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Loganin Prevents Hepatic Steatosis by Blocking NLRP3 Inflammasome Activation

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Abstract

Activation of the NLRP3 inflammasome is a necessary process to induce fibrosis in nonalcoholic fatty liver disease (NAFLD). Nonalcoholic steatohepatitis (NASH) is a kind of NAFLD that encompasses the spectrum of liver disease. It is characterized by inflammation and ballooning of hepatocytes during steatosis. We tested whether inhibiting the NLRP3 inflammasome could prevent the development and pathology of NASH. We identified loganin as an inhibitor of the NLRP3 inflammasome and investigated whether *in vivo* administration of loganin prevented NASH symptoms using a methionine-choline deficient (MCD) diet model in mice. We found that loganin inhibited the NLRP3 inflammasome activation triggered by ATP or nigericin, as shown by suppression of the production of interleukin (IL)-1β and caspase-1 (p10) in mouse primary macrophages. The speck formation of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) was blocked by loganin, showing that the assembly of the NLRP3 inflammasome complex was impaired by loganin. Administration of loganin reduced the clinical signs of NASH in mice fed the MCD diet, including hepatic inflammation, fat accumulation, and fibrosis. In addition, loganin reduced the expression of NLRP3 inflammasome components in the liver. Our findings indicate that loganin alleviates the inflammatory symptoms associated with NASH, presumably by inhibiting NLRP3 inflammasome activation. In summary, these findings imply that loganin may be a novel nutritional and therapeutic treatment for NASH-related inflammation.

Key Words: Phytochemical, Inflammasome, Liver, Steatosis, Fibrosis, Inflammation

INTRODUCTION

People's lifestyles are closely related to the prevalence of chronic disease (Loomba and Sanyal, 2013). In particular, diets high in sugar or lipids in the western diet are associated with an increased incidence of metabolic diseases (Schattenberg and Bergheim, 2019). Nonalcoholic fatty liver disease (NAFLD), which is characterized by lipid accumulation in the liver due to excess nutrients, is a metabolic disease in humans (Berna and Romero-Gomez, 2020). One of the main characteristics of NAFLD is low-grade chronic inflammation. A persistent inflammatory state in the liver can lead to progression from simple steatosis to nonalcoholic steatohepatitis (NASH) with severe inflammation and progression toward cirrhosis or liver cancer, making it difficult to return to the normal (predisease) state (Michelotti *et al*., 2013; Dhamija *et al*., 2019). Thus, resolving inflammatory responses and following injury in the liver is important to prevent the progression of NAFLD (Han *et al*., 2021). Investigation of the pathological pathways of liver diseases has been explored by many approaches using molecular and cellular experimental tools, knockout animal models, clinical samples, and recent data mining and analysis approaches to delineate biological networks in various liver injury statuses (Shin *et al*., 2022). However, the inflammatory mechanism underlying NAFLD is still unclear.

Recent evidence has established the significant role of the NLRP3 inflammasome in the pathogenesis of NAFLD (Seok *et al*., 2021). The NLRP3 inflammasome is a multiprotein assembly that identifies molecular patterns associated with pathogens or danger (Schroder and Tschopp, 2010). The levels of NLRP3 inflammasome components and pro-IL-1β in the livers of severe NASH patients were higher than those of non-NASH patients (Wree *et al*., 2014). The hepatic levels of the proinflammatory cytokine IL-1β increased when the NLRP3

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inflammasome was activated (Wree *et al*., 2014). Knockout of NLRP3 in mice resulted in protection from liver steatohepatitis and fibrosis induced by a choline-deficient diet (Wree *et al*., 2014). In addition, caspase-1 is activated and regulates high-fat diet-induced fibrogenesis in the liver upon NLPR3 inflammasome activation (Dixon *et al*., 2012; Seok *et al*., 2020). Caspase-1-deficient mice exhibited lower levels of hepatic steatosis, fibrosis, and inflammatory cytokines induced by a high-fat diet than wild-type mice (Dixon *et al*., 2012; Seok *et al*., 2020). Inhibiting the activation of the NLRP3 inflammasome by pharmacological agents alleviates NAFLD symptoms, which is associated with lowering the amount of IL-1β (Mridha *et al*., 2017; Yang *et al*., 2020a).

Loganin, as one of the principal iridoid glycosides, is derived from the herb Cornus officinalis Sieb. et Zucc. (Xu *et al*., 2006). Loganin exerts a variety of biological effects, including hepatic protection, renal protection, and anti-inflammatory activity (Huang *et al*., 2018). Loganin prevented renal mesangial cell growth induced by advanced glycation end products, possibly by its antioxidant activity (Xu *et al*., 2006). Treatment of mice with loganin in a diabetic nephropathy model improved pathological symptoms in kidney and serum parameters, accompanied by reduced malondialdehyde and increased superoxide dismutase (Liu *et al*., 2015). The biochemical parameters of lipid metabolism and inflammation in the type 2 diabetic db/db mice were improved by oral administration of loganin with regulation of lipid metabolism-related gene expression and NF-κB activation (Yamabe *et al*., 2010). However, it remains to be elucidated whether loganin is effective in preventing NASH. In this study, we investigated whether loganin exerted a beneficial effect on the pathological symptoms of NASH.

