Nobiletin alleviates palmitic acid-induced NLRP3 inflammasome activation in a sirtuin 1-dependent manner in AML-12 cells

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Abstract. The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome has been reported to contribute to palmitic acid (PA)-induced lipotoxicity. Nobiletin (Nob) is a polymethoxylated flavonoid derived from citrus fruits that has been reported to exert antioxidant and antitumor effects. However, its protective and regulatory mechanisms in PA-induced lipotoxicity remain unclear. Therefore, the aim of the present study was to investigate the protective effects of Nob in AML-12 cells against lipotoxicity and examine the underlying mechanism. Western blotting, reverse transcription-quantitative polymerase chain reaction and ELISA assays were performed to investigate the activation of the NLRP3 inflammasome. Sirtuin 1 (SIRT1) small interfering RNA was used to knockdown SIRT1 expression in AML-12 cells. The results demonstrated that PA effectively activated NLRP3 inflammasome and increased the expression and secretion of interleukin (IL)-1β and IL-18. Notably, the PA-induced inflammasome activation was reversed by Nob, as indicated by the decreased expression levels of NLRP3, Caspase-1, IL-1β and IL-18. Furthermore, Nob treatment with or without PA enhanced the expression of SIRT1 in AML-12 cells, while knockdown of SIRT1 with SIRT1-small interfering RNA reversed the anti-inflammatory effects of Nob. Overall, the results of the present study indicated that Nob alleviated PA-induced lipotoxicity in AML-12 cells via the suppression of NLRP3 inflammasome activation in a SIRT1-dependent manner. These results provide a possible basis of the underlying mechanism and, in turn, the potential application of Nob in the treatment of non-alcoholic fatty liver disease.

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Abbreviations: NLRP3, NOD-like receptor family pyrin domain containing 3; PA, palmitic acid; Nob, nobiletin; NAFLD, non-alcoholic fatty liver disease; SFA, saturated fatty acid; ER, endoplasmic reticulum; SIRT1, sirtuin 1; CCK-8, Cell Counting Kit-8; NASH, non-alcoholic steatohepatitis

Key words: lipotoxicity, nobiletin, NOD-like receptor family pyrin domain containing 3 inflammasome, sirtuin 1, hepatocyte

Introduction

Lipotoxicity is a key pathogenic feature of metabolic syndrome that results from the accumulation of saturated fatty acids (SFAs) and their toxic metabolites in lean tissues. SFAs induce hepatocyte lipotoxicity by increasing reactive oxygen species production, endoplasmic reticulum (ER) stress, inflammation and apoptosis (1,2). Nobiletin (Nob), a type of Chinese herbal medicine considered to have antioxidant and antitumor properties, is a polymethoxylated flavonoid present in citrus peels (3). There is a growing body of evidence that suggests that flavonoids can be used to attenuate lipotoxicity (4-6). For instance, baicalin, a flavonoid obtained from *Scutellaria baicalensis* Georgi, has been demonstrated to have significant anti-inflammatory and antibacterial properties (7). In addition, several studies have reported that the flavonoid silybin reduced lipid accumulation and oxidative stress *in vivo* and *in vitro* (8,9). However, to the best of our knowledge, there are no previous studies that have evaluated the effects of Nob on lipotoxicity.

The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is an intracellular multiprotein complex that can recognize pathogen- and danger-associated molecular patterns (10). NLRP3 interacts with apoptosis-associated speck-like protein containing a C-terminal Caspase-recruitment domain, and induces the cleavage and activation of Caspase-1. It further induces the expression and secretion of cytokines interleukin (IL)-18 and IL-1β cytokines (10,11). The NLRP3 inflammasome serves a critical role in metabolic syndromes and is also involved palmitic acid (PA)-induced lipotoxicity (12,13). In addition, flavonoid compounds have exhibited positive effects in alleviating lipotoxicity by targeting the NLRP3 inflammasome (14,15). Nevertheless, the role of Nob in the regulation of NLRP3 inflammasome activation remains unknown.

