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Hesperetin, a SIRT1 activator, inhibits hepatic inflammation via AMPK/CREB pathway

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ABSTRACT

Silent mating type information regulation 2 homolog 1 (SIRT1) is an important inflammatory regulator, which epigenetically reprograms inflammation by altering the acetylation of NF-κB. Hesperetin, as a common flavonoid, has been proven to have a significant effect on acute inflammatory diseases. However, the detailed molecular mechanism by which hesperetin alleviates inflammatory response and accompanied tissue injury is poorly understood. Our results show that SIRT1 is required for the inhibitory effect of hesperetin on inflammation. Hesperetin suppresses the acetylation of RelA/p65 to reduce NF-κB activity by inducing SIRT1 expression. Mechanistically, hesperetin increases SIRT1 expression through AMPK/CREB pathway. Additionally, the protective effect of hesperetin against LPS/D-GalN-induced hepatitis in mice is also dependent on SIRT1. Our study suggests that hesperetin is an SIRT1 activator and could be potential candidates for the treatments of inflammatory conditions.

1. Introduction

Most liver diseases are accompanied by inflammation and oxidative stress, regardless of the aetiology of the underlying disorder. Excessive and uncontrolled inflammation leads to irreversible damage to the hepatic parenchyma and loss of liver function [1]. There are currently no effective treatments to protect hepatic cells and improve liver function in acute and chronic inflammatory liver diseases. Co-administration of lipopolysaccharide (LPS) and D-galactosamine (D-GalN) in mice has been widely used as an animal model of inflammatory liver disease [2,3]. This model is characterized by the rapid activation of Kupffer cells (KCs), the inherent macrophages in liver tissue and the major source of pro-inflammatory mediators after liver injury [3,4]. Nuclear factor kappa-B (NF-κB) plays a pivotal role in LPS/D-GalN-induced inflammation and acute liver injury [3,5,6]. NF-κB activates the expression of a wide range of host genes that control the inflammatory and immune responses, such as TNF-α, IL-1β and IL-18 [7–9]. These inflammatory cytokines and chemokines aggravate LPS/D-GalN-induced liver injury.

Silent mating type information regulation 2 homolog 1 (SIRT1), an NAD^+-dependent protein deacetylase, is an important inflammatory regulator [10,11]. As a major metabolic regulator, SIRT1 epigenetically reprograms inflammation by altering the acetylation of histones and transcription factors such as NF-κB [12]. The transgenic mice with moderate overexpression of *Sirt1* under the control of its natural promoter show reductive activation of proinflammatory cytokines, such as TNFα and IL-6, and lowered lipid-induced inflammation via down-modulation of NF-κB activity [13]. Activation of SIRT1 by resveratrol

Abbreviations: Hes, hesperetin; SIRT1, Silent mating type information regulation 2 homolog 1; AMPK, AMP-activated protein kinase; CREB, cyclic AMP response-element-binding protein; NF-κB, nuclear factor kappa-B; NAM, Nicotinamide; Resv, resveratrol; LPS, lipopolysaccharide; D-GalN, D-galactosamine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, hematoxylin and eosin; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1beta; IL-18, interleukin-18; NLRP3, NLR family pyrin domain containing 3

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promotes deacetylation and proteasome degradation of RelA/p65 and suppression of transcriptional activation by NF-κB [10]. Conversely, decreased nuclear SIRT1 level/activity increase NF-κB RelA/p65 activity and amplify proinflammatory gene expression during chronic inflammation. When challenged with a high-fat diet, liver-specific *Sirt1* knockout mice develop hepatic steatosis and hepatic inflammation [14].

Hesperetin is a natural compound belonging to the flavanone class of flavonoids [1]. It widely exists in fruits, vegetables and Chinese traditional medicinal herbs in the form of glycosides like hesperidin and neohesperidin. Hesperetin has various of bioactivities, including antioxidant, anti-inflammatory and epigenetic modification capacity [15–17]. Previous studies revealed that hesperetin and its glycoside hesperidin had hepatoprotective effect on acute liver injury [1,2,18,19]. However, the detailed molecular mechanism by which hesperetin inhibits inflammation and attenuates liver injury is poorly understood. In our previous study, we found that Quzhou Fructus Aurantii, which was rich in hesperidin and neohesperidin, had an inhibitory effect on NF-κB activity [20]. Recently, we observed that hesperetin significantly induced SIRT1 expression *in vitro*. Therefore, we wonder if hesperetin plays its anti-inflammatory role via upregulation of SIRT1. In this study, we explored the underlying mechanism for hesperetin to alleviate hepatic inflammation. Our findings support the crucial role of SIRT1 in hesperetin associated hepatic protection in LPS/D-GalN challenged mouse model.

2. Materials and methods

2.1. Chemicals and reagents

Hesperetin (CAS# 520-33-2, HPLC ≥ 98%), Resveratrol (CAS# 501-36-0), EX-527 (CAS# 49843-98-3) and 666-15 (CAS# 1433286-70-4) were purchased from Shanghai Yuanye Biological Technology, China. Nicotinamide (NAM, #S1761) was purchased from Beyotime Biotechnology, China. Compound C (#B3252) was purchased from ApexBio, USA. Lipopolysaccharide (#L2630) and D-galactosamine (#12662) were purchased from Sigma, USA.

2.2. Cell culture

The RAW 264.7, AML12 and 293T cell line were all obtained from the Shanghai Bank of Cell Lines (Shanghai, China). RAW 264.7 cells were cultured in RPMI-1640, and AML12, 293T cells were in DMEM. All these were containing 10% fetal bovine serum (FBS, BBI Life Sciences Corporation, China), 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. The source of the cell line was Identified by STR profiling and tested for mycoplasma contamination.

2.3. Animal experiments

All Animal experiments were conducted in Zhejiang University of Traditional Chinese Medicine, China. Male BALB/c mice (22–25 g) were provided by the Experimental Animal Center of Zhejiang University of Traditional Chinese Medicine. All animals were kept under standard conditions with having free access to distilled water and common pelleted food. After one week of acclimation, the mice were randomly distributed into 4 groups of 10 mice: control group, which was orally administered saline for 5 days; LPS/D-GalN group, which was also orally administered saline for 5 days; LPS/D-GalN + Hes group, which was orally administered 50 mg/kg hesperetin for 5 days; LPS/D-GalN + Hes + EX-527 group, which was orally administered hesperetin and received intraperitoneal injection of EX-527 (2 mg/mL) for 5 days. Two hours after the last administration, the LPS/D-GalN, LPS/D-GalN + Hes and LPS/D-GalN + Hes + EX-527 group received a single intraperitoneal injection of LPS (6 μg/kg) and D-GalN (800 mg/kg), while control group received the same dose of saline injection. Animals were sacrificed for an experimental evaluation under anesthesia within 6 h of administration of LPS/D-GalN.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected by biochemical analyzer according to the manufacturer's instruction (DiaSys Diagnostic Systems, Shanghai, China).

Liver tissues were fixed in 10% formalinand and processed into paraffin sections. Then the sections were stained with hematoxylin and eosin (H&E) staining.

2.4. Immunohistochemical staining

The paraffin-embedded sections were deparrafinized and performed antigen retrieval as previously described [21]. MaxVision HRP-Polymer anti-Rabbit IHC Kit (MXB Biotechnologies, Fuzhou, China) was used to develop signal. The antibody against F4/80 (1:200 dilution) was purchased from Cell Signaling Technology (Cat #2370).

2.5. SIRT1 enzyme activity test

SIRT1 enzyme activity was measured using SIRT1 Inhibitor/Activator Screening Kit (Catalog # K325-100, BioVision, USA) according to the manufacturer's protocol. The fluorescent signal was measured by Synergy Microplate Reader (BioTek, USA).

2.6. Cell viability test

RAW264.7 or AML12 cells were treated with various concentrations Hes (0, 5, 10, 25, 50, 100 μM) overnight. Afterwards, 20 μL of 5 mg/mL MTT (Solarbio, China) was added to each well and incubated for 4 h at 37 °C. Then cell supernatant was abandoned and 100 μL of formanzan was added to each well. After shaking at room temperature for 15 min, spectrophotometric absorbance was measured by Synergy Microplate Reader (BioTek, USA) at 570 nm.