MATERIALS AND METHODS

Ethics statement

All animals were treated humanely in accordance with the National Academy of Sciences' "Guide for the Care and Use of Laboratory Animals" (NIH bulletin 86–23 amended 1985). All experimental methods were conducted in line with protocols approved by the Catholic University of Korea's Institutional Animal Care and Use Committee (IACUC) (permission# 2016-004-02).

Reagents

Purified lipopolysaccharide (LPS) from *Escherichia coli* from List Biological Laboratory (Campbell, CA, USA) was prepared in endotoxin-free water. Loganin was obtained from ChemFaces (Hubei, China). ATP and MCC950 were from InvivoGen (Carlsbad, CA, USA). Nigericin was purchased from Sigma–Aldrich (St Louis, MO, USA). Anti-mouse caspase-1 antibody and anti-ASC antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IL-1β antibody was obtained from R&D Systems (Minneapolis, MN, USA).

Animals

Male C57BL/6 mice were procured from Orient Bio (Seoul, Korea) and acclimated in pathogen-free settings in an animal facility. During the experiment, the mice were maintained with *ad libitum* access to their food and water in a temperature- and humidity-controlled room (23 ± 3°C, 40-60% humidity) with a 12/12 h light/dark cycle. Animals of similar age and weight in all groups were randomly assigned to treatment groups. Throughout the experiment, the researchers were blinded to the treatments.

Cell culture

Bone marrow-derived primary macrophages (BMDMs) were generated following the isolation of bone marrow from mice as previously described (Lee *et al*., 2016). Macrophages were cultured in Dulbecco's modified Eagle's medium supplied with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin.

Analysis of inflammasome activation

Purified lipopolysaccharide (LPS; 100 ng/mL) was used to prime BMDMs plated in 6-well plates (2×10^6 cells/mL) for 4 h. Phosphate-buffered saline (PBS) was used to remove the LPS before adding loganin. The cells were stimulated with the inflammasome activators ATP (5 mM) and nigericin (10 μM) in serum-free media. Cell supernatants and lysates were obtained and further processed for immunoblotting of pro-IL-1β, pro-caspase-1, IL-1β, and caspase-1 as previously described (Yang *et al*., 2020b).

Enzyme-linked immunosorbent assay

IL-1β levels were determined in culture media and liver homogenate supernatants with a DuoSet enzyme-linked immunosorbent assay kit (R&D Systems) following the manufacturer's instructions.

Confocal microscopy analysis

Microscopy was performed as described previously (Yeon *et al*., 2017). For the identification of ASC oligomers, BMDMs were fixed with cold methanol and stained with anti-ASC antibody and FITC-conjugated secondary antibody. An LSM710 confocal microscope was used to obtain images, which were then processed by the Zen2010 program (Zeiss, Oberkochen, Germany).

A mouse NASH model with a methionine-choline deficient diet

Eleven-week-old male C57BL/6 mice were randomly allocated to one of five weight-matched groups (n=8): normal chow diet (NOR), methionine-choline-deficient (MCD) diet, MCD diet+loganin 5 mg/kg, MCD diet+loganin 30 mg/kg, or MCD diet+MCC950 20 mg/kg. For two weeks, mice were fed an MCD diet with an intraperitoneal injection of loganin (5 or 30 mg/kg) or MCC950 (20 mg/kg) daily. At the conclusion of the two weeks, all animals were required to fast for 12 h. On the following day, mice were anesthetized with Zoletil (Virbac, Carros Cedex, France). Blood samples were collected by cardiac puncture. Liver tissues and serum were further analyzed as previously described (Yang *et al*., 2016).

Reverse transcription and quantitative real-time polymerase chain reaction (qRT−PCR) analysis

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. *qRT–PCR* analysis was performed using the following primers: F4/80, 5′-TGA CTC ACC TTG TGG TCC TAA-3′ and 5′- CTT CCC AGA ATC CAG TCT TTC C-3′; Ly6c, 5'-GCA