Sirtuin 1 (SIRT1), a class III histone deacetylase, serves a vital role in a variety of cellular processes, ranging from gene silencing, controlling the cell cycle and inflammation (16,17). A previous study has reported that SIRT1 interferes with the nuclear factor (NF)-κB signaling pathway and, thus, has an anti-inflammatory function (18). Furthermore, another study demonstrated that SIRT1 exerted an anti-inflammatory effect through regulation of the NLRP3 inflammasome in vascular endothelial cells (19). Notably, previous studies have also suggested that SIRT1 had protective effects in SFA-induced lipotoxicity (20,21). However, it is unknown whether the protective effect of SIRT1 is associated with the regulation of NLRP3 inflammasome activation.

Therefore, the aim of the present study was to investigate the potential effect of Nob on PA-induced NLRP3 inflammasome activation and examine the underlying mechanism.

Materials and methods

Reagents and materials. Nob (>99.05%) was purchased from Selleck Chemicals (S2333; Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The final concentration of DMSO was maintained at <0.1% in all experiments and treatments. PA (P0500; Sigma-Aldrich; Merck KGaA) was dissolved in 0.1 M NaOH at 70˚C and then complexed with 10% bovine serum albumin (BSA) at 55˚C for 10 min to achieve the final palmitate concentration (100 mM). The antibodies against SIRT1 (1:2,000; cat. no. ab110304), pro-Caspase-1 (1:1,000; cat. no. ab179515), Caspase-1 (1:5,000; cat. no. ab201476), IL-18 (1:500; cat. no. ab71495), IL-1β (1:1,000; cat. no. ab9722) and β-actin (1:5,000; cat. no. ab8226) were purchased from Abcam (Cambridge, UK), and the antibody against NLRP3 (1:1,000; cat. no. 15101) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-mouse and anti-rabbit IgG (1:5,000, cat. nos. ab205719 or ab205718) secondary antibodies were also purchased from Abcam. The small interfering RNA (siRNA) of SIRT1 (sc-40987) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. AML-12 cells, an immortalized normal mouse hepatocyte cell line, were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Science, Inc.) and insulin-transferrin-selenium (Sigma-Aldrich; Merck KGaA) at 37˚C under 5% CO_2.

Cell transfection. SIRT1-siRNA transfection was performed according to the manufacturer's protocol of the transfection reagent. Prior to transfection, the cells were seeded in 6-well plates at a concentration of $5x10^5$ cells/ml. Cells were transfected with 50 nM SIRT1-siRNA or 50 nM negative control using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) for 36 h at 37˚C.

Cell viability assay. AML-12 cells were suspended in DMEM/F12 and plated at a density of $1x10^4$ cells/well in 96-well plates. Cell viability was detected using a Cell Counting Kit-8 (CCK-8) kit (Dojindo Co., Kumamoto, Japan). Cells were treated with various concentrations (0, 10, 20, 50, 100, 200, 400 or 800 μM) of Nob for 12 h; or with 100 μM Nob for various times intervals (0, 2, 4, 8, 16, 24, 48 or 72 h), or different concentrations (0, 50, 100 or 200 μM) of Nob with 400 μM PA for 12 h. All cells were incubated at 37˚C. Subsequent to the indicated treatments, 10 μl CCK-8 solution was added to each well and then incubated for 4 h at 37˚C in 5% CO_2. The absorbance at 450 nm was measured with a spectrophotometer (Thermo Fisher Scientific, Inc., Shanghai, China).

ELISA assay. Cells were treated with various interventions, following which the culture medium was collected and the supernatant was obtained by centrifugation (3,000 x g for 10 min) at 4˚C. The concentrations of IL-1β and IL-18 in the supernatant were assayed with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The intra- and inter-assay coefficients of variation were 5.0 and 8% for IL-1β, and 6.8 and 7% for IL-18, respectively. Every sample was analyzed in triplicate, and absorbance values were read at 450 nm using a spectrophotometer.