2.7. siRNA and transfection

SIRT1 siRNA was purchased from GenePharma, Shanghai, China. The transfection was performed using Lipofectamine 2000 reagent from Life Technologies (Carlsbad, CA) according to the manufacturer's protocol. Briefly, 10 μL Lipofectamine 2000 was diluted with 250 μL Opti-MEM medium (Life Technologies, Cat #31985070) for 5 min at room temperature. 10 μL siRNA (20 μM) was diluted with 250 μL Opti-MEM medium. Then, the diluted Lipofectamine 2000 was incubated with the diluted siRNA for 20 min at room temperature. After that, the mixture was added to the 293T or RAW264.7 cells in a well of 6-well plate. 8 h later, cells were changed with fresh medium.

2.8. Luciferase reporter assay

pNF-κB-luc (#D2206) was purchased from Beyotime Biotechnology, China. The transfection was performed using Lipofectamine 2000 reagent from Life Technologies (Carlsbad, CA) according to the manufacturer's protocol. Luciferase activity was measured with a Dual-Luciferase Reporter Assay Kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. The fluorescent signal was measured by Synergy Microplate Reader (BioTek, USA).

2.9. ELISA assay

Tumor necrosis factor alpha (TNF-α), interleukin-18 (IL-18) and interleukin-1β (IL-1β) were determined using ELISA kits (MEIMIAN, China) according to the manufacturer's protocol.

Table 1
The primers used in this study for real time PCR.

Description	Sense primer (5′→3′)	Antisense primer (5′→3′)
Il-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
Il-1β	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC
Tnf-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
Nlrp3	TGGATGGGTTTGCTGGGAT	CTGCGTGTAGCGACTGTTGAG
Il-18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA
Sirt1	TGATTGGCACCGATCCTCG	CCACAGCGTCATATCATCCAG
Gapdh	TGAGGCCGGTGCTGAGTATGT	CAGTCTTCTGGGTGGCAGTGAT

2.10. Quantitative Real-time PCR

Total RNA from cells was collected using Trizol Reagent (#DP424, Tiangen Biotech Co. Ltd., Beijing, China) according to the instruction of the manufacturer. In brief, cDNA was generated by Maxima Reverse Transcriptases (#EP0751, Thermo Fisher Scientific, USA). Quantitative PCR was performed with SGExcel FastSYBR Mixture (#B532955-0005, Sangon Biotech Co., Ltd., Shanghai, China) on Roche LightCycler^{R} 480 Quantitative PCR System (Indianapolis, USA). All gene expression data were normalized to *Gapdh* expression level. Primers are listed in Table 1.

2.11. Western blotting

The protein extraction method was according to our previous study [21,22]. In total, the equal amounts of proteins were loaded onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Then the membranes were blocked with 1% casein at room temperature for 1 h and incubated overnight at 4 °C with the primary antibodies against anti-phospho-AMPKα (1: 1000, #2535, Cell Signaling Technology), anti-AMPKα (1: 1000, #5831, Cell Signaling Technology), anti-Sirt1 (1: 1000, #9457, Cell Signaling Technology), anti-Sirt1 (1: 500, ab110304, Abcam), anti-NF-κB p65 (1: 1000, #4764, Cell Signaling Technology), anti-acetyl-NF-κB p65 K310 (1: 200, ab19870, Abcam), anti-acetyl-NF-κB p65 K310 (1: 1000, #3045, Cell Signaling Technology), anti-NLRP3 (1: 1000, #15101, Cell Signaling Technology), anti-IL-1β (1: 1000, #12242, Cell Signaling Technology), anti-IL-18 (1: 500, ab71495, Abcam), anti-TNF-α (1: 500, ab1793, Abcam), anti-CREB (1: 1000, #9197, Cell Signaling Technology), anti-phospho-CREB (1: 1000, #9198, Cell Signaling Technology) and anti-β-actin (1: 3000, #3700, Cell Signaling Technology). Immunoreactive bands were visualized using Tanon 4200SF system (Tanon Biotechnology, Shanghai, China).

2.12. Statistical analysis

The quantitative data are presented as mean ± SD values. Analysis was performed using GraphPad Prism software, version 5.0 (GraphPad Software, La Jolla, CA). The statistical significance of the differences between groups was determined by ANOVA. P values of < 0.05 were considered statistically significant and P values of < 0.01 were considered statistically highly significant.

3. Results

3.1. Hesperetin stimulates SIRT1 expression and inhibits NF-κB p65 acetylation

More than a decade ago, flavonoids were found to have SIRT1 activation property [23]. Hesperetin (4-methoxy-3,5′,7′-trihydroxyflavanone), mainly derived from the immature fruit of Citrus L. (Rutaceae), is one kind of the dihydroflavonoids (Fig. 1A). To examine whether hesperetin could modulate SIRT1 activity, we incubated hesperetin at various concentrations with purified SIRT1 protein and measured the alteration of SIRT1 activity. The SIRT1 inhibitors EX-527 and NAM, and resveratrol, a well-known SIRT1-activator [24], were used for positive controls and benchmarks of the change of SIRT1 activity (Fig. 1B). Unexpectedly, neither dose of hesperetin can activate SIRT1 *in vitro* (Fig. 1B), suggesting that hesperetin may not be able to modulate SIRT1 activity directly. Next, we examined the effect of hesperetin on SIRT1 expression. As shown in Fig. 1C, hesperetin significantly promoted *Sirt1* mRNA expression in RAW 264.7 cells. Resveratrol has been known to promote intracellular SIRT1 activity through allosteric effect and upregulation of SIRT1 expression [25]. Our result revealed that the stimulatory effect of hesperetin on SIRT1 expression was comparable to resveratrol (Fig. 1C). In addition, we found hesperetin promoted the expression of SIRT1 in RAW 264.7 and AML12 cells in a dose-dependent manner (Fig. 1D), but had no significant effect on cell viability (Fig. S1).

In previous studies, hesperetin and its derivatives were reported to possess anti-inflammatory activity. The transcription factor NF-κB regulates multiple aspects of immune functions and serves as a pivotal mediator of inflammatory responses [26]. SIRT1 inhibits NF-κB activity by physically interacting with the RelA/p65 subunit of NF-κB and deacetylating RelA/p65 at lysine 310 [10,27]. RAW 264.7 cells treated with LPS dramatically induced NF-κB p65 acetylation at lysine 310, while the addition of hesperetin greatly reduced its acetylation (Fig. 1E). The change of NF-κB p65 acetylation by hesperetin was consistent to the expression of SIRT1 and the extent was similar to resveratrol treatment at the same dose (Fig. 1F).

3.2. Hesperetin suppresses NF-κB activation and inflammatory responses in macrophages

The acetylation of p65 at lysine 310 is necessary for the complete activation of NF-κB [28]. To determine whether hesperetin treatment suppresses the transcriptional activity of NF-κB, we conducted luciferase reporter assay in 293T and RAW 264.7 cells. As NF-κB activators, IL-1β, TNF-α and LPS could effectively stimulated luciferase activities in both 293T and RAW 264.7 cells (Fig. 2A). Pre-treatment with hesperetin significantly reduced NF-κB transcriptional activity with IL-1β, TNF-α or LPS stimulation reflecting by luciferase activities (Fig. 2A). Furthermore, the enhanced mRNA expression of *Il-1β*, *Il-18*, *Tnf-α*, *Il-6* and *Nlrp3*, which were NF-κB target genes, was significantly downregulated by hesperetin (Fig. 2B). Consistent with this, the intracellular amount of NLRP3, IL-18, IL-1β, TNF-α protein and the secretary IL-18, IL-1β, TNF-α were also inhibited by hesperetin (Fig. 2C and D), indicating decreased LPS-induced inflammatory responses.