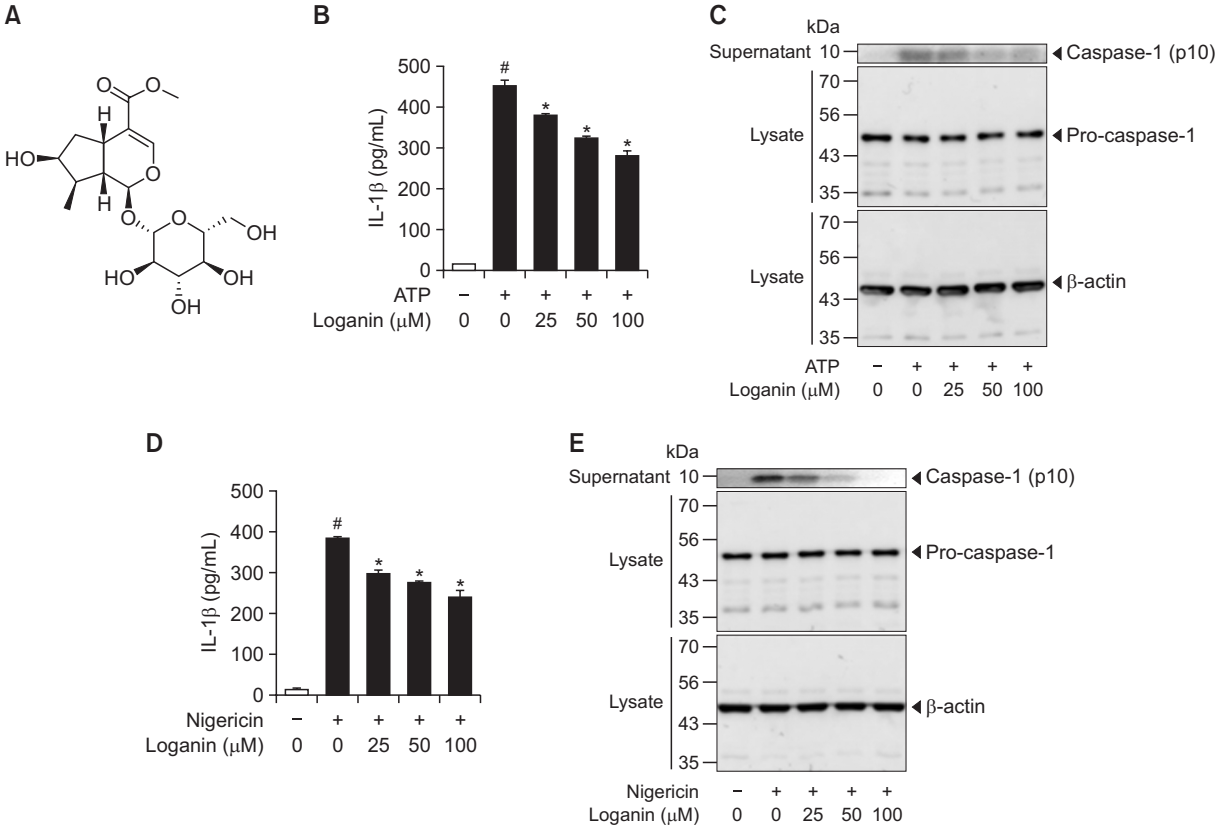

Fig. 1. Loganin suppresses NLRP3 inflammasome activation in primary macrophages. (A) Loganin's chemical structure. (B, D) Macrophages generated from bone marrow (BMDMs) were primed with LPS (100 ng/mL) for 4 h. After 1 h of loganin treatment, the cells were stimulated with (B) ATP (5 mM) for 2 h or (D) nigericin (10 μM) for 16 h. ELISA was employed to determine the amount of secreted IL-1β in cell culture supernatants. (C, E) BMDMs were primed with LPS (100 ng/mL) for 4 h. After 1 h of loganin treatment, the cells were stimulated with (C) ATP (5 mM) or (E) nigericin (10 μM) for 1 h. Immunoblotting was performed to detect caspase-1 (p10) and pro-caspase-1 in cell culture supernatants and cell lysates, respectively. For the bar graphs in (B) and (D), the values are the means ± SEMs (n=3). #Significantly different from vehicle alone, $p<0.05$. *Significantly different from (B) ATP alone or (D) nigericin alone, $p<0.05$.

GTG CTA CGA GTG CTA TGG-3′ and 5′-ACT GAC GGG TCT TTA GTT TCC TT-3′; Mpo, 5′-AGT TGT GCT GAG CTG TAT GGA-3′ and 5′-CGG CTG CTT GAA GTA AAA CAG G-3′; Col1a1, 5′-GCT CCT CTT AGG GGC CAC T-3′ and 5′-CCA CGT CTC ACC ATT GGG G-3′; Ctgf, 5′-GGG CCT CTT CTG CGA TTT C-3′ and 5′-ATC CAG GCA AGT GCA TTG GTA-3′; Timp1, 5′-CTT GGT TCC CTG GCG TAC TC-3′ and 5′-ACC TGA TCC GTC CAC AAA CAG-3′; Actin, 5′-TCA TGA AGTGT GAC GTT GAC ATC C-3′ and 5′-TTG CGG TGC ACG ATG GAG GGG CCG GA-3′.