Western blot analyses. Western blotting was performed as previously described (2,22). Total protein in hepatocytes was extracted using a protein extraction kit (cat. no. C510003; Sangon Biotech Co., Ltd., Shanghai, China). Briefly, the protein concentration in hepatocyte extracts was determined using a protein assay kit (Sangon Biotech Co., Ltd., Shanghai, China). A total of 30 μg of protein from each sample was separated by 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane. Next, the membranes were blocked in 3% BSA/Tris-buffered saline-Tween 20 (TBS-T) buffer at room temperature for 4 h. The blocked membranes were then incubated overnight at 4˚C with primary antibodies. The membranes were then washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG at room temperature for 45 min. Immunoreactive bands were visualized by enhanced chemiluminescence solution (EMD Millipore, Temecula, CA, USA). All bands were analyzed using Image-pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. The total RNA was extracted from AML-12 cells using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The RNA concentration and quality were measured using a K5500 micro-spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China) and by electrophoresis on 1% agarose gels, respectively. Next, 1 μg total RNA in each sample was reverse-transcribed into cDNA using an RT kit (Takara Biotechnology Co., Ltd.) according to the supplier's protocol. The mRNA expression levels were then evaluated using qPCR technology with the SYBR Green QuantiTect RT-PCR kit (Takara Biotechnology Co., Ltd.) and a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was conducted under the following

Table I. Primers used during quantitative polymerase chain reaction assay.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (˚C)
NLRP3	ATCAACAGGCGAGACCTCTG	GTCCTCCTGGCATACCATAGA	60
Caspase-1	CTTGGAGACATCCTGTCAGGG	AGTCACAAGACCAGGCATATTCT	60
IL-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC	60
IL-18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA	60
β-actin	CGATGCCCTGAGGCTCTTT	TGGATGCCACAGGATTCCAT	60
Caspase-3	TGGTGATGAAGGGGTCATTTATG	TTCGGCTTTCCAGTCAGACTC	60
Caspase-9	TCCTGGTACATCGAGACCTTG	AAGTCCCTTTCGCAGAAACAG	60
Bax	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG	60

NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin; Bax, B-cell lymphoma 2-associated X protein.

conditions: Initial denaturation at 94˚C for 2 min; 35 cycles of amplification, including denaturation at 94˚C for 10 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 30 sec; and final extension at 72˚C for 5 min. The primers used are shown in Table I. The relative expression of each target gene was normalized to β-actin and calculated using the $2^{-\Delta\Delta Cq}$ method (23).

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were conducted using IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). Statistical significance was calculated using Student's t-test in cases of comparisons between two groups, or using one-way ANOVA with subsequent Bonferroni correction in cases of comparisons among more than two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of Nob on cell viability. The chemical structure of Nob is presented in Fig. 1A. The direct cytotoxicity of Nob was assessed by CCK-8 assay. The results revealed that Nob at concentrations up to 400 μM did not affect cell viability, while cell viability was significantly reduced at 800 μM (Fig. 1B). Furthermore, as shown in Fig. 1C, exposing AML-12 cells to Nob for 72 h at a concentration of 100 μM did not influence the cell viability (Fig. 1C). In addition, PA (400 μM) alone caused significant cytotoxicity, while treatment with Nob exerted dose-dependently protective effects on the cell viability following PA stimulation (Fig. 1D). The expression of apoptosis-associated molecules was also examined. The results demonstrated that PA exposure alone significantly increased the mRNA expression levels of apoptosis-associated molecules, including Caspase-3, Caspase-9 and B-cell lymphoma 2-associated X protein, whereas co-treatment with Nob reversed the effects of PA on mRNA expression of these molecules (Fig. 1E-G). Taken together, the results indicated that Nob alleviated PA-induced cytotoxicity in AML-12 cells. Consequently, the present study selected the concentrations of 50, 100 and 200 μM Nob for treatments in subsequent experiments.

