an iridoid glycoside, one of the bioactive compounds isolated from the fruit Cornus officinalis Sieb. et Zucc., belonging to the family Cornaceae, which is frequently used in traditional medicine for the prevention and treatment of various diseases.14–16 A number of previous studies have shown that loganin has a variety of beneficial pharmacological
effects, including anti-inflammatory, antioxidant, neuroprotective, immune regulatory and anti-tumor effects.\textsuperscript{17-24} Recently, Cheng et al.\textsuperscript{25} reported that loganin can ameliorate pyroptosis, an inflammatory programmed cell death, by mitigating oxidative stress through inhibition of ROS generation and inactivation of NF-κB in high glucose-stimulated Schwann cells. In addition, it has been proposed that the effectiveness on anti-inflammation and antioxidant of loganin could be mediated by modulating the TLR4/NF-κB signaling pathway in an experimental model of loganine burn injury.\textsuperscript{26,27} and the inhibition of TLR4/NF-κB activity was also attributed to suppress the inflammatory response upon LPS in the intestinal epithelium.\textsuperscript{27} Moreover, the exogenous addition of loganin has been shown to induce the expression of nuclear factor-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), thereby inhibiting hepatic oxidative stress caused by type 2 diabetes.\textsuperscript{28} HO-1, a representative cytoprotective and inducible enzyme, is one of the downstream phase II enzymes dependent upon transcription factor Nrf2.\textsuperscript{29,30} These observations well-support previous findings that loganin plays a promising role as an anti-inflammatory and antioxidant. However, the correlation between HO-1 induction by loganin and anti-inflammatory efficacy is still unknown. Therefore, in this study, we investigated the inhibitory effect of loganin on inflammatory and oxidative responses in LPS-stimulated mouse RAW264.7 macrophages and investigated how increased HO-1 affected the anti-inflammatory efficacy of loganin.

MATERIALS AND METHODS

Cell Culture The monocyte-macrophage lineage RAW264.7 cells (Korea Cell Line Bank, Seoul, Republic of Korea) were maintained in in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U/mL penicillin and streptomycin, and 10% fetal bovine serum (all from WelGENE Inc., Daegu, Republic of Korea) in humidified air at 37 °C, and 5% CO\textsubscript{2}. To make the stock solution, loganin (Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) and LPS (Sigma-Aldrich Chemical Co.) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) and then exposed to light for 1 h and then incubated for 1 h in the absence or presence of loganin (Fig. 1C).

Cell Viability Assay The cytotoxicity of loganin against RAW264.7 cells in the presence or absence of LPS was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) reduction assay as previously described.\textsuperscript{31} To observe cell morphology changes, the cell images were captured using an inverted-phase contrast microscope (Carl Zeiss, Oberkochen, Germany).

Phagocytosis Analysis The phagocytic activity was measured using a commercially available phagocytosis assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer’s instruction. The extent of phagocytosis was evaluated using flow cytometry (BD Biosciences, San Jose, CA, U.S.A.) as previously described.\textsuperscript{32}

Measurement of NO, PGE\textsubscript{2}, and Cytokines RAW264.7 macrophages were treated with various concentrations of loganin for 1 h and then stimulated with 100 ng/mL LPS for 24 h. The culture supernatants were collected and assayed the amounts of NO, PGE\textsubscript{2}, and cytokines. The NO level was evaluated by the amount of nitrite measured using the Griess reagent (Sigma-Aldrich Chemical Co.) as previously described.\textsuperscript{33} To investigate the PGE\textsubscript{2} and cytokine levels, the cell supernatants were assayed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN, U.S.A.) according to the instructions from the manufacturer. The absorbance was measured at a wavelength of 450 nm using an ELISA reader as previously described.\textsuperscript{33}

Western Blot Analysis The cells were harvested and lysed with lysis buffer, as previously described.\textsuperscript{34} Total protein was extracted using Pro-prep protein extraction solution (Intron Biotechnology, Gyeonggi-do, Korea). Nuclear and cytoplasmic proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the gel were subsequently transferred to polyvinylidene difluoride membranes (Schleicher and Schuell GmbH, Keene, NH, U.S.A.). The protein-transferred membranes were blocked with non-fat dry milk solution (5%), and then reacted with primary anti-bodies and their corresponding secondary antibodies. The information on the antibodies used is provide in supplementary Table S1. The membrane was reacted with an enhanced chemiluminescent solution purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.) and then exposed to X-ray film to visualize the corresponding proteins.