Histological assessment

Tissues were fixed in 10% formalin, embedded in paraffin, cut into slices (3-mm), and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed to detect tissue protein expression. To visualize fibrosis, liver sections were stained for 2 h with Masson's trichrome reagent (Mridha *et al*., 2017).

Statistical analysis

Data are presented as the mean ± SEM. One-way ANOVA followed by Tukey's multiple range test was employed to compare data across groups. Significant values were defined as $p<0.05$. Representative results from two or three separate experiments are shown.

RESULTS

Loganin suppresses NLRP3 inflammasome activation in macrophages

Mature IL-1β secretion was measured in mouse bone marrow-derived mouse macrophages (BMDMs) to assess whether loganin (Fig. 1A) reduced NLRP3 inflammasome activation. Loganin inhibited the release of IL-1β from BMDMs activated with ATP, a typical NLRP3 inflammasome agonist, as assessed by ELISA (Fig. 1B). Another sign of NLRP3 inflammasome activity is pro-caspase-1 cleavage to caspase-1 (p10). Loganin inhibited the ATP-induced degradation of pro-caspase-1 into caspase-1 (p10) in BMDMs, as determined by immunoblotting (Fig. 1C). Similarly, loganin inhibited the release of IL-1β generated by another NLRP3 agonist, nigericin, in BMDMs (Fig. 1D). Consistently, loganin inhibited caspase-1 (p10) production from pro-caspase-1 degradation caused by nigericin in BMDMs (Fig. 1E). These findings demonstrate that loganin inhibits NLRP3 inflammasome activation elicited