3.3. SIRT1 is required for the inhibitory effect of hesperetin to NF-κB activity

To determine whether SIRT1 is indispensable to the reduction of NF-κB transcriptional activity by hesperetin, we inhibited SIRT1 via RNAi or chemical inhibitors in 293T cells expressing luciferase reporter which was driven by promotor containing typical NF-κB binding motifs. As shown in Fig. 3A, hesperetin did not suppressed NF-κB activity in

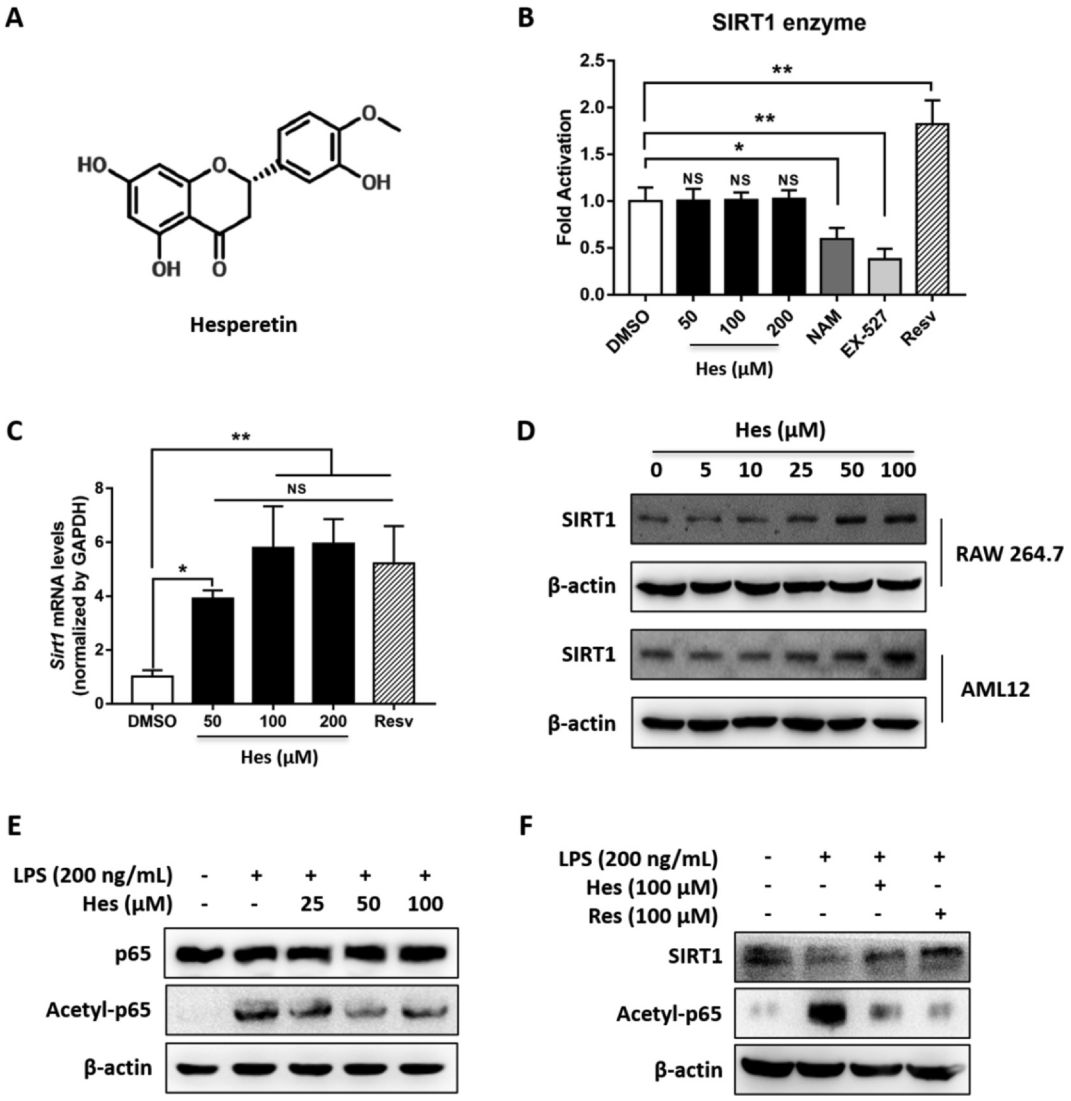

Fig. 1. Hesperetin stimulates SIRT1 expression and inhibits the acetylation of NF-κB p65. (A) Chemical structure of hesperetin (Hes). Its molecular weight is 302.29. (B) A colorimetric assay was performed to measure SIRT1 enzyme activity in the presence of DMSO, Hes (50, 100, 200 μM), NAM (1 μM), EX-527 (100 nM) and Resv (50 μM). Values were expressed as mean ± SD (n = 6). (C) RAW 264.7 cells were treated with various concentrations of Hes (50, 100, 200 μM) or resveratrol (Resv, 50 μM) overnight. The mRNA expression of *Sirt1* were determined by RT-PCR. Values were expressed as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$; NS, not statistically significant. (D) RAW 264.7 and AML12 cells were treated with various concentrations of Hes (0, 5, 10, 25, 50, 100 μM) overnight. The amount of SIRT1 was measured by western blotting. (E) RAW 264.7 cells were treated with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (25, 50 or 100 μM) overnight. The amount of p65, acetyl-p65 and β-actin were measured by western blotting. (F) RAW 264.7 cells were treated with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (100 μM) and LPS (200 ng/mL) + Resv (100 μM) overnight. The amount of SIRT1, acetyl-p65 and β-actin were measured by western blotting.

SIRT1 knockdown 293T cells. Similarly, SIRT1 inhibitor EX-527 and NAM abrogated the inhibitory effect of hesperetin on NF-κB activity (Fig. 3B). Moreover, we inhibited SIRT1 activity in RAW 264.7 cells by inhibitor or siRNA to examine the effect of hesperetin on p65 acetylation. As shown in Fig. 3D, LPS-induced p65 acetylation was repressed by hesperetin in RAW 264.7 cells, while EX-527 and NAM counteracted this inhibitory effect (Fig. 3C). Likewise, hesperetin, no matter the low or high dose, can't suppress of in LPS-induced p65 acetylation in SIRT1 knockdown cells (Fig. 3D). The inhibitory effect of hesperetin on NF-κB activity through SIRT1 mediated deacetylation of RelA/p65 can also be verified by changes in the expression level of NF-κB downstream target genes. The qPCR results showed that the down-regulation of *Il-1β*, *Il-18*, *Tnf-α*, *Il-6* and *Nlrp3* mRNA expression by hesperetin in LPS-treated RAW 264.7 cells were reversed by EX-527 and NAM (Fig. 3E). These results indicate that hesperetin-mediated inhibition of inflammatory response is dependent on SIRT1.

3.4. Hesperetin increases SIRT1 expression through AMPK/CREB pathway

SIRT1 expression changes in various physiological conditions, which is induced during low energy status, and repressed during energy excess states [29]. Cyclic AMP response-element-binding protein (CREB) is the most well-studied transcription factor modulating Sirt1 expression, which increases SIRT1 level upon nutrient starvation [30]. CREB is activated by phosphorylation in response to various signals. As shown in Fig. 4A, hesperetin increased the phosphorylation of CREB after LPS stimulation in RAW264.7. 666-15, a potent and selective CREB inhibitor [31], counteracted hesperetin-induced activation of CREB and upregulation of SIRT1 in both mRNA and protein levels (Fig. 4A and B), suggesting CREB was involved in the modulation of *Sirt1* expression by hesperetin.