RESULTS

Effect of Loganin on the Proliferation of RAW264.7 Macrophages The cytotoxic effect of loganin on RAW264.7 cells was evaluated by the MTI assay. At concentrations below 30 μM, loganin did not affect cytotoxicity, but significant cytotoxicity was observed in cells treated with 50 μM (Fig. 1A).

As shown in Fig. 1B, subsequent experiments did not show any adverse effect on cell viability when 30 μM or less loganin was administered to 100 ng/mL LPS-stimulated macrophages. In addition, polygonal spindle-shaped pseudopodia, a macrophage activation signal, were formed in LPS-stimulated cells, while the original round shape largely remained in the presence of loganin (Fig. 1C).

Loganin Prevents Phagocytosis in LPS-Treated RAW264.7
Macrophages Since one of the key characteristics of abnormally activated macrophages is an excessive increase in phagocytic capacity, we evaluated phagocytic activity to investigate whether loganin could modulate the physiological activity of macrophages. As expected, LPS highly stimulated phagocytosis in RAW264.7 cells, which was significantly prevented by loganin (Fig. 2). Although the phagocytic activity of cells treated with loganin alone was slightly higher than that of control cells, there was no statistical difference between these two groups.

Loganin Reduces NO and PGE2 Production in LPS-Stimulated RAW264.7 Macrophages In order to investigate the anti-inflammatory effects of loganin, changes in the levels of released inflammatory mediators were detected. As shown in Figs. 3A and B, LPS markedly enhanced the release of NO and PGE2, but this increase was substantially reduced by pretreatment of loganin in a concentration-dependent manner. Next, we assessed whether loganin could inhibit the expression of iNOS and COX-2 by LPS. As results of Western blot, up-regulation of iNOS and COX-2 by LPS was significantly suppressed in the presence of loganin (Fig. 3C).

Loganin Inhibits the Production and Expression of LPS-Induced Pro-inflammatory Cytokines in RAW264.7 Macrophages Next, we investigated the effect of loganin on the release and expression of inflammatory cytokines increased by LPS treatment. Our results showed that the released amount of TNF-α and IL-1β were markedly enhanced after stimulation with LPS. However, the increased production of these cytokines by LPS was significantly decreased by loganin in a dose-dependent manner (Figs. 4A, B). Subsequently, we investigated whether the inhibition of inflammatory cytokine release by loganin was associated with the expression of these genes. As shown in Fig. 4C, LPS treatment significantly increased the expression of these cytokines, but their expression was reduced in cells pre-treated with loganin.

Loganin Alleviates LPS-Mediated Generation of ROS in RAW264.7 Macrophages Next, we evaluated whether loganin could suppress LPS-induced oxidative stress that due to involved in the inflammatory response. The flow cytometry results using the DCF-DA probe showed that LPS significantly increased ROS content, but this increment was notably suppressed by pre-treatment of loganin in a concentration-dependent manner (Figs. 5A, B). In concord with the results from the flow cytometry, the increase of DCF-DA intensity by LPS was markedly weakened in presence of loganin (Fig. 5C).

Loganin Increases the Activation of Nrf2/HO-1 Signaling Pathway in RAW264.7 Macrophages In order to evaluate whether the inhibitory effect of loganin on oxidative stress by LPS was involved in Nrf2/HO-1 signaling pathway, the level of Nrf2 and HO-1 expression was analyzed by Western blot. The expression of Nrf2 and HO-1 was increased by loganin treatment in concentration-dependent manner, which was associated with an increase in the expression of phosphorylated Nrf2 (p-Nrf2) (Fig. 6A). Although LPS slightly increased the expression of Nrf2, p-Nrf2, and HO-1, the expression in cells co-treated with LPS and loganin was much higher that of cells.
treated with LPS and loganin alone (Fig. 6B). Since activated Nrf2 should be translocated to the nucleus to initiate the expression of antioxidant enzymes including HO-1, we further investigated whether loganin can regulate nuclear translocation of Nrf2. According to the immunoblotting results obtained using cytoplasmic and nuclear proteins, the Nrf2 protein expression was markedly up-regulated in the nucleus of the loganin intervention cells compared to the loganin or LPS alone treatment cells, but the expression of HO-1 was observed only in the cytoplasm (Figs. 6C, D).

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**Fig. 2. Effect of Loganin on Phagocytosis Activity in LPS-Stimulated RAW264.7 Macrophages**

The phagocytic uptake of FITC-labeled dextran was measured by flow cytometry. RAW264.7 macrophages were treated with various concentrations of loganin for 1 h and then stimulated with 100 ng/mL LPS for 24 h. (A) The images shown represent representative plots of three replicate experiments. (B) The histogram results of phagocytic uptake of FITC-dextran were statistically analyzed. Data were expressed as the mean ± S.D. (n = 3). ***p < 0.001, vs. control cells; ###p < 0.001, vs. LPS-stimulated cells.