Nob inhibits NLRP3 inflammasome activation in the presence of PA stimulation. Initially, the effects of PA on NLRP3 inflammasome activation were measured, and it was revealed that PA significantly increased NLRP3 inflammasome activation, and IL-1β and IL-18 secretion in AML-12 cells (Fig. 2A-G). Notably, Nob decreased the mRNA and protein expression levels of NLRP3, Caspase-1, IL-1β and IL-18 in a dose-dependent manner in PA-treated AML-12 cells (Fig. 2A-E). Furthermore, Nob also suppressed the secretion of IL-1β and IL-18 cytokines in a dose-dependent manner in PA-treated AML-12 cells (Fig. 2F and G). Taken together, these results indicated that Nob inhibits PA-induced NLRP3 inflammasome activation in AML-12 cells.

Nob upregulated SIRT1 expression in AML-12 cells. As shown in Fig. 3A, Nob increased SIRT1 protein expression in a dose-dependent manner. PA treatment alone evidently decreased the protein expression of SIRT1; however, Nob reversed the inhibitory effect of PA on SIRT1 expression (Fig. 3B). These results demonstrated that Nob upregulates SIRT1 expression in AML-12 cells.

Nob suppresses PA-induced NLRP3 inflammasome activation in a SIRT1-dependent manner. To investigate whether SIRT1 mediated the protective effect of Nob, the present study used SIRT1-siRNA to knockdown SIRT1. NC siRNA did not affect SIRT1 expression (Fig. 4A) or cell viability (data not shown) compared with the blank control. The results revealed that SIRT1-siRNA significantly reduced the mRNA and protein expression levels of SIRT1 compared with with NC group in AML-12 cells (Fig. 4A and B). Notably, Nob was unable to suppress the PA-induced NLRP3 inflammasome activation in the presence of SIRT1-siRNA (Fig. 4C-J). Specifically, Nob decreased the mRNA and protein expression levels of NLRP3, Caspase-1, IL-1β and IL-18 in the presence of PA, while this effect was reversed by SIRT1-siRNA (Fig. 4D-H). The concentrations of IL-1β and IL-18 exhibited the same pattern (Fig. 4I and J). Furthermore, Nob was unable to reverse the PA-induced reduction in cell viability in the presence of SIRT1-siRNA (Fig. 4K). In summary, these results demonstrated that Nob suppresses PA-induced NLRP3 inflammasome activation in a SIRT1-dependent manner.

