Apigenin Alleviates Non-Alcoholic Fatty Liver Disease by Downregulating the NLRP3/NF-κB Signaling Pathway in Mice

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Research Article

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Abstract

Background: Apigenin, a flavone found in several plant foods with various biological properties including anti-inflammatory and other abilities, alleviated non-alcohol fatty liver disease (NAFLD) induced by a high fat diet (HFD) in mice. However, the mechanisms underlying this protection of inflammation and NAFLD has not been known clearly.

Methods: Low density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice were fed with HFD diet to induce NAFLD model and were treated with apigenin (50 mg/kg/day) for eight weeks. Hepatic lipid accumulation and inflammation in the livers were analyzed and quantified. In vitro experiments, HepG2 cells were stimulated by LPS plus oleic acid (OA) in the absence of presence of apigenin (50μM). Lipid accumulation and the effect of apigenin on NLRP3/NF-κB signaling pathway was investigated.

Results: Apigenin administration reduce the weight, plasma lipid levels in *Ldlr*$^{-/-}$ mice when fed an HFD. Apigenin (50 mg/kg/day) treated mice displayed reduced hepatic lipid accumulation and inflammation in the livers of mice given the HFD diet. Treating the HepG2 cells with apigenin reduced lipid accumulation. And, apigenin also inhibited activation of NLRP3/NF-κB signaling pathway stimulated by OA together with LPS.

Conclusions: Our results indicated that apigenin supplementation prevented NAFLD via down-regulating the NLRP3/NF-κB signaling pathway in mice, and suggested apigenin might be a potential therapeutic agent for the prevention of NAFLD.

Introduction

Non-alcohol fatty liver disease (NAFLD) is the most common of liver-related morbidity and mortality disease [1, 2], which characterized by the ectopic fat accumulation in the liver caused by injurious factors other than alcohol. It is now widely accepted that such factors include inflammation, mitochondrial dysfunction, oxidative stress and adipose disorder [3–5]. Such factors cause serious liver injury, eventually leading to NAFLD, liver steatosis, liver fibrosis, liver cirrhosis and even hepatocellular carcinoma.

Many studies showed that inflammation plays a critical role in the initiation and progression of NAFLD [6]. The inflammasome NLRP3 is a multiprotein scaffold which can mediate the activation of inflammation reactions [7, 8]. Activation of NLRP3 triggers local and systemic inflammation and has been related to pathogenesis of NAFLD [7]. And, NF-κB is the critical transcriptional factor for the activation of NLRP3 inflammasome and then up-regulate transcription of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and IL-18 [9]. Many studies reported that inhibition of NLRP3 inflammasome reduced inflammation and fibrosis in mice livers [10, 11]. Therefore, exploring inhibitor of the inflammatory regulators such as NLRP3 might represent a promising strategy of the treatment of NAFLD.

Apigenin (APN) is a naturally bioflavonoid and is abundant in fruits, vegetables, herbs and spices [12]. APN has been shown to exert diverse pharmacological activities such as anti-inflammation activity [13], anti-oxidation ability [14] and antitumor activity [15]. Moreover, many studies have demonstrated that APN ameliorated diabetes [16], obesity [17], atherosclerosis [18] and hepatic lipid accumulation [19] in mice. However, the effects of APN in NAFLD, as well as the underlying mechanism, have not been fully explored.

In our study, we investigated the possible prevention effect by APN of NAFLD in vivo, using low density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice fed with a high fat diet (HFD) to induce NAFLD model. And in vitro on HepG2 cells stimulated by LPS plus oleic acid (OA). We further clarified the mechanisms of APN's hepatoprotective effect against NAFLD. Our findings have demonstrated that APN has beneficial effects on NAFLD. The possible mechanism may be involved in inhibition of NF-κB/NLRP3 signaling pathway.