**Fig. 3. Effect of Loganin on the Production and Expression of Pro-inflammatory Mediators in LPS-Stimulated RAW264.7 Macrophages**

RAW264.7 macrophages were treated with various concentrations of loganin for 1 h and then stimulated with 100 ng/mL LPS for 24 h. (A) The NO concentration in the culture supernatants was evaluated by the Griess reaction. (B) The PGE2 concentration was determined using a PGE2 ELISA kit. (A, B) Data were expressed as the mean ± S.D. (n = 3). ***p < 0.001, vs. control cells; ##p < 0.01 and ###p < 0.001, vs. LPS-stimulated cells. (C) The expression levels of iNOS and COX-2. β-Actin was used as an internal control.
HO-1 Inhibitor Abolishes the Anti-inflammatory and Antioxidant Effects of Loganin in RAW264.7 Macrophages

To further investigate the Nrf2/HO-1 signaling pathway involved in the anti-inflammatory effect of loganin, we used zinc protoporphyrin IX (ZnPP), a potent competitive HO-1 inhibitor. To achieve this, we pre-incubated with ZnPP with or without loganin, followed by LPS, and found that ZnPP partially counteracted the suppressive effect of loganin on the
increased release of TNF-α and IL-1β by LPS (Figs. 7A, B). As shown in Figs. 7C and D, we also found that ZnPP significantly partially reversed the effect of loganin on the decrease in ROS generation in LPS-treated cells.

**DISCUSSION**

In the present study, we established that loganin has the anti-inflammatory and antioxidant potential in LPS-treated RAW264.7 macrophage model. Loganin attenuated the phenotype and phagocytic activity of RAW264.7 macrophages activated by LPS, evidencing the inhibitory effect on the activation of macrophages by loganin. Furthermore, loganin down-regulated the pro-inflammatory mediators and cytokines, as well as suppressed the accumulation of ROS, which was involved in the activation of the Nrf2/HO-1 signaling pathway.

In order to investigate the anti-inflammatory efficacy of loganin, we assessed the effect of loganin on the production of NO and PGE₂. Among them, NO plays a key role in normal physiological conditions such as neurotransmission, vasodilation, and immune defense. However, excessive NO formation due to increased iNOS expression promotes the inflammatory response and increases oxidative stress and tissue damage. COX enzymes catalyze the conversion of arachidonic acid to prostaglandins, including PGE₂, a group of hormone-like substances that participate in various body functions. However, excessive PGE₂ production, promoted by the increased activity of COX-2 following various inflammatory stimuli, plays an important role as an inflammatory mediator. Our data indicated that the up-graduated secretion of NO and PGE₂ by LPS was progressively inhibited at increasing concentrations of loganin, which was attributed to inhibition of the expression of iNOS and COX-2 mRNA and protein. These data demonstrated that the anti-inflammatory effect of loganin was at least in part due to the reduced expression of iNOS and COX-2, which are involved in pro-inflammatory mediators, and support the results of previous studies.

Cytokines are secreted primarily by macrophages in response to inflammation, which are involved in various signaling pathways producing autocrine and/or paracrine effects. All of these are essential components for the initiation and improvement of the inflammatory response, and their expression is also increased by the LPS stimulation of macrophages. Furthermore, they can accelerate the inflammatory response by activating or increasing the expression of pro-inflammatory mediators as well as other pro-inflammatory cytokines.

In the current study, we found that loganin reduced the production of TNF-α and IL-1β in LPS-stimulated RAW264.7 macrophages by suppressing their expression. Consistent with our results, Cui et al. also reported similar effects in BV-2 microglia stimulated with amyloid β, and these results support the anti-inflammatory efficacy of loganin found in several experimental models. In many previous studies, along with various intracellular signaling pathways, NF-κB has been identified as a critical transcription factor in regulation of pro-inflammatory mediators by LPS stimulation. Therefore, as mentioned in previous studies, it is presumed that the inactivation of NF-κB was probably led to the inhibition of these pro-inflammatory mediators and cytokines by loganin.

