

SIRT1 upregulation protects against liver injury induced by a HFD through inhibiting CD36 and the NF- κ B pathway in mouse kupffer cells

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Abstract. Sirtuin 1 (SIRT1) is an NAD(+)-dependent deacetylase, and a critical regulator in various metabolic processes, such as non-alcoholic fatty liver disease (NAFLD). The present study aimed to investigate whether activating SIRT1 could modulate the CD36 and nuclear factor (NF)- κ B pathways to protect against liver injury induced by a high-fat diet (HFD) in mice. A mouse NAFLD model was established by administration of a HFD for 8 weeks. During the last 4 weeks, SIRT1720, a specific SIRT1 activator, was added daily to the HFD feed. The hepatic morphological structure was observed using hematoxylin and eosin staining, and the ultrastructures in the liver tissue were observed using transmission electron microscopy. Protein expression of SIRT1, CD36 and P65 in liver tissues was detected by immunohistochemistry. Kupffer cells (KCs) from the livers of the mouse models were isolated to determine the mRNA and protein expression of SIRT1, CD36 and P65. SIRT1 activation attenuated the HFD-induced liver injury and significantly reduced the body weight and the levels of alanine transaminase, aspartate aminotransferase, triglyceride, tumor necrosis factor- α and interleukin-6. We observed an increased expression of SIRT1 in the liver tissues from the HFD+SIRT1720 group compared with the HFD

group. Simultaneously, the expression of CD36 and P65 in the liver tissues was downregulated in the HFD+SIRT1720 group. The mRNA and protein expression of SIRT1 was elevated in the HFD+SIRT1720 group, whereas the mRNA and protein expression of CD36 and P65 in KCs was significantly decreased in the HFD+SIRT1720 group. The present study demonstrated that SIRT1 activation attenuated HFD-induced liver steatosis and inflammation by inhibiting CD36 expression and the NF- κ B signaling pathway.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common hepatic disorder worldwide, and will be the most common reason for liver transplantation by 2020 (1). NAFLD begins with simple hepatic steatosis that may progress to non-alcoholic steatohepatitis (NASH), potentially leading to liver cirrhosis and hepatic cell carcinoma (1). Excessive fat accumulation is the initiating factor in the development of NAFLD, which is associated with diabetes, obesity and hyperlipidemia. Immune and inflammatory responses are secondary processes in the development of NAFLD (2). However, the specific pathogenesis of NAFLD remains unclear.

Fatty acid translocase (known as FAT or CD36), which may be induced in obesity, is a fatty acid receptor that plays an essential role in modulating lipid and glucose use; the upregulation of CD36 is associated with NASH (3). The mRNA expression of nuclear factor (NF)- κ B, a key regulator involved in the induction of inflammatory mediators, can be significantly influenced by the manipulation of CD36 expression (4). Nevertheless, the results of studies regarding the function of CD36 in NAFLD have been conflicting, and the mechanisms are not well understood.

Sirtuin 1 (SIRT1) is an NAD(+)-dependent deacetylase, and a critical regulator in various metabolic processes. SIRT1 overexpression reduces the level of oxygen consumption in NAFLD. It has been reported that activation of SIRT1 by SIRT1720 can alleviate high-fat diet (HFD)-induced liver

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steatosis (5). SIRT1 can interact with RelA/P65, subunits of NF- κ B, directly, and inhibit NF- κ B by deacetylating RelA/P65 at lysine 310, leading to the I κ B α -dependent nuclear export of NF- κ B (6).

Hepatic macrophages, known as Kupffer cells (KCs), account for 80-90% of the inherent macrophages. KCs release various inflammatory cytokines and play a critical role in the pathogenesis of liver inflammation disease (7). It has been reported that SIRT1 can control hepatic CD36 expression and triglyceride accumulation. However, other studies have suggested that SIRT1 increased the expression of proteins in the CD36 metabolic pathway. Therefore, whether SIRT1 could control the expression of CD36 in the KCs of free fatty acid (FFA)-reduced NASH liver remains unknown. In the present study, we investigated the effects of SIRT1 on the expression of CD36 and NF- κ B, and the protective effect of SIRT1 against NAFLD.