Flavonoids have always been considered as natural AMP-activated protein kinase (AMPK) activators [32]. Indeed, we confirmed that hesperetin was an effective AMPK activator, which promoted the

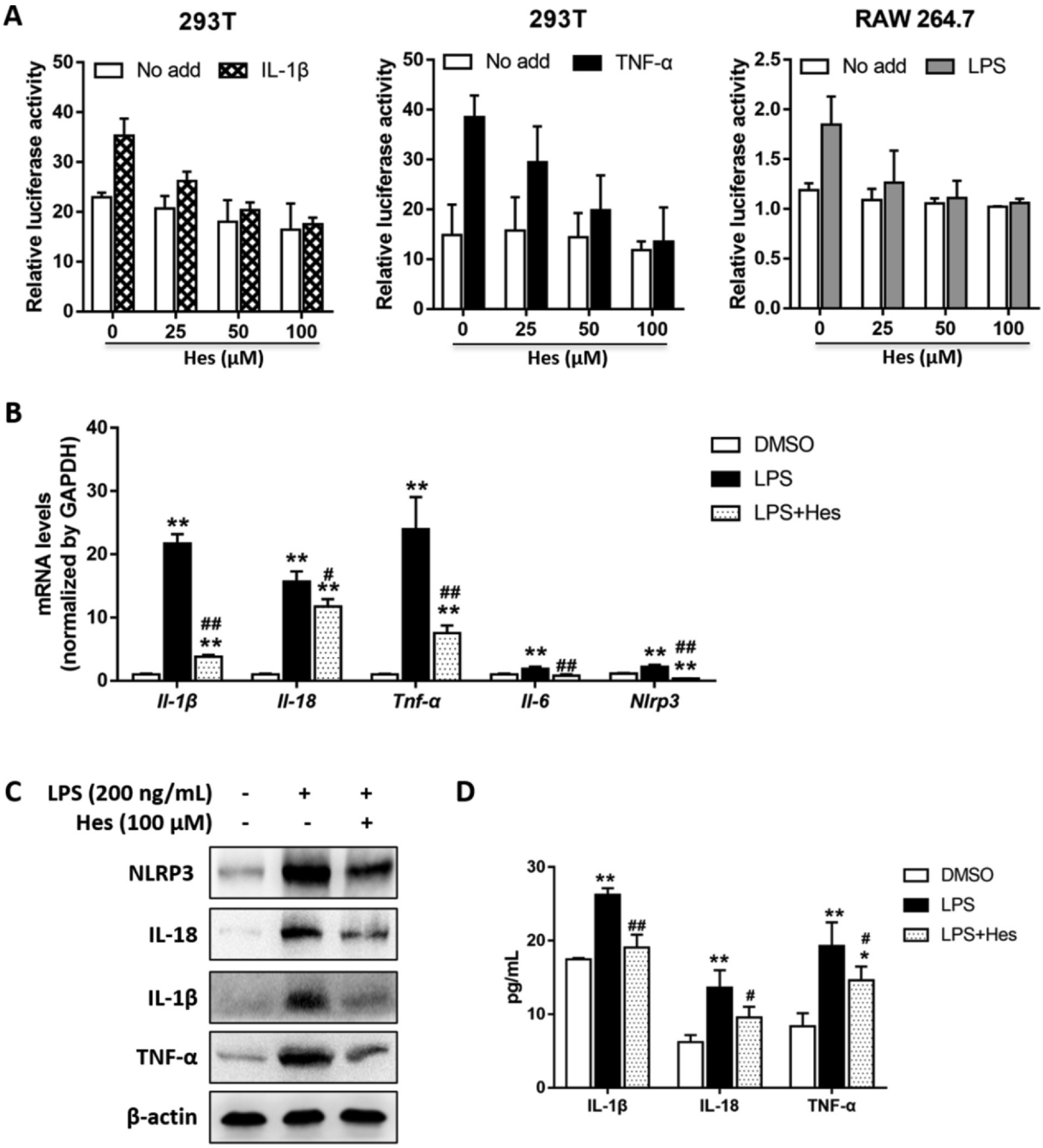

Fig. 2. Inhibitory effect of hesperetin on NF-κB activation and inflammatory responses *in vitro*. (A) 293T and RAW 264.7cells were transiently transfected with the pNFκB-luc. 293T cells were left untreated (No Add) or treated overnight with Hes (25, 50, 100 μM), Hes (25, 50, 100 μM) + IL-1β (50 ng/mL) or TNF-α (20 ng/mL). RAW 264.7 cells were left untreated (No Add) or treated overnight with Hes (25, 50, 100 μM), Hes (25, 50, 100 μM) + LPS (200 ng/mL). Values were expressed as mean ± SD (n = 6). (B) RAW 264.7 cells were treated with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (100 μM) overnight. The mRNA expression of *Il-1β*, *Il-18*, *Tnf-α*, *Il-6* and *Nlrp3* were determined by RT-PCR. Values were expressed as mean ± SD (n = 3). (C) The amount of NLRP3, IL-18, IL-1β, TNF-α and β-actin were measured by western blotting. (D) The IL-18, IL-1β and TNF-α concentrations in cell supernatant were determined by ELISA. Values were expressed as mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, versus DMSO group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus LPS group.

phosphorylation of AMPK in a time- and dose-dependent manner (Fig. 4C). CREB is the downstream target of AMPK [33]. AMPK phosphorylates CREB and activates its transcriptional activity. Inhibition of AMPK activation by a selective inhibitor Compound C abolished hesperetin-induced phosphorylation of CREB (Fig. 4D). Meanwhile, it also diminished the upregulation of SIRT1 expression in both mRNA and protein levels by hesperetin treatment (Fig. 4D and E). These data suggested that hesperetin enhanced SIRT1 expression through AMPK/CREB pathway.

3.5. SIRT1 is required for the remission of LPS/D-GalN-induced hepatic inflammation in hesperetin-treated mice

To determine the role of SIRT1 in hesperetin derived alleviation of inflammation *in vivo*, we examined the effect of hesperetin on LPS/D-GalN-induced hepatic inflammation with or without SIRT1 inhibitor. Consistent with previous study [1], the administration of hesperetin protected liver from inflammatory injury, reflecting by reduced levels of liver injury markers such as ALT and AST in LPS/D-GalN-induced mice (Fig. 5A). The serum pro-inflammatory cytokines including IL-1β