Methods

Materials

Apigenin ($C_{15}H_{10}O_5$, MW: 270.24) was purchased from Sigma-Aldrich (St. Louis, MO). Total cholesterol (TC), triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and Oil Red O kit were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Trizol reagent and SYBR Green Master Mix were purchased from Vazyme (Nanjing, China). Total protein Extraction kit were purchased from TRANSGEN BIOTECH (Beijing, China). BCA protein kit were purchased from Thermo (USA). Antibiotic-antimycotic solution (10,000 units/ml of penicillin, 10,000 ug/ml of streptomycin) was obtained from Sigma Chemical Co. (St Louis, MO, USA). OA were purchased from Energy Chemical (Shanghai, China). MTT was purchased from Solarbio (Beijng, China). Rabbit anti-NLRP3 (ab-270449), Rabbit anti-NF-κB/p-65 (ab-76302), rabbit anti-GAPDH (ab-181602) were purchased from Abcam (Cambrige, UK).

Animals

Ldlr$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All experimental procedures were approved by the Animal Care Ethics Committee of the Henan Provincial People's Hospital, and were conducted in accordance with the American Physiology Institutes of Health. All mice were maintained on a 12:12-h light-dark cycle and have free access to water and food. Mice were randomly divided into three groups (8 mice/group), including control group, HFD group, HFD plus APN (50 mg/kg) (HFD+APN) group. Control group mice were fed with a normal chow diet. HFD group mice were fed with the HFD diet, containing 20% fat and 0.5% cholesterol. HFD+APN group (50mg/kg/day, dispersed in 0.5% sodium carboxymethyl cellulose) was administrated by gavage every day. Control group and HFD group mice were treated with the same volume of 0.5% sodium carboxymethyl cellulose as vehicle group.

Blood metabolite analysis

Blood was obtained by retro-orbital bleeding. Plasma total cholesterol (TC), and triglyceride (TG) were enzymatic methods (Sigma Kits, USA).

Cell culture

HepG2 cells were cultured in DMEM supplemented with 15% FBS and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37℃. Cells were starved in serum-free DMEM for 12 hour followed by incubation with OA for additional 24 hour in the absence or presence of APN (50 μM).

Histological analysis

The livers were fixed in 10% formalin and embedded in paraffin, and then cut into 5μm serial sections. Tissue sections were subjected to standard haematoxylin-eosin (H&E) staining for determination of hepatic fat accumulation. OCT-embedded_frozen livers were sectioned at 7μm for Oil Red O staining.

Methylthiazolyl tetrazolium assay for cell viability

Cells were cultured at a density of 4-5 x 10^4 cells per well in 96-well plates for 24 h. The cells were treated with different concentrations of APN for 24 h. And then cell viability was determined by the MTT reduction assay. Cells were incubated with MTT solution (5 mg/mL) for 4 h at 37 °C. The dark blue formazan crystals that formed in intact cells were solubilized with 150 μL of DMSO, and the absorbance at 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Western blotting

Total protein were extracted from cells and liver tissues using RIPA lysis buffer and phenylmethylsulfonyl fluoride (Beyotime, China). The protein concentration was detected by using a BCA protein assay kit. Equal amounts of protein (20μg) were separated using 10 or 12% SDS-PAGE and were transferred onto a polyvinylidene difluoride membrane (PVDF). Next, PVDF membranes were blocked with 5% fat-free milk and incubated with primary antibodies to NLRP3 (Abcam, Inc., CA, USA, Cat. No. ab-270449), NF-κB/p-65 (Abcam, Inc., CA, USA, Cat. No. ab-76302), GAPDH (Abcam, Inc., CA, USA, Cat. No.ab-181602) overnight at 4℃. Subsequently, the membranes were washed and incubated with secondary antibodies at room temperature. The optical density of the bands was visualized by an ECL system (Pierce). GAPDH was used as an endogenous control. Data was normalized to GAPDH levels.

RNA isolation and qPCR analysis

Total RNA was extracted from the frozen tissues or treated HepG2 cells using Trizol reagent. First strand cDNA was synthesized using an RT kit. Amplifications were performed using an opticon continuous fluorescence detection system with SYBR green fluorescence. A single melting curve peak confirmed the presence of a single product. Results were expressed as fold differences relative to GAPDH using the $2^{-\Delta\Delta CT}$ method. All the primers were synthesized by Sangon Biotech (Shanghai, China) and the sequence are listed in Table 1.