Materials and methods

Animals and diets. Male C57BL/6 mice (weighing 18-22 g) were provided by the laboratory animal research center of Chongqing Medical University (Chongqing, China). All the mice were housed in a ventilated and temperature-controlled specific pathogen-free room. Water and food were accessed *ad libitum*. Mice were randomized into three groups, with 10 mice in each group: Normal diet (ND) group, mice fed with an ND for 8 weeks; HFD group, mice fed with a HFD (cat. no. D12492; Research Diets, Inc., New Brunswick, NJ, USA) for 8 weeks; and HFD+SRT1720 group, mice fed with a HFD for 8 weeks, and SRT1720 (30 mg/kg/d) in the last 4 weeks. Ether inhalation was used for anesthetizing mice prior to the experiment. Sections of the liver tissues were formalin-fixed and embedded in paraffin for analysis by hematoxylin and eosin (H&E) staining and immunohistochemistry. Mouse serum was stored at -80°C for ELISA. This study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Committee for Animal Subjects of Chongqing Medical University. Animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture. Primary KCs were isolated from mouse livers as previously described (8) for use in qPCR and western blot analyses. Cells were cultured in DMEM medium (high glucose) supplemented with 10% fetal bovine serum (both Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 100 nM penicillin/streptomycin.

H&E staining and immunohistochemistry. The paraffin embedded tissues were sectioned and stained with H&E, and immunohistochemically stained with specific antibodies against SIRT1, CD36 and P65, as follows: The fixed liver samples were cut into 5- μ m sections, which were treated with 3% H₂O₂ and blocked with 3% normal goat serum. Then, the sections were incubated with SIRT1, CD36 and P65 (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) rabbit primary antibodies overnight at 4°C. A biotinylated secondary antibody was detected by liquid aminoethylcarbazole or

diaminobenzidine, and hematoxylin was used for counterstaining. The presence of brown particles in cells when observed under an optical microscope was considered positive staining.

Western blotting. Total protein was extracted with RIPA buffer, and the concentration of protein was determined using a Bradford Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equivalent amounts of protein were separated by electrophoresis on 10% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% bovine serum albumin for 1 h, and were incubated with primary antibodies against β -actin, SIRT1, CD36 and P65 (all Santa Cruz Biotechnology, Inc.) overnight at 4°C, followed by a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. Using an enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), protein bands on the membranes were exposed on autoradiographic film (Kodak, Rochester, NY, USA) and quantified by an image analysis system (Bio-Rad Gel Doc 2,000; Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative PCR analysis. Total RNA was extracted from KCs using the RNAiso Plus kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's instructions. The concentration of RNA was quantitated by measuring A₂₆₀/A₂₈₀ with a spectrophotometer. Equivalent amounts of RNA were reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.). cDNA was amplified by quantitative real-time PCR using the Bio-Rad iCycler (Bio-Rad Laboratories, Inc.) with SYBR-Green DNA fluorescent dye (Takara Bio, Inc.) as previously described (9). β -actin served as endogenous normalization control. Bio-Rad CFX Manager software (version 2.0; Bio-Rad Laboratories, Inc.) was utilized for data analysis. Primers were designed by Sangon Biotech Co., Ltd. (Shanghai, China), with sequences as follows: SIRT1 forward, 5'-CGGCTACCGAGGTCCATATAC-3' and reverse, 5'-ACAATCTGCCACAGCGTCAT-3'; TNF forward, 5'-CCTCACACTCACAAACCACCA-3' and reverse, 5'-ACAAGGTACAACCCATCGGC-3'; IL-6 forward, 5'-CCAGTTGCCTTCTTGGACT-3' and reverse, 5'-GGTCTGTTGGGAGTGGTATCC-3'; CD36 forward, 5'-TTGTGGAGCTCAAAGACCTG-3' and reverse, 5'-TGCAAGAAGCGGATGTAGTC-3'; β -actin forward, 5'-CATTGTGATGGACTCCGGAG-3' and reverse, 3'-ATATGATGACCTGGCCGTC-5'. The relative mRNA expression was calculated by the Vandesompele method.