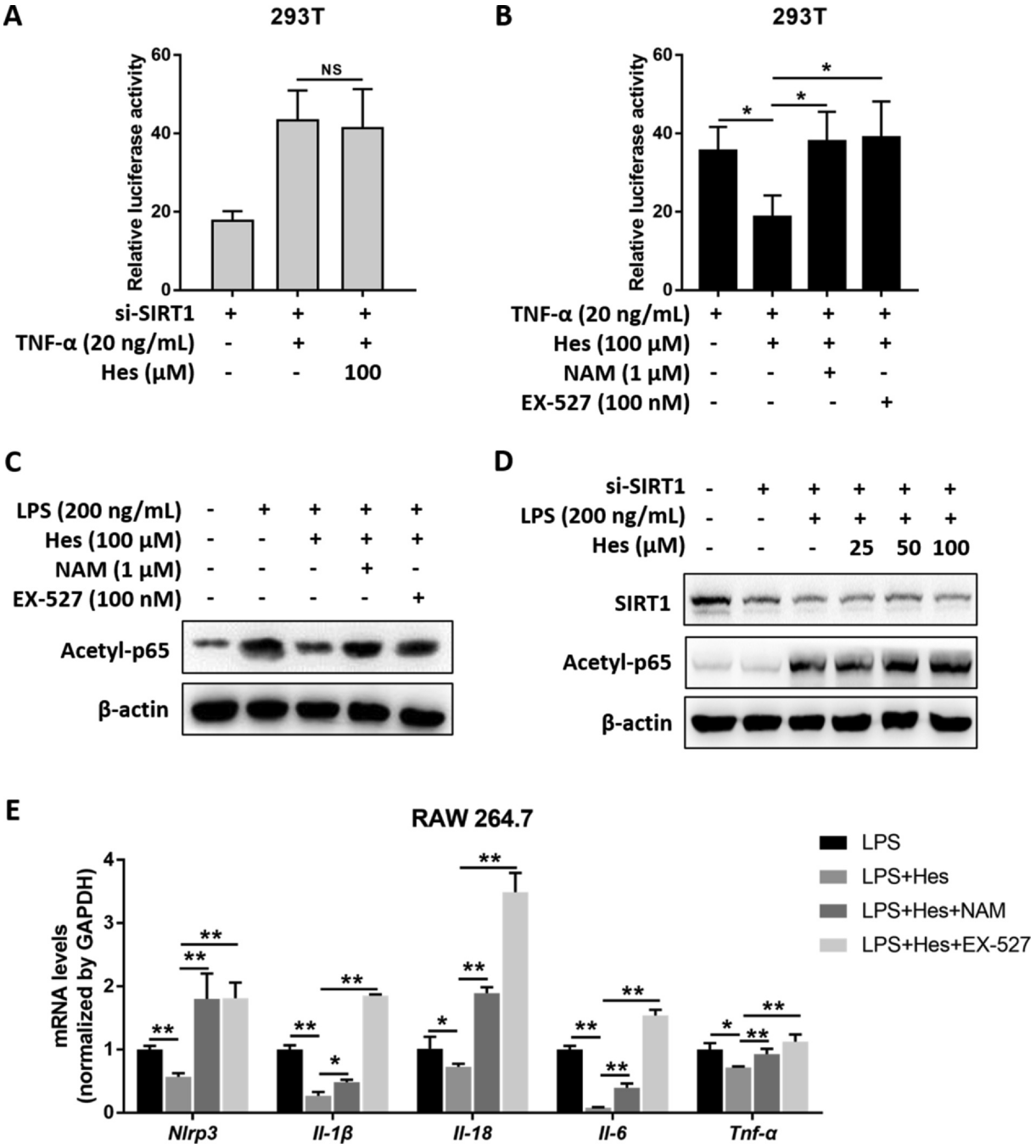

Fig. 3. Role of SIRT1 in hesperetin inhibition of NF-κB transcriptional activity *in vitro*. 293T were transiently transfected with the pNFκB-luc. (A) 293T cells were transiently transfected with siRNA-Sirt1 (siSIRT1). Then the cells were left treated overnight with DMSO, TNF-α (20 ng/mL), TNF-α (20 ng/mL) + Hes (100 μM). (B) 293T cells were treated overnight with TNF-α (20 ng/mL), TNF-α (20 ng/mL) + Hes (100 μM), TNF-α (20 ng/mL) + Hes (100 μM) + NAM (1 μM) and TNF-α (20 ng/mL) + Hes (100 μM) + EX-527 (100 nM). Values were expressed as mean ± SD (n = 6). (C) RAW 264.7 cells were treated with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (100 μM), LPS (200 ng/mL) + Hes (100 μM) + NAM (1 μM) and LPS (200 ng/mL) + Hes (100 μM) + EX-527 (100 nM) overnight. The amount of acetyl-p65 and β-actin were measured by western blotting. (D) RAW 264.7 cells were transiently transfected with siRNA-Sirt1 (siSIRT1). Then the cells were left treated overnight with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (25, 50 or 100 μM). The amount of SIRT1, acetyl-p65 and β-actin were measured by western blotting. (E) RAW 264.7 cells were treated with LPS (200 ng/mL), LPS (200 ng/mL) + Hes (100 μM), LPS (200 ng/mL) + Hes (100 μM) + NAM (1 μM) and LPS (200 ng/mL) + Hes (100 μM) + EX-527 (100 nM) overnight. The mRNA expression of *Il-1β*, *Il-18*, *Tnf-α*, *Il-6* and *Nlrp3* were determined by RT-PCR. Values were expressed as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$; NS, not statistically significant.

and TNF-α were also lowered by hesperetin (Fig. 5B). These effects were largely attenuated by co-administration of SIRT1 inhibitor EX-527 (Fig. 5A and B). The H&E and F4/80 immunohistochemical staining of liver tissue section revealed that the alleviation of LPS/D-GalN-induced liver damage and inflammatory cell infiltration by hesperetin was counteracted by SIRT1 inhibition (Fig. 5C). The inhibition of SIRT1 by EX-527 also invalidated the inhibitory effect of hesperetin on the mRNA expression of *Il-1β*, *Il-18*, *Tnf-α* and *Nlrp3* upon LPS/D-GalN challenge (Fig. 5D).

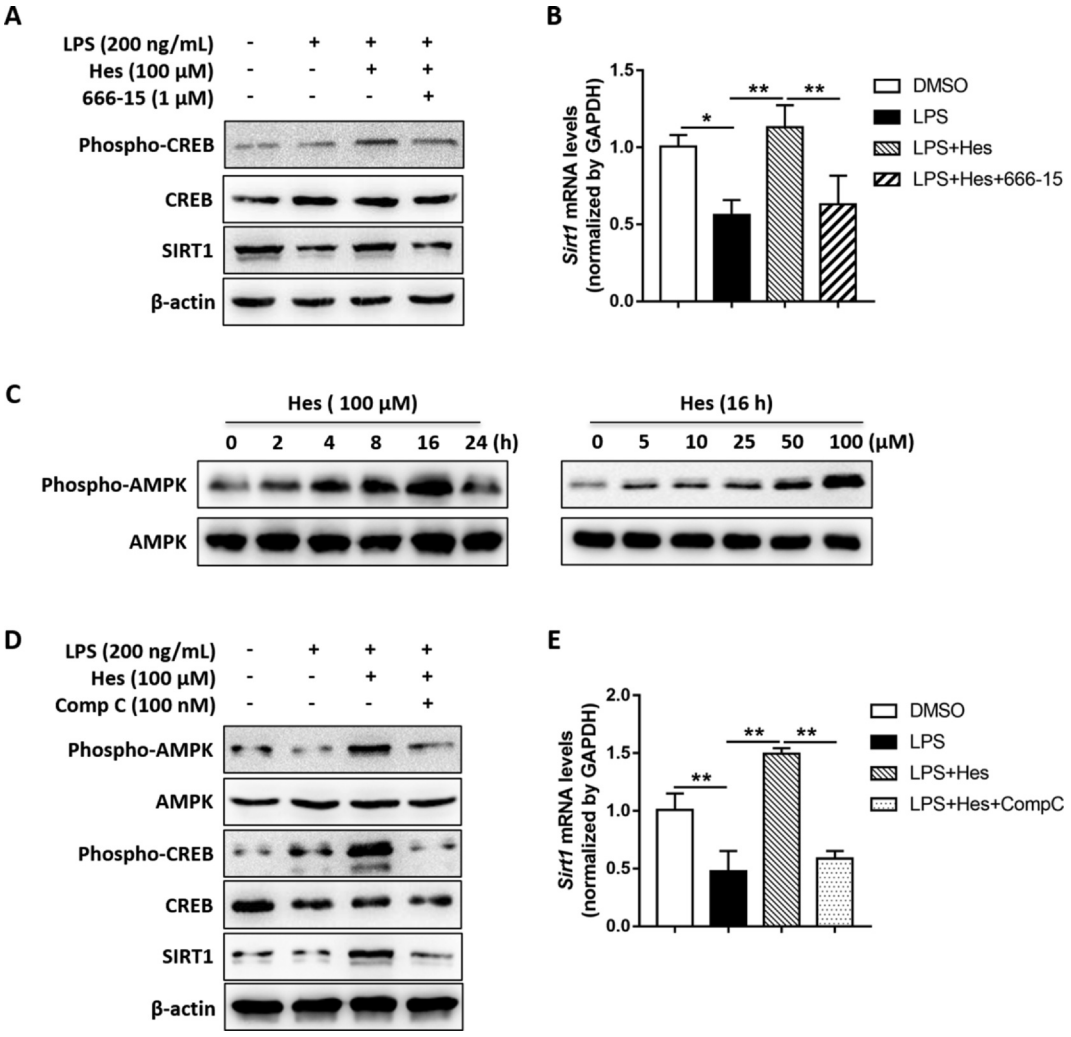

Fig. 4. Hesperetin increases SIRT1 expression through inducing AMPK activity and CREB transcriptional activity in RAW 264.7 cells. (A) RAW 264.7 cells were treated with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (100 μM), LPS (200 ng/mL) + Hes (100 μM) + 666-15 (1 μM) for 16 h. The amount of phospho-CREB, CREB, SIRT1 and β-actin was measured by western blotting. (B) The mRNA expression of *Sirt1* were determined by RT-PCR. Values were expressed as mean ± SD (n = 3). (C) RAW 264.7 cells were treated with Hes (100 μM) at different time (0, 2, 4, 8, 16, 24 h) or treated with various concentrations of Hes (0, 5, 10, 25, 50, 100 μM) for 16 h. The amount of phospho-AMPK and AMPK was measured by western blotting. (D) RAW 264.7 cells were treated with DMSO, LPS (200 ng/mL), LPS (200 ng/mL) + Hes (100 μM), LPS (200 ng/mL) + Hes (100 μM) + Compound C (CompC, 100 nM) for 16 h. The amount of phospho-AMPK, AMPK, phospho-CREB, CREB, SIRT1 and β-actin was measured by western blotting. (E) The mRNA expression of *Sirt1* were determined by RT-PCR. Values were expressed as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$.