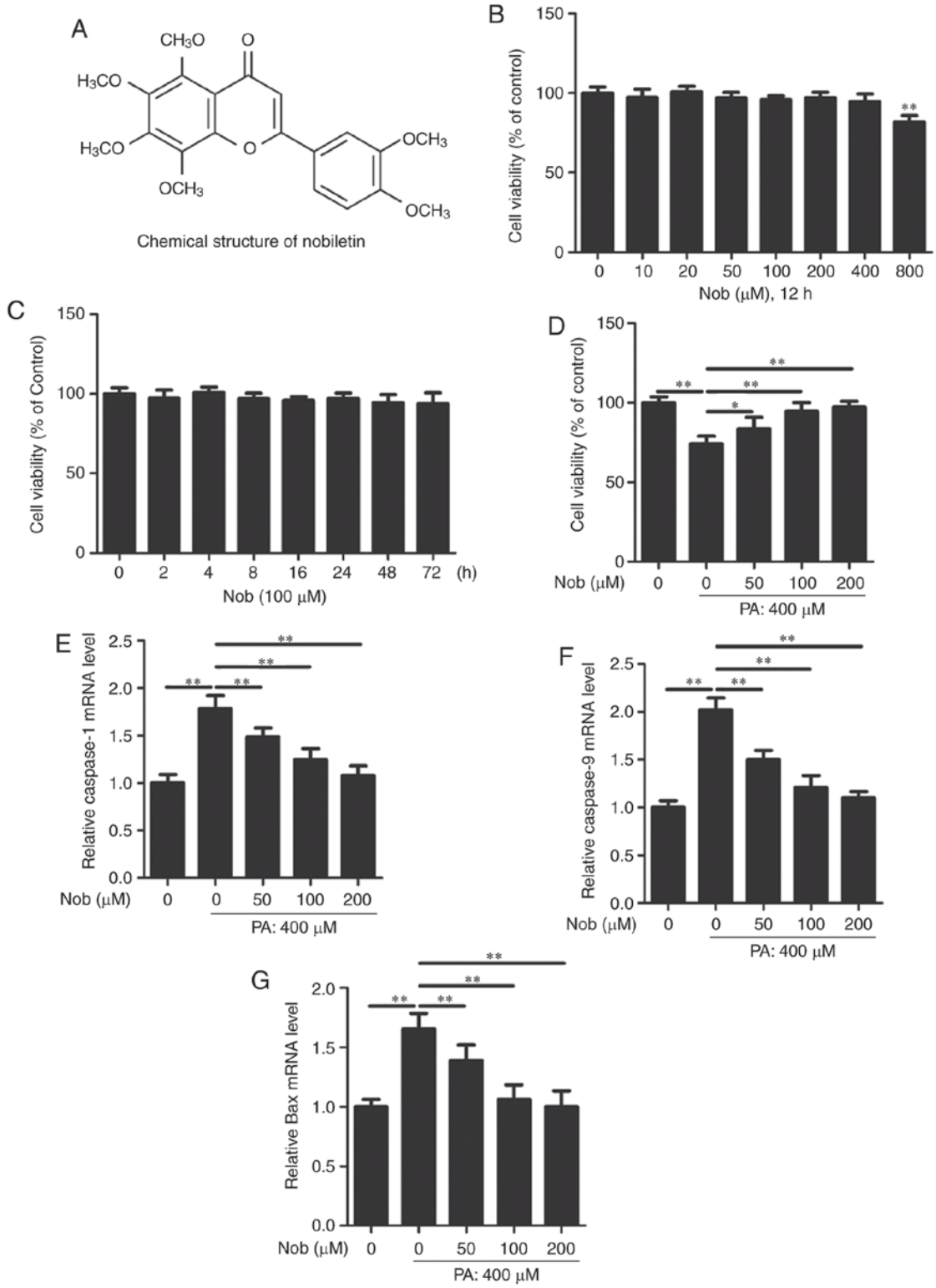

Figure 1. Effects of Nob on cell viability. (A) Chemical structure of Nob. (B) Viability of AML-12 cells treated with different concentrations of Nob (0, 10, 20, 50, 100, 200, 400 or 800 μM) for 12 h. **P<0.01 vs. untreated group. (C) Viability of AML-12 cells treated with 100 μM Nob for different durations (0, 2, 4, 8, 16, 24, 48 or 72 h). (D) Viability of AML-12 cells treated with different concentrations of Nob (0, 50, 100 or 200 μM) along with 400 μM PA. Reverse transcription-quantitative polymerase chain reaction was used to measure the mRNA expression levels of (E) Caspase-3, (F) Caspase-9 and (G) Bax. All data are presented as the mean ± standard deviation. **P<0.01. Nob, nobiletin; PA, palmitic acid; Bax, B-cell lymphoma 2-associated X protein.

Discussion

Lipotoxicity induced by SFAs serves a pivotal role in the origin and pathological development of metabolic syndrome, particularly in non-alcoholic fatty liver disease (NAFLD) (24). Non-alcoholic steatohepatitis (NASH) is the progressive subtype of NAFLD and is characterized by hepatocyte injury and inflammation, which can lead to liver cirrhosis