ELISA. The serum levels of TNF- α and IL-6 were measured by specific ELISA kits (Abcam, Hong Kong, China) according to the manufacturer's protocols. Briefly, samples and standards were inserted into a pre-coated 96-well plate. The inflammatory mediators were bound to the wells by immobilized antibodies. A biotinylated antibody for TNF- α or IL-6 was added separately. HRP-conjugated streptavidin and TMB substrate solution were added successively after washing, and the addition of Stop Solution changed the color to yellow. Finally, the absorbance was measured at 450 nm, and the levels of TNF- α and IL-6 were calculated by the optical density and standard curve.

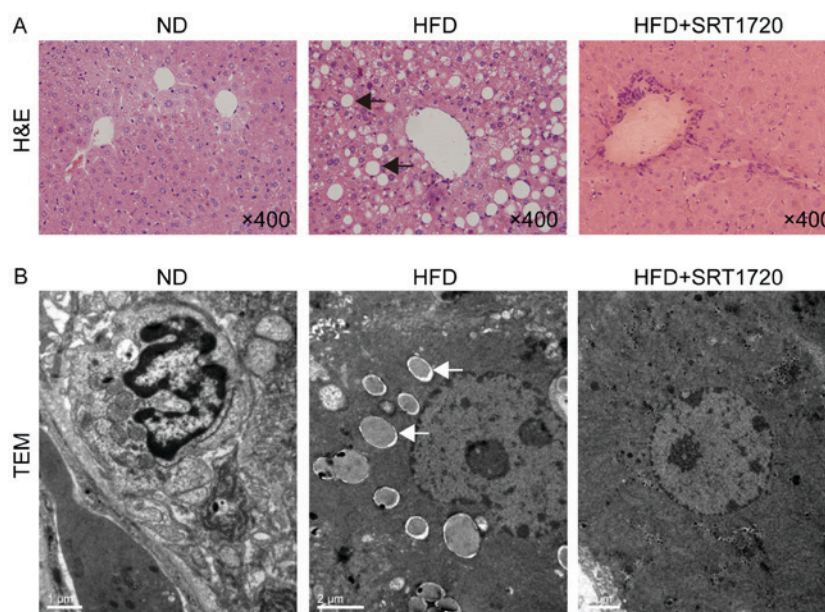


Figure 1. H&E staining and TEM images of liver tissues. (A) Histopathologic changes of liver tissues in each group (magnification, x400). There was no obvious liver injury in control group. Extensive ballooning degeneration of hepatocytes and infiltration of inflammatory cells were displayed in HFD group. Fewer ballooning degeneration of hepatocytes and infiltration of inflammatory cells were observed in HFD+SRT1720 group. (B) The ultrastructure of mice liver from HFD+SRT1720 group was less severe than in HFD group. Arrows, steatosis and lipid droplet. H&E, hematoxylin and eosin; HFD, high-fat diet; ND, normal diet.

Statistical analysis. Experimental data were presented as the mean \pm standard deviation of at least three independent experiments, and the differences between groups were compared by Student's t test or one way ANOVA with the Student-Newman-Keuls post-hoc test in SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SIRT1 activation alleviates hepatic steatosis and inflammation. H&E-stained sections of liver were observed under a light microscope to assess the effect of SRT1720 on the hepatic histopathology. The normal-appearing hepatic lobule structures and hepatocytes were evident in the ND group, while the morphological changes were clearly observable in the HFD group (Fig. 1A). Hepatic lobule structures were swollen and severely damaged, with the ballooning degeneration of hepatocytes and inflammatory cell infiltration. SRT1720 administration alleviated the histological presentation of edema, ballooning degeneration and inflammation in the liver (Fig. 1A).

The ultrastructures of the liver samples were examined by TEM, demonstrating that the number of lipid droplets were significantly increased in the hepatocyte cytoplasm, accompanied by a compressed hepatic sinusoid, in the HFD group (Fig. 1B). The ultrastructures were less abnormal in the HFD+SRT1720 group than in the HFD group (Fig. 1B). These results indicated that SRT1720 can attenuate hepatic steatosis and inflammation induced by HFD, and the upregulation of SIRT1 may be a protective event in NAFLD.