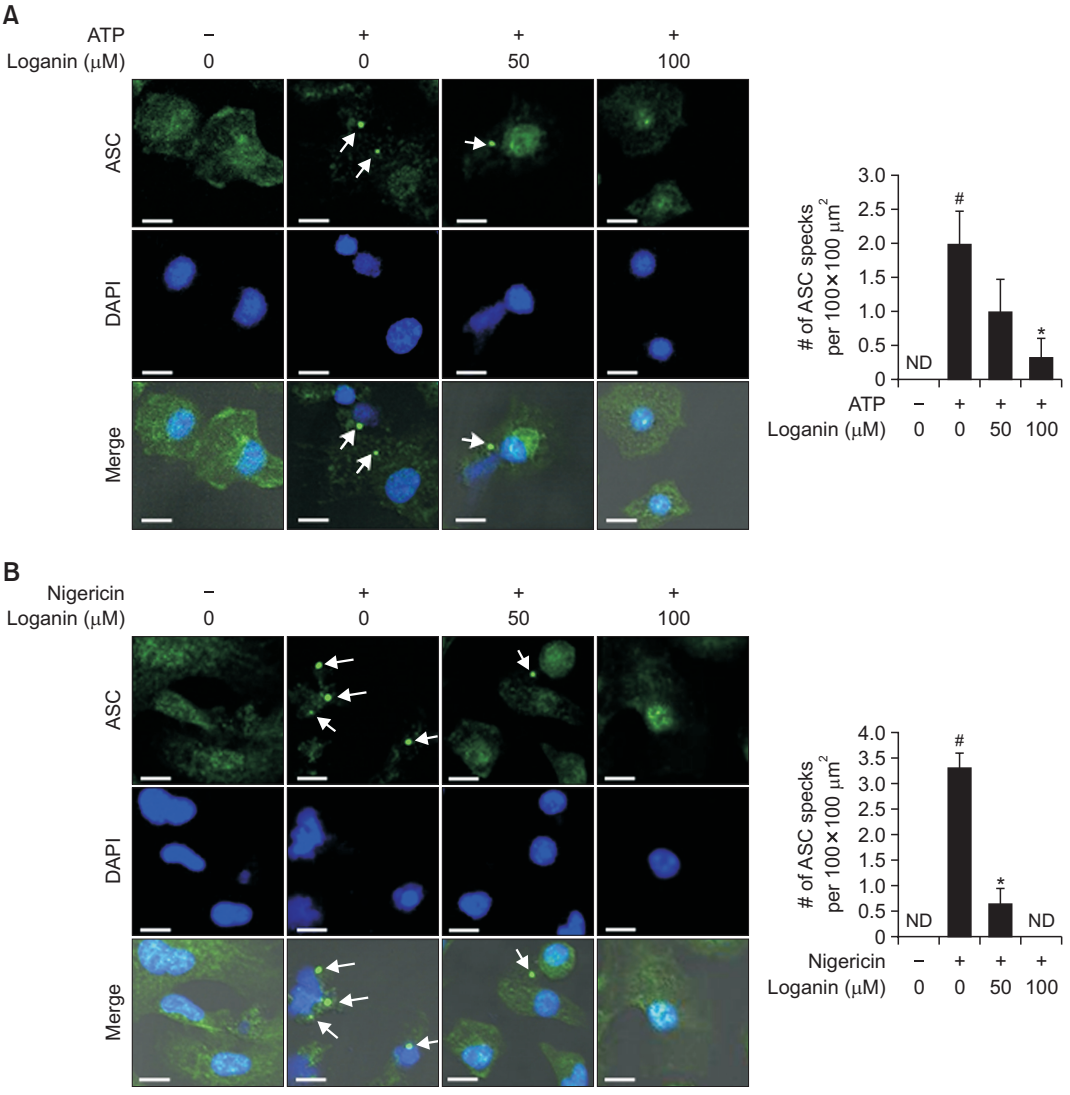

Fig. 2. Loganin disrupts the formation of ASC specks in primary macrophages. (A, B) Bone marrow-derived macrophages (BMDMs) were primed with LPS (100 ng/mL) for 4 h. After 1 h of loganin treatment, the cells were stimulated with (A) ATP (5 mM) for 1 h or (B) nigericin (10 μM) for 1 h. BMDMs were stained for ASC (green). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI: blue). ASC specks are marked with arrows. Scale bar=10 μm. Representative images for each group are presented. The numbers of ASC specks were counted from different, randomly selected fields of view and are presented as bar graphs. For bar graphs, the values are the means ± SEMs (n=3). #Significantly different from vehicle alone, $p<0.05$. *Significantly different from (A) ATP alone or (B) nigericin alone, $p<0.05$. ND, not detected.

by NLRP3 activators in primary macrophages.

Loganin disrupts the formation of the NLRP3/ASC complex induced by NLRP3 activators in macrophages

Upon stimulation with NLRP3 activators, NLRP3 associates with its adaptor protein ASC to form the NLRP3-ASC complex and ASC oligomers. Confocal imaging studies showed ATP-induced formation of ASC oligomers as condensed ASC specks (Fig. 2A). Loganin inhibited the development of ASC specks caused by ATP in BMDMs (Fig. 2A). Consistently, loganin inhibited the nigericin-induced formation of ASC aggregates in BMDMs (Fig. 2B). These results demonstrate that loganin disrupts ASC oligomerization and possibly the association of NLRP3 and ASC. These findings corroborate loganin's inhibitory effect on NLRP3 inflammasome activation.

Loganin attenuates hepatic inflammation and fat accumulation in a NASH mouse model fed a methionine-choline-deficient diet

The aim of this study was to examine whether loganin-induced suppression of the NLRP3 inflammasome may result in the prevention of liver inflammation in patients with NASH. We utilized the MCD diet paradigm, which is frequently used to generate NASH [10]. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels dramatically increased in mice fed the MCD diet for two weeks (Fig. 3A). Intraperitoneal injections of loganin (5 and 30 mg/kg) into mice fed the MCD diet significantly decreased AST and ALT levels