4. Discussion

Flavonoids has attracted increasing interest recently, especially for their activation of SIRT1 in inflammation and related diseases. However, the mechanism by which flavonoids activate SIRT1, and whether it is important to their inhibitory effect on inflammation remains largely unknown. Here, we report that SIRT1 is required for the inhibitory effect of hesperetin on inflammation. Hesperetin suppresses the acetylation of RelA/p65 to reduce NF-κB activity by inducing SIRT1 expression. Mechanistically, hesperetin increases SIRT1 expression through AMPK/CREB pathway. Furthermore, we demonstrate that the protective effect of hesperetin against LPS/D-GalN-induced hepatitis in mice is dependent on SIRT1 (Fig. 6).

It has been shown that the complementary and alternative medical therapies, together with dietary supplements is very beneficial to relieve hepatitis [34]. Hesperetin, as a common flavonoid, has been proven to have a significant effect on acute inflammatory diseases [35,36]. NF-κB is the major regulator for LPS induced hepatitis *in vivo* and activated inflammatory response of RAW264.7 cells *in vitro* [5,37]. Hesperetin and its glycosides can alleviate LPS-induced hepatitis [1,2,38]. Of importance, this effect is related to the effect of hesperetin on inhibiting NF-κB activity. We confirmed that hesperetin is an effective inhibitor for NF-κB transcriptional activity *in vitro* by luciferase reporter assay. However, it remains elusive how hesperetin inhibits NF-κB activity. In current study, we found that SIRT1 was necessary for hesperetin to inhibit NF-κB activity. Hesperetin modulates NF-κB transcriptional activity by promoting the expression and activity of SIRT1 and deacetylating RelA/p65 on lysine residue K310. Meanwhile, hesperetin relieves LPS-induced hepatitis, mainly through SIRT1 as well.

In recent years, it has been reported that many small molecular natural compounds, such as resveratrol, quercetin, etc., can activate SIRT1 [23]. Resveratrol is the most reported and well-defined natural small molecular SIRT1 activator [23]. SIRT1 activity is modulated at several levels, including transcriptional control, post-translational modifications, the interaction with other proteins, and substrate abundance [30]. Our study shows that hesperetin promotes the expression of intracellular SIRT1, but has no direct effect on SIRT1 activity in cell-free system. In terms of transcription regulation, CREB and carbohydrate response element binding protein (ChREBP) have been

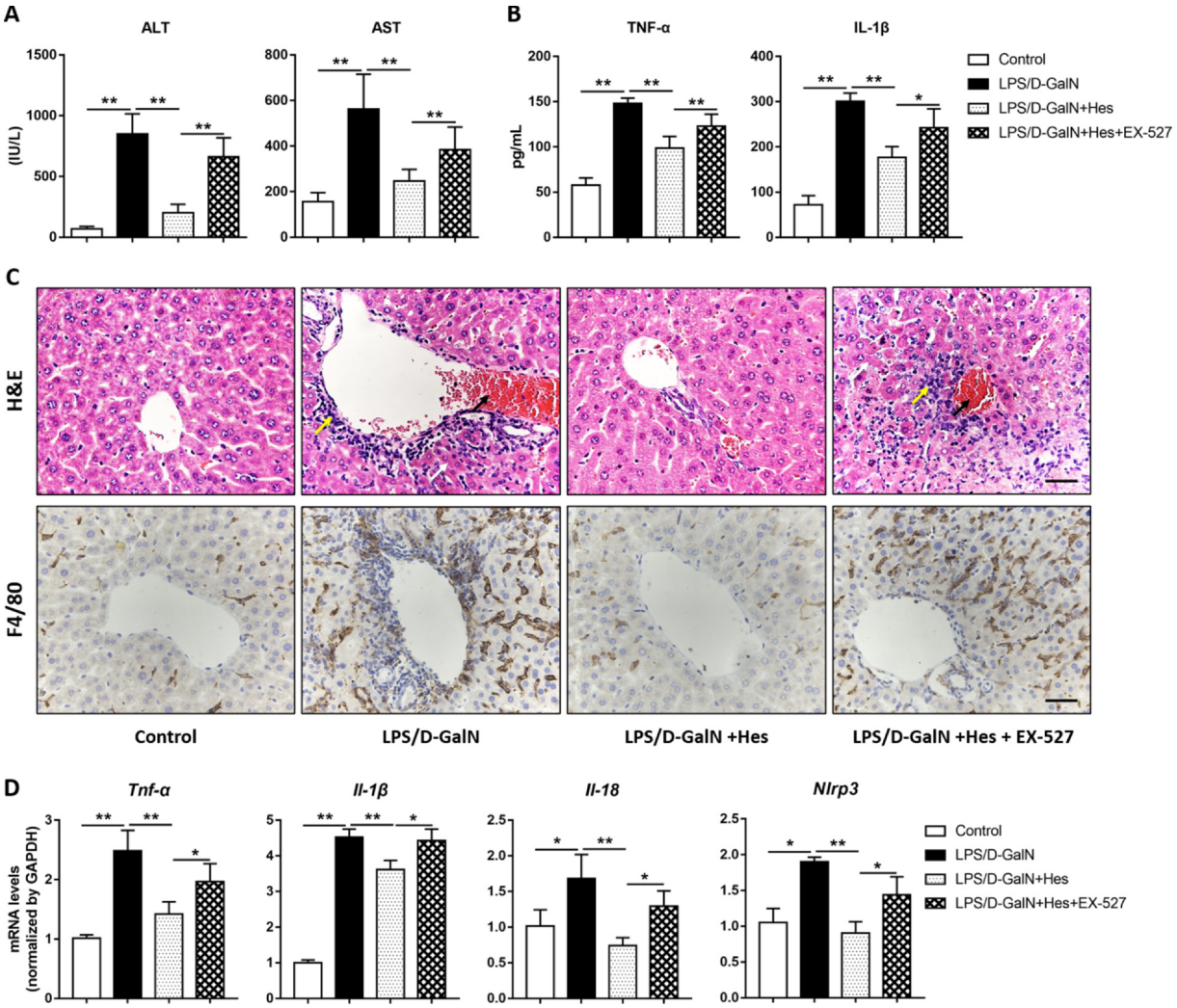

Fig. 5. Role of SIRT1 in remission of LPS/D-GalN induced hepatic inflammation in hesperetin-treated mice. Male BALB/c mice were injected single i.p. with LPS (6 μg/kg) and D-GalN 800 mg/kg, after oral Hes (50 mg/kg/day) alone or oral Hes (50 mg/kg/day) + i.p. EX-527 (1 mg/mL/day) for 5 days. (A) Serum ALT and AST levels in each group. (B) Serum TNF-α and IL-1β levels in each group. Values were expressed as mean ± SD (n = 10). (C) The representative images of H&E and F4/80 immunohistochemical staining in livers from each group (Magnification 400 ×). Black arrow denotes central venous hyperemia; yellow arrow denotes inflammatory cell infiltration; white arrow denotes the loss of hepatic architecture-vacuolation. Scale bar = 300 μm. (D) The mRNA expression of *Il-1β*, *Il-18*, *Tnf-α* and *Nlrp3* were determined by RT-PCR. Values were expressed as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$.