SRT1720 improves liver function and decreases inflammatory factor levels in serum. The levels of ALT and AST were

significantly increased in the HFD group compared with the ND group. However, the levels of ALT and AST were significantly decreased in the HFD+SRT1720 group, suggesting that SRT1720 played a protective role in the progression of NAFLD (Fig. 2A and B). Additionally, the mouse body weight and triglyceride levels in the HFD+SRT1720 group were lower than in the HFD group (Fig. 2C). The serum triglyceride level in the HFD group was significantly higher than those in the control and HFD+SRT1720 group (Fig. 2D). Afterwards, we examined the expression of the inflammatory factors, IL-6 and TNF- α , in the mouse serum from each group. IL-6 and TNF- α remained at low levels in the ND group, and were significantly increased in the HFD group compared with the ND group ($P < 0.05$). Meanwhile, the levels of IL-6 and TNF- α were significantly decreased in the HFD+SRT1720 group compared with the HFD group ($P < 0.05$) (Fig. 2E and F). These data suggested that SRT1720 effectively inhibited the production of inflammatory factors induced by a HFD, further inhibiting the progression of NAFLD.

SIRT1 upregulation suppresses the protein expression of CD36 and P65. The expression levels of SIRT1, P65 and CD36 were identified by immunohistochemistry. As shown in Fig. 3, a HFD reduced the SIRT1 protein expression level, and induced the upregulation of CD36 and P65, indicating that the protection of SIRT1 was lost, leading to the expression of more lipid receptors, and inflammation. As expected, the SIRT1 level was significantly upregulated following the treatment with SRT1720, and P65 was simultaneously decreased. Interestingly, the level of CD36 was also downregulated, compared with the HFD group.

Furthermore, western blotting was performed to quantify the protein expression levels of SIRT1, P65 and CD36; the results were consistent with the immunohistochemistry results

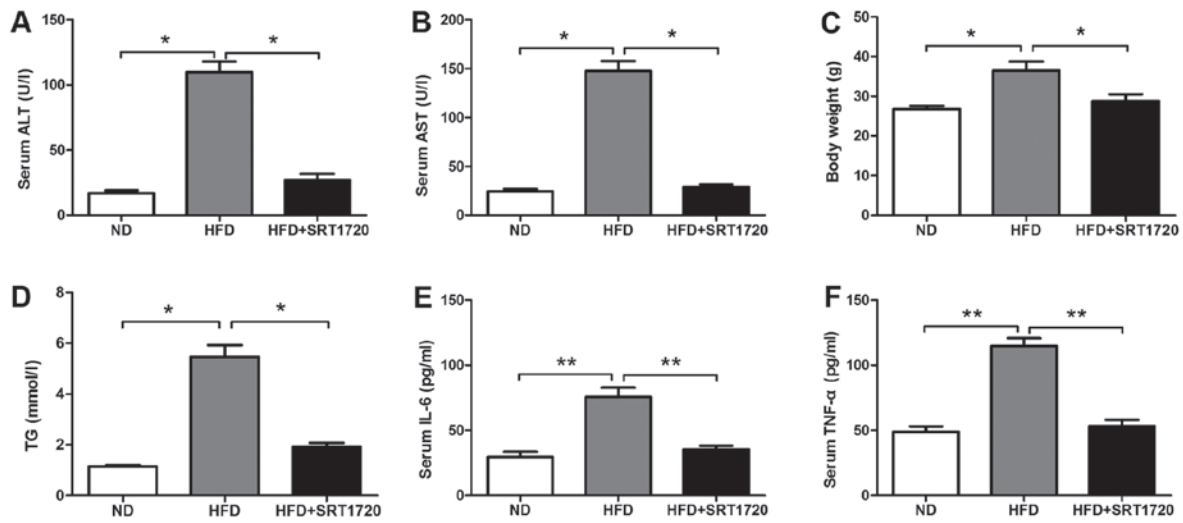


Figure 2. The serum levels of aminotransferase and pro-inflammatory cytokines. SRT1720 decreased the serum levels of ALT (A) and AST (B) (* $P<0.05$). (C) The body weight levels ($P<0.05$). (D) The serum triglyceride levels ($P<0.05$). SRT1720 decreased the serum concentrations of TNF- α (E) and IL-6 (F) (** $P<0.01$).