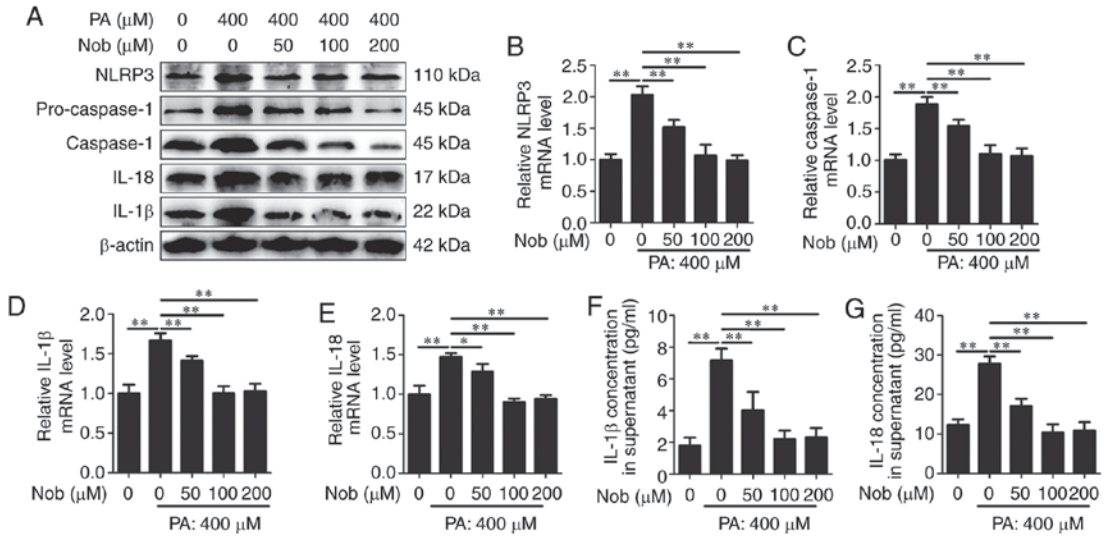

Figure 2. Nob inhibits NLRP3 inflammasome activation. Cells were treated with different concentrations of Nob (0, 50, 100 or 200 μM) along with 400 μM PA. (A) Western blots of protein expression of NLRP3, pro-Caspase-1, Caspase-1, IL-18 and IL-1β. mRNA expression levels of (B) NLRP3, (C) Caspase-1, (D) IL-1β and (E) IL-18. (F) IL-1β and (G) IL-18 concentrations in the supernatant. All data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. Nob, nobiletin; PA, palmitic acid; NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin.

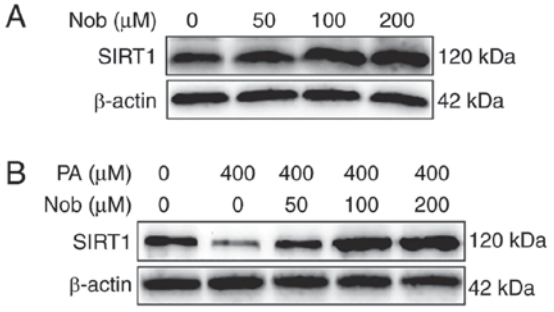

Figure 3. Nob upregulates SIRT1 expression in AML-12 cells. Western blots demonstrating the protein expression of SIRT1 in cells treated with (A) different concentrations of Nob (0, 50, 100 or 200 μM) for 12 h, or (B) different concentrations of Nob (0, 50, 100 or 200 μM) along with 400 μM PA. Nob, nobiletin; PA, palmitic acid; SIRT1, sirtuin 1.

and hepatocellular carcinoma (25). The absence of approved pharmacological therapies for NAFLD has inspired research into developing effective therapeutic agents for this condition (26). In the present study, it was demonstrated that Nob protects AML-12 cells from PA-mediated hepatotoxicity via the inhibition of NLRP3 inflammasome activation in a SIRT1-dependent manner.