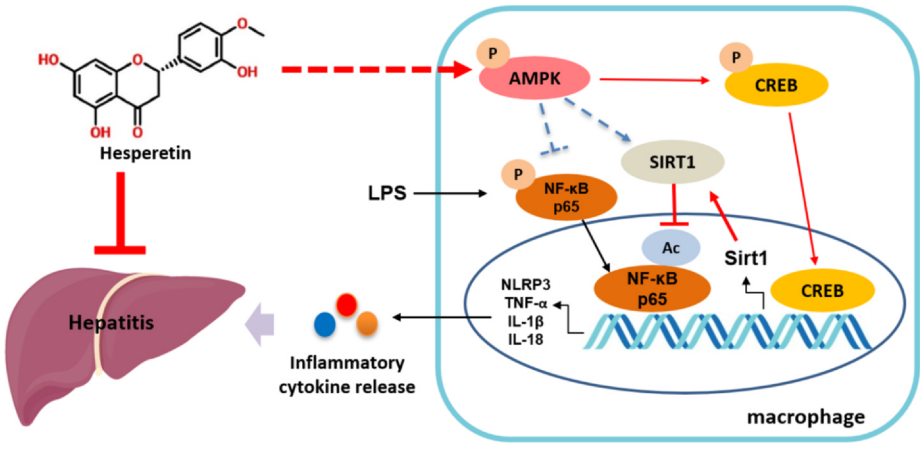

Fig. 6. Schematic diagram of the mechanism. Hesperetin inhibits hepatic inflammation by promoting SIRT1 expression. In depth, hesperetin activate AMPK to phosphorylate CREB, which regulates *Sirt1* transcription. Sirt1 affects NF-κB activity by inhibiting its acetylation, resulting in reduced production of inflammatory factors. AMPK can affect SIRT1 activity through two approaches. On one hand, AMPK regulates SIRT1 activity indirectly by modulating the ratio of NAD^+ and NADH. On the other hand, AMPK controls SIRT1 expression by phosphorylating CREB and promoting its nuclear translocation and transcriptional activity. The NF-κB subunits are not direct phosphorylation targets of AMPK, while the inhibition of NF-κB signaling is mediated by several downstream targets of AMPK, e.g., SIRT1, PGC-1α, p53, and FoxO factors.

proved to be crucial transcription factors regulating *SIRT1* expression [30]. CREB can promote SIRT1 expression by binding to the CREB binding site on the SIRT1 promoter. Conversely, the binding of ChREBP on SIRT1 promoter inhibits its expression. In this study, we find that hesperetin promotes SIRT1 expression by increasing CREB transcriptional activity.

AMPK, as a central intracellular energy sensor, plays a key role in maintaining cellular metabolic homeostasis [39]. AMPK regulates bunch of gene transcription via modification of a variety of transcription factors closely associated with cell metabolism [40]. AMPK promotes the phosphorylation of CREB and ChREBP, enhancing or inhibiting their transcriptional activity respectively [33]. Flavonoids have always been considered as natural AMPK agonists, and many of their pharmacological activities are bound to the activation of AMPK [41]. In current study, our findings indicate that hesperetin induces SIRT1 expression in inflammatory cells mainly through activation of AMPK. Certainly, AMPK can also elevate SIRT1 activity by enhancing the mitochondrial metabolism and thereafter increasing the abundance of intracellular NAD^+ [42]. Our study does not rule out this possibility. However, we can see that after hesperetin treatment, SIRT1 expression increased significantly. Obviously, this increase will also have a significant effect on the total activity of SIRT1 in the cells. Therefore, we can infer reasonably that hesperetin may increase these two variables, namely expression and activity of SIRT1, through AMPK to enhance the deacetylase activity of SIRT1 on NF-κB. Another evidence supporting SIRT1′s dominant role in inflammatory response in macrophage is that the addition of SIRT1 inhibitors (especially EX-527) not only completely counteracted the inhibitory effect of hesperetin on inflammatory gene expression, but also further promoted their expression. The increased part of inflammatory gene expression should be interpreted as the inflammatory effect which was caused by EX-527 induced inhibition of SIRT1 on its basal expression level.

In conclusion, our findings indicate that the regulation of SIRT1 is one of the most important mechanisms of hesperetin's anti-inflammatory activity. SIRT1 is a critical metabolic regulator. The regulatory effect of hesperetin on SIRT1 is not only effective in the treatment of acute hepatitis, but it may have a greater potential application in chronic metabolic diseases and accompanying inflammatory conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.107036.

References

[1] X. Bai, P. Yang, Q. Zhou, B. Cai, M. Buist-Homan, H.e. Cheng, J. Jiang, D. Shen, L. Li, X. Luo, K.N. Faber, H. Moshage, G. Shi, The protective effect of the natural compound hesperetin against fulminant hepatitis in vivo and in vitro: hesperetin protects against fulminant hepatitis, British J. Pharmacol. 174 (1) (2017) 41–56.

[2] Y. He, Z. Xia, D. Yu, J. Wang, L. Jin, D. Huang, X. Ye, X. Li, B. Zhang, Hepatoprotective effects and structure-activity relationship of five flavonoids against lipopolysaccharide/d-galactosamine induced acute liver failure in mice, Int. Immunopharmacol. 68 (2019) 171–178.

[3] S. Yang, et al., Mangiferin attenuates LPS/D-GalN-induced acute liver injury by promoting HO-1 in kupffer cells, Front Immunol. 11 (2020) 285, https://doi.org/10.3389/fimmu.2020.00285.

[4] H. Tsutsui, S. Nishiguchi, Importance of Kupffer cells in the development of acute liver injuries in mice, Int. J. Mol. Sci. 15 (2014) 7711–7730, https://doi.org/10.3390/ijms15057711.

[5] M. Hu, Q. Luo, G. Alitongbieke, S. Chong, C. Xu, L. Xie, X. Chen, D. Zhang, Y. Zhou, Z. Wang, X. Ye, L. Cai, F. Zhang, H. Chen, F. Jiang, H. Fang, S. Yang, J. Liu, M.T. Diaz-Meco, Y. Su, H.u. Zhou, J. Moscat, X. Lin, X.-K. Zhang, Celastrol-induced nur77 interaction with TRAF2 alleviates inflammation by promoting mitochondrial ubiquitination and autophagy, Mol. Cell 66 (1) (2017) 141–153.e6.

[6] H. Lv, et al., Asiatic acid exhibits anti-inflammatory and antioxidant activities against lipopolysaccharide and d-galactosamine-induced fulminant hepatic failure, Front Immunol. 8 (2017) 785, https://doi.org/10.3389/fimmu.2017.00785.

[7] B. Huang, X.D. Yang, A. Lamb, L.F. Chen, Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway, Cell. Signal. 22 (2010) 1282–1290, https://doi.org/10.1016/j.cellsig.2010.03.017.

[8] J. Wu, et al., Loss of PDK4 switches the hepatic NF-kappaB/TNF pathway from pro-survival to pro-apoptosis, Hepatology 68 (2018) 1111–1124, https://doi.org/10.1002/hep.29902.

[9] E.-K. Jo, J.K. Kim, D.-M. Shin, C. Sasakawa, Molecular mechanisms regulating NLRP3 inflammasome activation, Cell. Mol. Immunol. 13 (2) (2016) 148–159.

[10] H. Yang, et al., SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF-kappaB activity, PLoS ONE 7 (2012) e46364, https://doi.org/10.1371/journal.pone.0046364.

[11] T.T. Schug, Q. Xu, H. Gao, A. Peres-da-Silva, D.W. Draper, M.B. Fessler, A. Purushotham, X. Li, Myeloid deletion of SIRT1 induces inflammatory signaling in response to environmental stress, MCB 30 (19) (2010) 4712–4721.

[12] V.T. Vachharajani, T. Liu, X. Wang, J.J. Hoth, B.K. Yoza, C.E. McCall, Sirtuins link inflammation and metabolism, J. Immunol. Res. 2016 (2016) 1–10.

[13] P.T. Pfluger, D. Herranz, S. Velasco-Miguel, M. Serrano, M.H. Tschop, Sirt1 protects against high-fat diet-induced metabolic damage, Proc. Natl. Acad. Sci. 105 (28) (2008) 9793–9798.