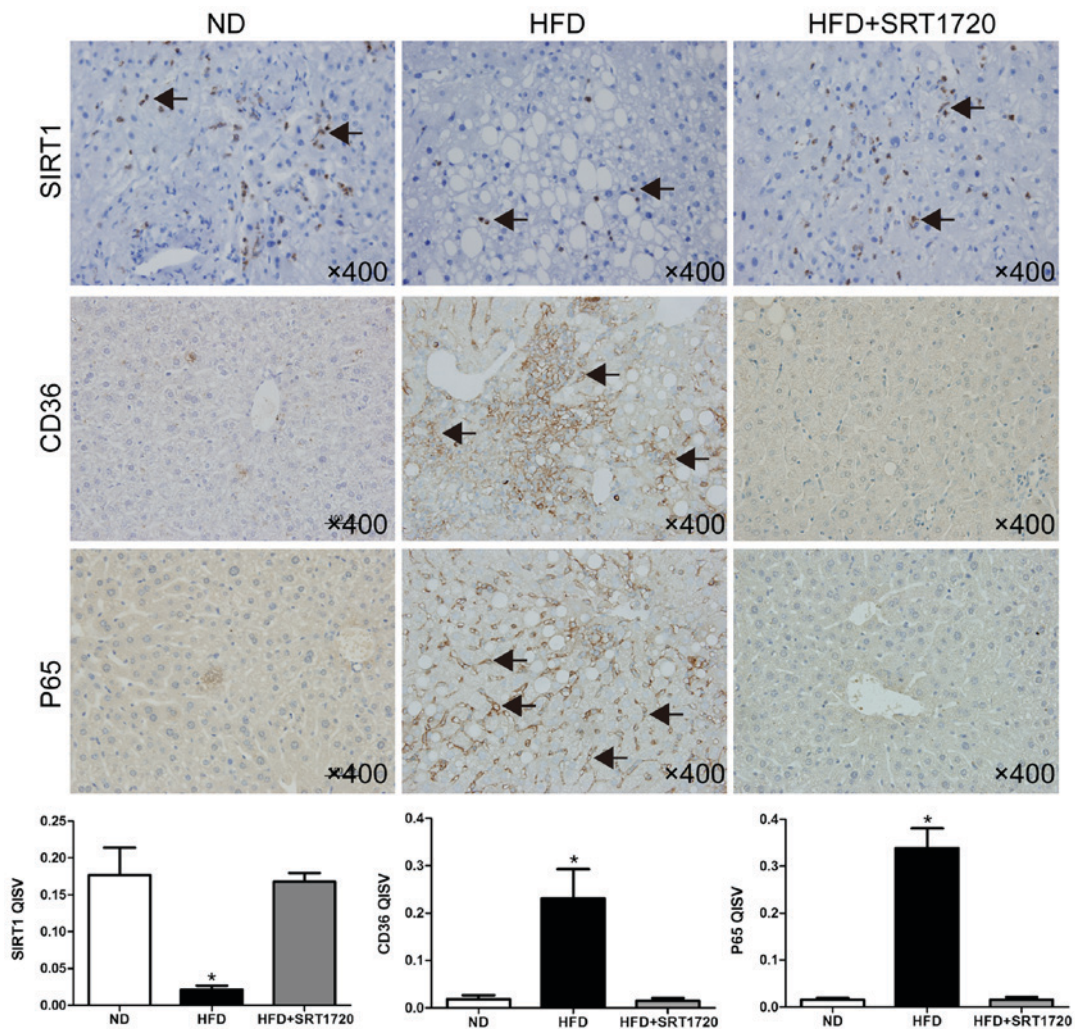


Figure 3. Immunohistochemical analysis of SIRT1, CD36 and P65 protein in each group. Strong expression of CD36 and P65 were observed in HFD group, accompanied with weak expression of SIRT1, comparing with the ND group. SIRT1 expression was enhanced, while the expressions of CD36 and P65 were decreased in HFD+SRT1720 group (magnification, x400). Arrows, positive cells. HFD, high-fat diet; ND, normal diet.