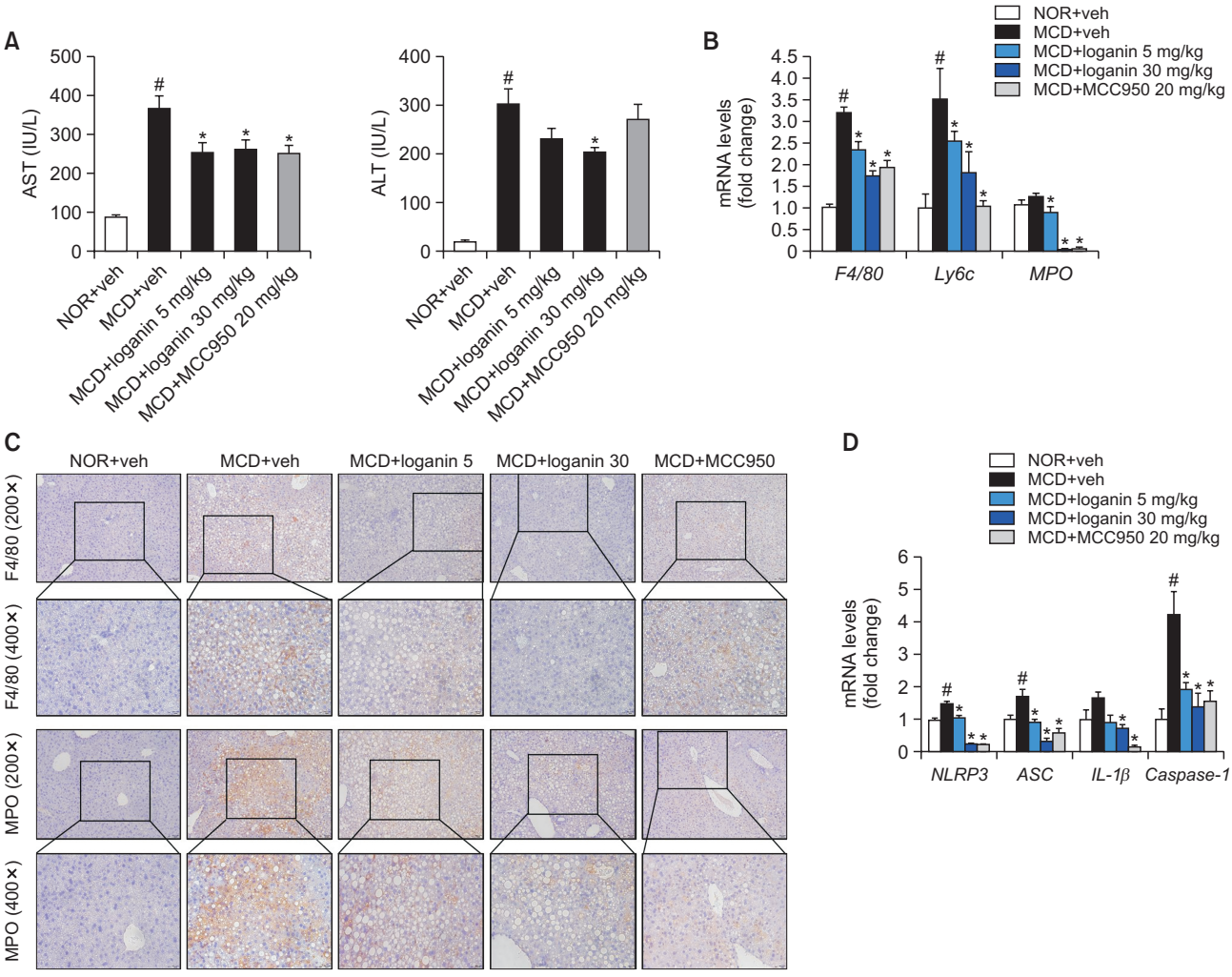

Fig. 3. Loganin reduces hepatic inflammation in MCD diet-fed mice. For two weeks, C57BL/6 mice were given either a normal diet (NOR) or a methionine-choline deficient diet (MCD). Loganin (5 or 30 mg/kg) or MCC950 (20 mg/kg) was administered intraperitoneally with the meals on a daily basis. (A) Serum levels of AST and ALT were measured. (B, D) Quantitative PCR was performed to evaluate hepatic mRNA levels for total macrophages (F4/80), inflammatory macrophages (Ly6c), neutrophils (MPO), NLRP3, ASC, IL-1β, and caspase-1. For (A), (B), and (D), the values are the means ± SEMs (n=8). #Significantly different from the NOR+vehicle group, $p<0.05$. *Significantly different from MCD+vehicle, $p<0.05$. (C) Representative immunohistochemistry liver sections stained for F4/80 and MPO (200× and 400×).

(Fig. 3A). An inhibitor of the NLRP3 inflammasome, MCC950, was utilized as a positive control. MCC950 (20 mg/kg) intraperitoneal injection decreased the AST levels caused by the MCD diet but did not affect the ALT levels (Fig. 3A). The infiltration of total macrophages, inflammatory macrophages, and neutrophils into the liver was assessed by quantifying F4/80, Ly6c, and myeloperoxidase (MPO) mRNA levels in the liver. Total macrophages (F4/80) and inflammatory macrophages (Ly6c) infiltrated the liver substantially more in MCD diet-fed mice than in NOR diet-fed mice, although neutrophils (MPO) infiltrated the liver only slightly more (Fig. 3B). Interestingly, loganin treatment at doses of 5 and 30 mg/kg inhibited the infiltration of total macrophages (F4/80), inflammatory macrophages (Ly6c), and neutrophils (MPO) (Fig. 3B). Similarly, MCC950 therapy decreased F4/80, Ly6c, and MPO mRNA levels in the liver, which had been increased by the MCD diet (Fig. 3B). The MCD diet enhanced hepatic infiltration of total macrophages (F4/80) and neutrophils (MPO) as determined by immunohistochemistry (Fig. 3C). In contrast, loganin therapy decreased macrophage and neutrophil infiltration, as seen by decreased immunohistochemical staining for F4/80 and MPO (Fig. 3C). Similarly, MCC950 inhibited macrophage and neutrophil infiltration generated by the MCD diet (Fig. 3C). Administration of loganin reduced the expression of NLRP3 inflammasome components such as NLRP3, ASC, and caspase-1 in livers (Fig. 3D). These findings demonstrate that loganin therapy diminishes hepatic inflammation generated in mice by an MCD diet, together with the downregulation of the NLRP3 inflammasome components.