[14] A. Purushotham, T.T. Schug, Q. Xu, S. Surapureddi, X. Guo, X. Li, Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation, Cell Metab. 9 (4) (2009) 327–338.

[15] C. Busch, M. Burkard, C. Leischner, U.M. Lauer, J. Frank, S. Venturelli, Epigenetic activities of flavonoids in the prevention and treatment of cancer, Clin. Epigenet. 7 (1) (2015), https://doi.org/10.1186/s13148-015-0095-z.

[16] M. Iranshahi, R. Rezaee, H. Parhiz, A. Roohbakhsh, F. Soltani, Protective effects of flavonoids against microbes and toxins: the cases of hesperidin and hesperetin, Life Sci. 137 (2015) 125–132.

[17] H. Parhiz, A. Roohbakhsh, F. Soltani, R. Rezaee, M. Iranshahi, Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: an updated review of their molecular mechanisms and experimental models: hesperidin and hesperetin as antioxidant and anti-inflammatory agents, Phytother. Res. 29 (3) (2015) 323–331.

[18] G. Li, M.-J. Chen, C. Wang, H. Nie, W.-J. Huang, T.-D. Yuan, T. Sun, K.-G. Shu, C.-f. Wang, Q. Gong, S.-Q. Tang, Protective effects of hesperidin on concanavalin A-induced hepatic injury in mice, Int. Immunopharmacol. 21 (2) (2014) 406–411.

[19] G. Kaur, N. Tirkey, K. Chopra, Beneficial effect of hesperidin on lipopolysaccharide-induced hepatotoxicity, Toxicology 226 (2-3) (2006) 152–160.

[20] Y.-F. Bai, S.-W. Wang, X.-X. Wang, Y.-Y. Weng, X.-y. Fan, H. Sheng, X.-T. Zhu, L.-J. Lou, F. Zhang, The flavonoid-rich Quzhou Fructus Aurantii extract modulates gut microbiota and prevents obesity in high-fat diet-fed mice, Nutr. Diab. 9 (1) (2019), https://doi.org/10.1038/s41387-019-0097-6.

[21] S.-W. Wang, Y.-F. Bai, Y.-Y. Weng, X.-y. Fan, H. Huang, F. Zheng, Y.i. Xu, F. Zhang, Cinobufacini ameliorates dextran sulfate sodium–induced colitis in mice through inhibiting M1 macrophage polarization, J. Pharmacol. Exp. Ther. 368 (3) (2019) 391–400.

[22] S.-W. Wang, Y.i. Xu, Y.-Y. Weng, X.-y. Fan, Y.-F. Bai, X.-Y. Zheng, L.-J. Lou, F. Zhang, Astilbin ameliorates cisplatin-induced nephrotoxicity through reducing oxidative stress and inflammation, Food Chem. Toxicol. 114 (2018) 227–236.

[23] K.T. Howitz, K.J. Bitterman, H.Y. Cohen, D.W. Lamming, S. Lavu, J.G. Wood, R.E. Zipkin, P. Chung, A. Kisielewski, L.-L. Zhang, B. Scherer, D.A. Sinclair, Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan, Nature 425 (6954) (2003) 191–196.

[24] N. Price, A. Gomes, A.Y. Ling, F. Duarte, A. Martin-Montalvo, B. North, B. Agarwal, L. Ye, G. Ramadori, J. Teodoro, B. Hubbard, A. Varela, J. Davis, B. Varamini, A. Hafner, R. Moaddel, A. Rolo, R. Coppari, C. Palmeira, R. de Cabo, J. Baur, D. Sinclair, SIRT1 Is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function, Cell Metab. 15 (5) (2012) 675–690.

[25] Q. Zhao, Z. Tian, G. Zhou, Q. Niu, J. Chen, P. Li, L. Dong, T. Xia, S. Zhang, A. Wang, SIRT1-dependent mitochondrial biogenesis supports therapeutic effects of resveratrol against neurodevelopment damage by fluoride, Theranostics 10 (11) (2020) 4822–4838.

[26] T. Liu, L. Zhang, D. Joo, S.C. Sun, NF-kappaB signaling in inflammation, Signal Transduct. Target Ther. 2 (2017), https://doi.org/10.1038/sigtrans.2017.23.

[27] F. Yeung, et al., Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase, EMBO J. 23 (2004) 2369–2380, https://doi.org/10.1038/sj.emboj.7600244.

[28] L.F. Chen, Y. Mu, W.C. Greene, Acetylation of RelA at discrete sites regulates

distinct nuclear functions of NF-kappaB, EMBO J. 21 (2002) 6539–6548, https://doi.org/10.1093/emboj/cdf660.
[29] J.R. Revollo, X. Li, The ways and means that fine tune Sirt1 activity, Trends Biochem. Sci. 38 (3) (2013) 160–167.
[30] L.G. Noriega, J.N. Feige, C. Canto, H. Yamamoto, J. Yu, M.A. Herman, C. Mataki, B.B. Kahn, J. Auwerx, CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability, EMBO Rep. 12 (10) (2011) 1069–1076.
[31] X. Guan, et al., Cerebrolysin ameliorates focal cerebral ischemia injury through neuroinflammatory inhibition via CREB/PGC-1alpha pathway, Front. Pharmacol. 10 (2019) 1245, https://doi.org/10.3389/fphar.2019.01245.
[32] C.L. Lyons, H.M. Roche, Nutritional modulation of AMPK-impact upon metabolic-inflammation, Int. J. Mol. Sci. 19 (2018), https://doi.org/10.3390/ijms19103092.
[33] D.M. Thomson, S.T. Herway, N. Fillmore, H. Kim, J.D. Brown, J.R. Barrow, W.W. Winder, AMP-activated protein kinase phosphorylates transcription factors of the CREB family, J. Appl. Physiol. 104 (2) (2008) 429–438.
[34] L. Zhang, D. Schuppan, Traditional Chinese Medicine (TCM) for fibrotic liver disease: hope and hype, J. Hepatol. 61 (1) (2014) 166–168.
[35] T. Muhammad, M. Ikram, R. Ullah, S.U. Rehman, M.O. Kim, Hesperetin, a citrus flavonoid, attenuates LPS-induced neuroinflammation, apoptosis and memory impairments by modulating TLR4/NF-kappaB signaling, Nutrients 11 (2019), https://doi.org/10.3390/nu11030648.
[36] J. Ye, M. Guan, Y. Lu, D. Zhang, C. Li, Y. Li, C. Zhou, Protective effects of hesperetin on lipopolysaccharide-induced acute lung injury by targeting MD2, Eur. J. Pharmacol. 852 (2019) 151–158.
[37] H. Ren, et al., Hesperetin suppresses inflammatory responses in lipopolysaccharide-induced RAW 264.7 cells via the inhibition of NF-kappaB and activation of Nrf2/HO-1 pathways, Inflammation 39 (2016) 964–973, https://doi.org/10.1007/s10753-016-0311-9.
[38] X. Chen, et al., Hesperetin derivative-14 alleviates inflammation by activating PPAR-gamma in mice with CCl4-induced acute liver injury and LPS-treated RAW264.7 cells, Toxicol. Lett. 274 (2017) 51–63, https://doi.org/10.1016/j.toxlet.2017.04.008.
[39] D. Garcia, R.J. Shaw, AMPK: mechanisms of cellular energy sensing and restoration of metabolic balance, Mol. Cell 66 (6) (2017) 789–800.
[40] S. Herzig, R.J. Shaw, AMPK: guardian of metabolism and mitochondrial homeostasis, Nat. Rev. Mol. Cell Biol. 19 (2) (2018) 121–135.
[41] D. Gentile, M. Fornai, C. Pellegrini, R. Colucci, C. Blandizzi, L. Antonioli, Dietary flavonoids as a potential intervention to improve redox balance in obesity and related co-morbidities: a review, Nutr. Res. Rev. 31 (2) (2018) 239–247.
[42] C. Cantó, Z. Gerhart-Hines, J.N. Feige, M. Lagouge, L. Noriega, J.C. Milne, P.J. Elliott, P. Puigserver, J. Auwerx, AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity, Nature 458 (7241) (2009) 1056–1060.