Increasing evidence has suggested that metabolic syndrome may be associated with the overproduction of inflammatory cytokines, including IL-1β and IL-18 (27,28). In addition, patients with NAFLD or NASH have been observed to have increased inflammation levels. Recent studies have highlighted the role of the NLRP3 inflammasome in NAFLD and NASH, and have demonstrated that NLRP3 inflammasome activation resulted in hepatocyte pyroptosis, liver inflammation, and fibrosis, as well as the blockade of NLRP3 inflammasome-reduced liver inflammation and fibrosis (29,30). The application of natural compounds and medicinal plants to treat various diseases has become a novel trend in clinical medicine research. Flavonoids exert a wide range of health benefits, including anti-inflammatory, antitumor and antioxidant activities (31,32). Nob, a polymethoxylated flavonoid, was previously reported to attenuate cardiac dysfunction, oxidative stress and inflammation in streptozotocin-induced diabetic cardiomyopathy (33). He *et al* (34) reported that Nob reduced lipopolysaccharide-induced liver injury in mice by inhibiting nuclear factor-κB-mediated cytokine production. In the present study, the effect of Nob on NLRP3 inflammasome activation was investigated. The results demonstrated that PA, a model of lipotoxicity, increased the activation of NLRP3 inflammasome and the secretion of IL-1β and IL-18. Notably, Nob markedly reduced the expression of NLRP3 inflammasome-associated molecules, including NLRP3, pro-Caspase-1 and Caspase-1. Furthermore, Nob significantly reduced the mRNA and protein secretion of IL-1β and IL-18 in PA-treated AML-12 cells. These results suggested that Nob effectively protected AML-12 cells against PA-induced lipotoxicity by inhibiting NLRP3 inflammasome activation. These results also demonstrated that Nob may be an effective natural compound to combat NAFLD.

SIRT1 serves key roles in the regulation of lipid and glucose homeostasis, in anti-inflammatory processes and in the control of oxidative stress. A previous study indicated that hepatocyte-specific deletion of SIRT1 altered the fatty acid metabolism, and resulted in hepatic steatosis and inflammation (35). In addition, hepatic overexpression of SIRT1 in mice attenuated high-fat diet-induced hepatic ER stress, insulin resistance and lipid accumulation (36). Therefore, pharmacologic activation of SIRT1 may be a potential therapeutic strategy for combating PA-induced lipotoxicity. In the present study, it was also demonstrated that Nob increased SIRT1 expression, with or without PA treatment, in AML-12 cells. To further investigate the association between SIRT1 and Nob, SIRT1-siRNA was employed to knockdown SIRT1 *in vitro*. Notably,