(Fig. 4A and B). We found that the KC levels of P65 and CD36 were significantly increased in the HFD group, accompanied

with a decrease in SIRT1, compared with the ND group ($P<0.01$). Similarly, SRT1720 treatment remarkably upregulated the

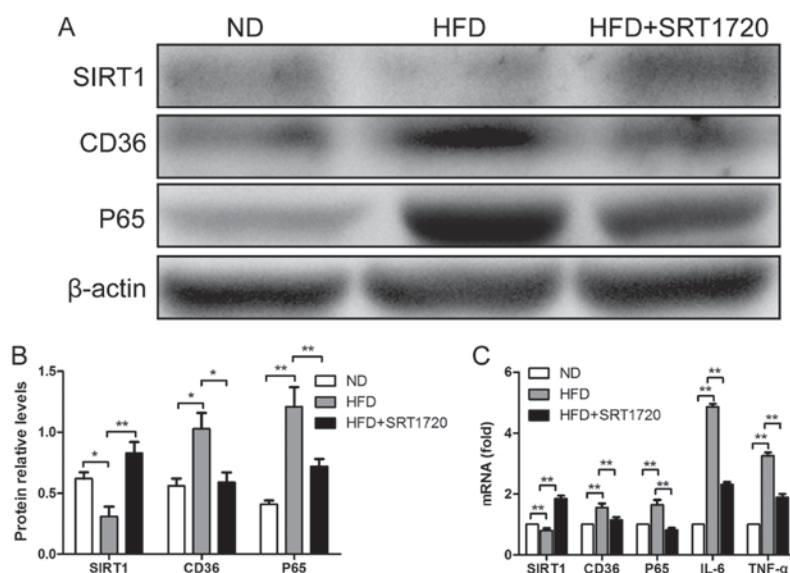


Figure 4. Up-regulation of SIRT1 by SRT1720 decreased the mRNA and protein expressions of CD36 and P65 in KCs. (A and B) Western blot analysis the protein expressions of SIRT1, CD36 and P65 in KCs. (C) Analyses of mRNA expressions of SIRT1, CD36, P65 and proinflammatory cytokines by qPCR in KCs (* $P < 0.05$, ** $P < 0.01$). KCs, Kupffer cells; HFD, high-fat diet; ND, normal diet.

SIRT1 level, leading to reductions in P65 and CD36 compared with the HFD group ($P < 0.05$). Our results demonstrated that the upregulation of SIRT1 by SRT1720 treatment can reduce the P65 and CD36 protein expression levels induced by a HFD.

SIRT1 upregulation by SRT1720 suppresses mRNA expression levels of CD36 and NF-κB (P65). To determine whether SRT1720 could attenuate hepatic steatosis and inflammation by modulating SIRT1, P65 and CD36 at the gene expression level, the mRNA expression of these genes in KCs were detected by qPCR. As demonstrated in Fig. 4C, the mRNA levels of P65 and CD36 were elevated in the HFD group compared with the ND group, whereas SIRT1 followed the opposite trend ($P < 0.01$). However, the elevation of P65 and CD36 levels was inhibited by SRT1720 treatment. P65 and CD36 were significantly decreased in the HFD+SRT1720 group compared with the HFD group ($P < 0.05$ and $P < 0.01$, respectively). These data revealed that the upregulation of SIRT1 by SRT1720 inhibited the P65 and CD36 mRNA levels in KCs.

Discussion

In the present study, we confirmed that the activation of SIRT1 by SRT1720 treatment can protect against liver injury induced by a HFD. Using a mouse NAFLD model fed with a HFD, we observed that the liver morphology was damaged by swollen hepatocytes and infiltrating inflammatory cells, and TNF- α and IL-6 in serum were significantly increased. SRT1720 treatment performed favorably in relieving liver injury, and suppressing the levels of TNF- α and IL-6 induced by a HFD through increasing the expression of SIRT1, which resulted in the inhibition of NF- κ B and CD36 expression in KCs.