As hepatic steatosis is another characteristic of NASH, we investigated whether loganin therapy might diminish hepatic fat accumulation in mice fed the MCD diet. The buildup of triglycerides in the livers of mice given the MCD diet was dramatically elevated (Fig. 4A). Intraperitoneal administration of loganin, as well as MCC950, significantly decreased hepatic lipid buildup generated by the MCD diet (Fig. 4A). H&E stain-

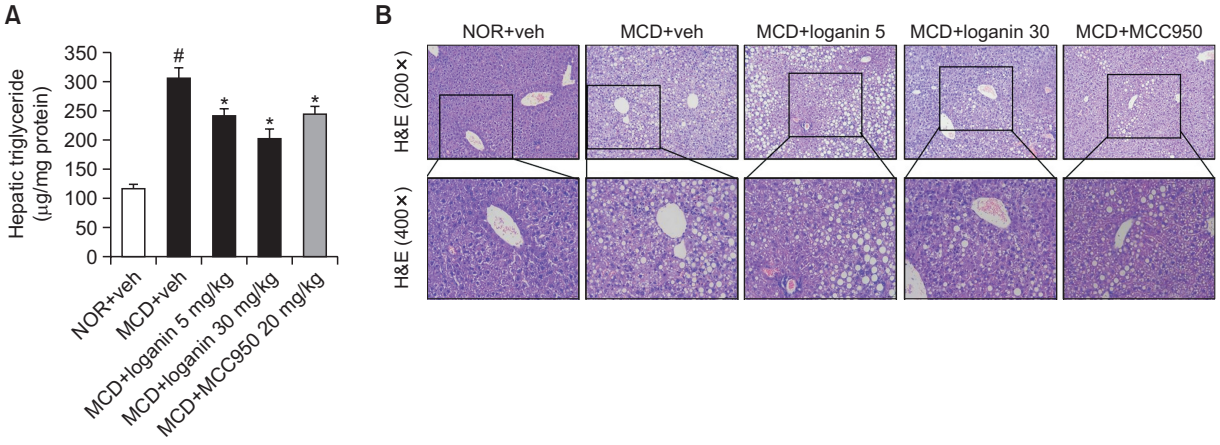

Fig. 4. Loganin lowers triglyceride levels in MCD diet-fed mice. Liver samples were obtained from the mice mentioned in the legend of Fig. 3. (A) Hepatic triglyceride levels. The values are the means ± SEMs (n=8). #Significantly different from NOR+vehicle, $p<0.05$. *Significantly different from MCD+vehicle, $p<0.05$. (B) Representative H&E-stained sections of liver tissue (200× and 400×). Hepatic steatosis and infiltrated neutrophils were identified via H&E staining. The purple dots indicate neutrophils.

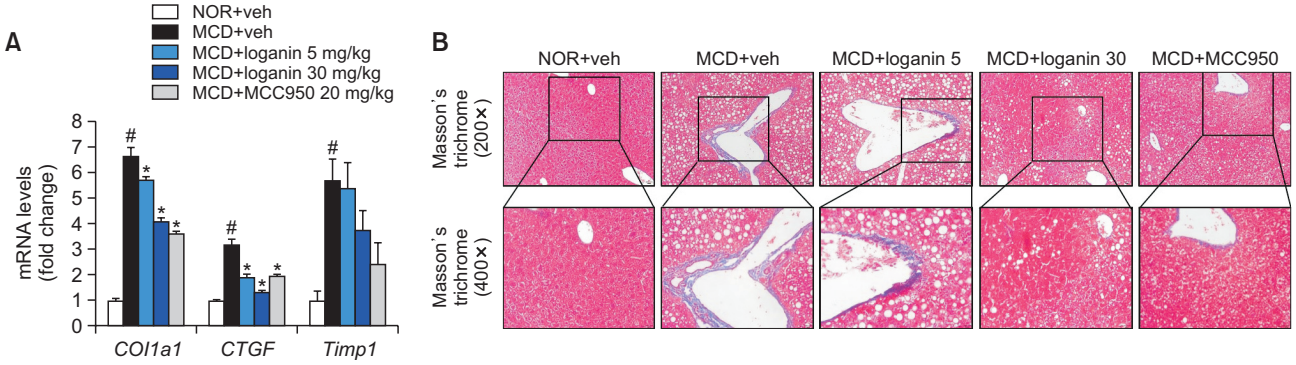

Fig. 5. Loganin reduces hepatic fibrosis in MCD diet-fed mice. Liver samples were obtained from the mice mentioned in the legend of Fig. 3. (A) Hepatic mRNA expression of collagen type 1 (Col1a1), connective tissue growth factor (CTGF), and a matrix metalloproteinase 1 tissue inhibitor (Timp1). The values are the means ± SEMs (n=8). #Significantly different from NOR+vehicle, $p<0.05$. *Significantly different from MCD+vehicle, $p<0.05$. (B) Representative pictures of Masson's trichrome-stained liver sections (200× and 400×).

ing indicated that the MCD diet promoted hepatic steatosis by increasing the number of lipid droplets in the liver (Fig. 4B). Loganin and MCC950 both decreased the quantity of hepatic lipid droplets by small amounts (Fig. 4B).