Meanwhile, endogenous free radicals like ROS play a core role in host defense. However, excess ROS cause oxidative damage to cellular macromolecules, and has been shown to play a critical role in initiating and promoting inflammation-
related diseases by upregulating the pro-inflammatory mediators.\(^2,44\) ROS also contribute to the activation of macrophages, and ROS generation is enhanced in overactive macrophages.\(^2,10\) According to our results, loganin strongly inhibited LPS-induced ROS formation in RAW264.7 macrophages. Although the antioxidant potential of loganin has been reported in previous studies,\(^18,25,26\) the results of this study support the use of loganin as an antioxidant for the management of oxidative stress associated with inflammatory responses. Certain antioxidant signaling pathways negatively modulate excessive inflammatory responses to maintain homeostasis in the body.\(^30,45\) Our results mentioned above indicated that loganin could increase the activity of the intracellular antioxidant enzyme system to reduce oxidative damage. Accumulated evidence indicates that HO-1, along with its by-products, can stimulate the generation of anti-inflammatory mediators, while suppression of pro-inflammatory mediators.\(^30,46,47\) Nrf-2 is a major transcription factor that maintains cellular homeostasis involved in inflammation and oxidative stress by activating detoxifying genes, including HO-1. For Nrf-2 present in the cytoplasm to act as a transcription factor, it must be liberated from Kelch-like ECH-associated protein 1, a negative regulator of Nrf2, and phosphorylated before translocation to the nucleus.\(^29,30\) Therefore, we evaluated whether the effect of loganin regulates Nrf2/HO-1 signaling pathway in LPS-stimulated macrophages. Our finding showed that loganin up-regulated the levels of Nrf2 and HO-1 in the presence of LPS, and the degree of phosphorylation and nuclear translocation of Nrf-2 was also markedly enhanced by loganin in LPS-stimulated RAW264.7 cells, indicating that the activity of Nrf-2 as a transcription factor was improved. Our present result corresponded to Zhang et al.’s recent finding that loganin suppressed the oxidative stress of LPS-stimulated human proximal renal epithelial cell line HK2 by activating the Nrf2/HO-1 signaling pathway.\(^48\) Therefore, the present results suggest that loganin increased the expression of HO-1 by promoting the phosphorylation and nuclear translocation of Nrf2 in the presence of LPS, and support the previous results that the protective effect on hepatic oxidative stress by administration of loganin was associated with increased Nrf-2 and HO-1 expression.\(^28\) Moreover, ZnPP, a selective HO-1 inhibitor, partially counteracted the inhibitory effects of loganin on LPS-mediated pro-inflammatory cytokine production and the increase in ROS generation. Although the limitation of the present study is additional mechanistic studies are needed to interpret the mechanisms related to the anti-inflammatory and antioxidant efficacy of loganin, these observations demonstrated that the anti-inflammatory and antioxidant effects of

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**Fig. 7.** The Role of Nrf2/HO-1 Signaling in the Anti-inflammatory and Antioxidant Efficacies of Loganin

Cells were pre-incubated with 10\(\mu\)M ZnPP for 2h, followed by 1h of incubation with 30\(\mu\)M loganin and exposure to 100ng/mL LPS for 24h (A, B) or 1h (C, D). (A, B) The concentrations of TNF-\(\alpha\) (A) and IL-1\(\beta\) (B) in the culture medium. (C) The DCF-DA-stained cells were analyzed by flow cytometry. (A, B, D) Data are expressed as the mean \(\pm\) S.D. (\(n=3\)). *\(p<0.05\), **\(p<0.01\) and ***\(p<0.001\), vs. control cells; *\(p<0.01\) and ***\(p<0.001\), vs. LPS-stimulated cells; *\(p<0.05\) and $$$\(p<0.001\), vs. loganin and LPS-stimulated cells.
loganin are, in part, related to the activation of the Nrf2/HO-1 axis in LPS-stimulated RAW264.7 macrophages.

CONCLUSION

In this study, we established the inhibitory effects of loganin on LPS-mediated inflammatory and oxidative responses in RAW264.7 macrophages. According to our findings, loganin inhibited morphological changes and phagocytic activity in LPS-stimulated RAW264.7 macrophages. Notably, loganin significantly attenuated LPS-induced ROS generation, and enhanced the expression of Nrf2-mediated HO-1. However, when HO-1 activity was artificially blocked, the beneficial effects of loganin were negated. Taken together, the current results demonstrate that loganin promotes anti-inflammatory and antioxidant capacity by blocking LPS-mediated macrophage activation, and at least Nrf2-mediated activation of HO-1 is involved in this process. However, further studies are required to assess the relationship between the Nrf2/HO-1 signaling pathway and other intracellular signaling pathways that may lead to the beneficial efficacy of loganin.

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Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials.

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