SIRT1 has been shown to have a protective effect against the pathophysiological mechanisms of NAFLD, and is a candidate therapeutic target to prevent the development and progression of NAFLD (10). It was reported that treatment with SRT1720, an SIRT1 agonist, was associated with a decreased prevalence

of NAFLD (11). Mounting evidence has indicated that SIRT1 is a protective factor, which maintained insulin sensitivity, adjusted lipid homeostasis through antihyperlipidemic activity, inhibited inflammation, positively influenced autophagy and apoptosis, and promoted resistance to aging (12). Our study also demonstrated the protective effects of SIRT1 against inflammation and steatosis, which may be mediated by regulating NF- κ B and CD36. However, previous studies have reported that SIRT1 mediated cell survival by deacetylating K382 on p53, and mediated cell death by deacetylating K310 on P65, resulting in diminished NF- κ B transcription and a decrease in pro-survival gene products (13). Thus, the conclusions regarding the function of SIRT1 are still controversial, and the mechanisms are not fully understood.

Previous findings have demonstrated that the excessive fat accumulation in the liver induced by a HFD results from an increased rate of hepatic *de novo* lipogenesis and impaired fatty acid oxidation due to the inhibition of 5'-AMP-activated protein kinase (AMPK) activation through SIRT1 (14). Researchers have speculated that in addition to the deacetylation of P65 K310, SIRT1 may bind histone H3 by the CD36 promoter, leading to the downregulation of CD36 in hepatocytes, which controls triglyceride accumulation (15). In our experiments, a HFD induced the upregulation of CD36 while inhibiting SIRT1 expression in an NAFLD mice model, which was reversed by SRT1720 treatment. These data were consistent with previous studies. In addition, another study reported that SIRT1 expression was decreased in aged mice, whereas CD36 was upregulated, which could explain the increasing prevalence and progression of NAFLD associated with age in the general human population (16). The AMPK-mediated effects of SIRT1 have been reported to promote the deacetylation of peroxisome proliferator-activated receptor gamma coactivator 1 α , which then stimulates peroxisome proliferator-activated receptor- α , leading to the initiation of CD36 transcription (17).

Studies have shown that CD36 contributes to the development of NAFLD by modulating the rate of FFA uptake (18).

Serum CD36, which is significantly correlated with hepatic CD36 expression, is an independent factor for the diagnosis of advanced steatosis in NAFLD (19). A decrease in hepatic CD36 level in HFD-fed animals has been shown to be protective against inflammation and insulin resistance (20). However, other experiments revealed that CD36 depletion released LKB1, resulting in the constitutive activation of AMPK, further impairing the AMPK lipid-sensing ability (17). In addition, a recent study reported that CD36 over-expression unexpectedly attenuated hepatic steatosis, increased very low-density lipoprotein secretion, and improved glucose tolerance and insulin sensitivity (21). Some researchers regard CD36 as a protective metabolic sensor in the liver during lipid overload or metabolic stress.

Due to the complexity of their functions, the relationship between CD36 and SIRT1 is quite intricate. In a previous study, H9c2 rat cardiomyoblasts were treated with palmitic acid (PA); during the experiment, CD36 and SIRT1 protein expression levels were altered by PA treatment in a dose- and time-dependent manner. A SIRT1 activator was then administered, significantly increasing the expression of the CD36 metabolic pathway proteins to counter the PA-induced switching from the SIRT1-CD36-fatty acid pathway to the PKC zeta-GLUT4-glucose pathway (22).

At present, there is still no effective treatment for NAFLD. Our results indicate that SRT1720 may be a promising candidate for the treatment of NAFLD. However, before it can be used in humans, its mechanisms of action need to be further studied. In conclusion, we identified that the anti-steatosis and anti-inflammatory properties of SRT1720-induced SIRT1 during NAFLD may be associated with the inhibition of the NF- κ B signaling pathway and regulation of CD36.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BN, KH, XZ and PL designed and performed the experiments. BN, GR, SY and ZO analyzed the data. KH, PL, XZ, SY and JG prepared all the figures. BN, KH and SY wrote the paper.

Ethics approval and consent to participate

The present study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Committee for Animal Subjects of Chongqing

Medical University. Animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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