Loganin reduces hepatic fibrosis in a NASH mouse model fed a methionine-choline-deficient diet

As NASH progresses, liver fibrosis featuring collagen deposition occurs. As a result, we investigated whether loganin could be used to treat liver fibrosis. The mRNA levels of liver fibrosis markers, such as collagen type 1 (Col1a1), connective tissue growth factor (CTGF), and tissue inhibitor of matrix metalloproteinase 1 (Timp1), were determined as indicators of liver fibrosis. These mRNA levels were significantly increased in livers from MCD diet-fed mice compared to livers from normal diet (NOR)-fed mice (Fig. 5A). Loganin treatment significantly decreased liver fibrosis marker expression, while the MCD diet increased the expression (Fig. 5A). Masson's trichrome staining was used to assess collagen deposition in the liver. Loganin treatment significantly reduced collagen deposition in MCD diet-fed mice (Fig. 5B). Likewise, MCC950 decreased the mRNA levels of liver fibrosis markers, and the MCD diet increased collagen deposition (Fig. 5). The results indicate that loganin inhibits hepatic fibrosis in mice fed an MCD diet.

DISCUSSION

Our results demonstrate that NLRP3 inflammasome inhibition could provide a potentially effective therapeutic tactic for treating or preventing the pathological events of NASH. The inhibitory effects of loganin on the NLRP3 inflammasome in both macrophages and liver tissues are well correlated with its relieving effects on the pathological symptoms of NASH, including hepatic inflammation, triglyceride accumulation, and fibrosis. The activation of the NLRP3 inflammasome consists of a two-step event, a priming stage and an activation stage. The priming stage is a prerequisite event to express pro-IL-1β, so that caspase-1 activated in the activation step degrades pro-IL-1β to mature IL-1β. Previous studies reported that lo-

ganin can inhibit NF-κB activation (Hu *et al*., 2020; Wang *et al*., 2020; Wen *et al*., 2020), which is required for the priming step. Loganin reduces intestinal epithelial inflammation via the TLR4/NF-κB and JAK/STAT3 signaling pathways (Wang *et al*., 2020). Loganin has anti-inflammatory properties in a murine acute colitis model by inhibiting the STAT3/NF-κB pathway (Yuan *et al*., 2020). Loganin treatment reduced intracellular reactive oxygen species and inhibited the expression of the NF-κB/P2RX7/TXNIP proteins, thereby protecting RSC96 Schwann cells from injury caused by NLRP3 inflammasome activation (Cheng *et al*., 2020). These previous reports suggest that loganin may affect the priming step, which requires NF-κB activation. In this study, we aimed to investigate whether loganin regulates the activation step of the NLRP3 inflammasome. Therefore, we treated macrophages with loganin after the priming event. Our results show that loganin can suppress the activation step, disrupting the formation of ASC specks.

ASC is expressed in both the cytosol and nucleus and is redistributed to the cytosol upon inflammatory stimulation, resulting in the formation of cytosolic ASC aggregates (Bryan *et al*., 2010). Translocation of ASC from the nucleus to the cytosol is a critical event to bridge NLRP3 and pro-caspase-1 localized in the cytosol to form the NLRP3 inflammasome complex and to release IL-1β (Bryan *et al*., 2009). Consistently, our confocal microscopic data showed that a condensed form of ASC aggregates was distinctly observed in the cytosol when the cells were stimulated with ATP or nigericin, while ASC was distributed in both the cytosol and nucleus in the control group. In addition, loganin treatment reduced the formation of condensed ASC aggregates, possibly leading to decreased assembly of the NLRP3 inflammasome complex and a reduction in IL-1β release.

As the relationship between the NLRP3 inflammasome and NAFLD has been revealed, targeting the activity of the NLRP3 inflammasome has become a promising therapeutic strategy to pursue NASH treatment. Treatment with MCC950, which targets the NLRP3 NACHT domain, exerted pharmacological efficacy against liver inflammation and fibrosis in MCD diet-induced NASH in mice (Mridha *et al*., 2017). Oral administration of sulforaphane protected against hepatic steatosis and inflammation in mice fed a high-fat diet, accompanied by suppression of the NLRP3 inflammasome in the liver (Yang *et al*., 2016). Our results also demonstrate that loganin alleviates hepatic inflammation and fibrosis, accompanied by inhibition of the NLRP3 inflammasome. These results clearly suggest that the continuous discovery and development of NLRP3 inflammasome inhibitors that exert pharmacological activity with lower toxicity would be beneficial for the treatment of NASH or NAFLD.

This study focuses on NLRP3 inflammasome activation in NASH and shows that regulating the NLRP3 inflammasome products IL-1β and caspase-1 may play an essential role in preventing the pathology of NASH. Loganin reduced the activation of the NLRP3 inflammasome *in vitro* and improved the inflammation induced *in vivo* by the MCD diet. In addition, loganin affected inflammation by decreasing the infiltration of immune cells, one of the characteristics of NASH. Thus, modulation of NLRP3 inflammasome activation by loganin represents a possible prevention and treatment strategy for inflammation in NASH.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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