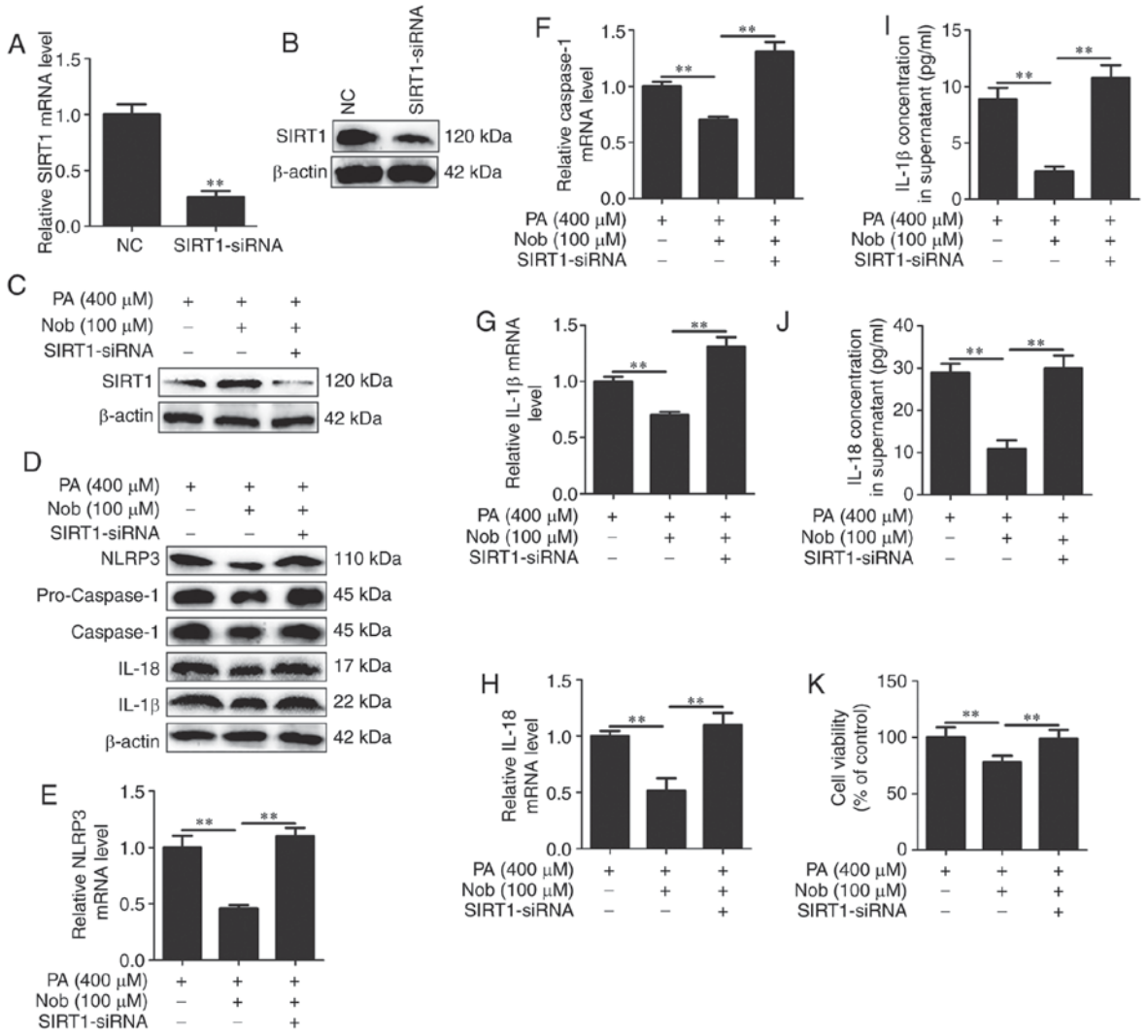

Figure 4. Nob suppresses PA-induced NLRP3 inflammasome activation in a SIRT1-dependent manner. (A) mRNA and (B) protein expression levels of cells transfected with SIRT1-siRNA or NC. **P<0.01 vs. NC group. Next, cells were divided into three groups, and treated as follows: PA (400 μM) alone for 12 h; PA (400 μM) + Nob (100 μM) for 12 h; or transfected with SIRT1-siRNA and then treated with PA (400 μM) + Nob (100 μM) for 12 h. (C) Protein expression of SIRT1. (D) Protein expression levels of NLRP3, pro-Caspase-1, Caspase-1, IL-18 and IL-1β. (E) NLRP3, (F) Caspase-1, (G) IL-1β and (H) IL-18 mRNA expression levels. (I) IL-1β and (J) IL-18 concentrations in the supernatant. (K) Viability of AML-12 cells. All data are presented as the mean ± standard deviation. **P<0.01. Nob, nobiletin; PA, palmitic acid; SIRT1, sirtuin 1; NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin; siRNA, small interfering RNA; NC, negative control.

SIRT1-siRNA reversed the anti-inflammatory effects of Nob, as indicated by the increased expression levels of NLRP3, Caspase-1, IL-1β and IL-18. Therefore, the results suggested that Nob increased the expression of SIRT1 and that the protective effects of Nob were associated with SIRT1. Furthermore, based on these results, it is suggested that SIRT1 has potential as a therapeutic target to prevent the progression and development of NAFLD.

In conclusion, the present study demonstrated that Nob ameliorated PA-induced lipotoxicity by suppressing NLRP3 inflammasome activation in a SIRT1-dependent manner in AML-12 cells. Notably, the results provided the basis for effective complementary and alternative therapeutic strategies for the treatment of NAFLD.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

ZP and XBL performed the experiments, prepared the figures and wrote the manuscript. XWL designed and supervised the study. DX, XD, ZW and GL performed the statistical

analyses and edited of the manuscript. All authors approved the manuscript prior to submission.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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