Statistical analysis

All data are presented as means ± SEM. SPSS 21.0 was used to perform statistical analysis of the data. Statistical differences were calculated with the 2-tailed Student t test when comparing 2 conditions, and ANOVA was used when comparing ≥2 conditions. A value of $P < 0.05$ was considered statistically significant.

Results

Apignin ameliorates metabolic abnormalities in *Ldlr*$^{-/-}$ mice fed a HFD diet

To investigate whether apigenin (APN) has a protective role on the development of liver steatosis, NAFLD were induced by HFD diet in *Ldlr*$^{-/-}$ mice. Treated *Ldlr*$^{-/-}$ mice with HFD diet for eight weeks, significant increase in body weight and liver weight were observed in HFD group (Figure 1A and B), which were significantly reversed in the HFD + APN group (Figure 1A and B). Plasma lipid levels were then analyzed. As shown in Figure 1C and D, the TG and TC levels in HFD + APN group mice were significantly lower than those of the HFD group mice. These results demonstrated that APN treatment could reduce body weight and plasma lipid level in HFD fed *Ldlr*$^{-/-}$ mice.

Apigenin attenuated liver steatosis induced by HFD diet in *Ldlr*$^{-/-}$ mice

We further analyzed hepatic lipid accumulation in HFD group and APN treatment group. HFD diet induced obvious hepatic lipid accumulation in the livers of the HFD group mice (Figure 2A and B) and a nearly 2-fold increase in liver TG accumulation (Figure 2C). However, APN treatment induced a significant decrease of hepatic lipid deposition in the HFD + APN group mice (Figure 2A, B, C and D).

Apigenin ameliorates hepatic inflammation in HFD fed *Ldlr*$^{-/-}$ mice

Inflammation is one of the main reasons that promote hepatic lipid steatosis. Then we examined inflammatory cytokine gene expression in liver in each group of mice. As shown in Figure 3A, HFD-fed mice had significantly increased mRNA levels of inflammatory genes (F4/80, MCP-1, TNF-α、IL-6、NLRP3, NF-κB, TGF-β1, IL-1β and IL-18) expression, and APN treatment could correct the upregulation of these inflammatory genes expression.

To explore novel mechanisms underlying the protection effects of APN on NAFLD, we focused on NLRP3/NF-κB signaling pathway which has attracted a lot of attention in NAFLD progression. As shown in Figure 3C, the hepatic protein level of nuclear NF-κB were significantly upregulated in HFD mice, and APN supplementation significantly reversed HFD-induced increase of nuclear NF-κB protein level. Consistent with the change of nuclear NF-κB protein level, the hepatic protein expression of NLRP3 were significantly enhanced in the HFD group mice. And, APN administration could lower greatly the hepatic level of NLRP3 as shown in Figure 3B.

Apigenin prtotection against NAFLD through inhibiting NLRP3/NF-κB signaling pathway

Furthermore, we established in vitro NAFLD model in a fat overload profile in HepG2 cells by stimulation with LPS plus OA. Firstly, we detected the cytotoxicity of APN on HepG2 cells. As shown in Figure 4A, the results of MTT assay showed that APN had no cytotoxic effects on HepG2 cells at concentrations less than 200μM. Thus, in the following experiments, APN concentrations of 50μM was chosen. HepG2 cells were cultured in medium containing LPS plus OA and lipid accumulation in cells were analyzed by Oil Red O staining. Treatment with APN markedly decreased OA induced lipid accumulation in HepG2 cells, as revealed by cellular triglyceride content and Oil red O staining (Figure 4B and C). Consistent with in vivo results, NLRP3 and NF-κB was activated after stimulation of LPS plus OA in HepG2 cells, and treatment with APN significantly reduced NLRP3 and NF-κB activation (Figure 5A and B).

Discussion

Nonalcoholic fatty liver disease (NAFLD) has become one of the most prevalent liver disease worldwide [20]. A better understanding of the mechanisms underlying NAFLD is important for developing more effective diagnostic and therapeutic strategies. In the present study, we evaluated the effect of apigenin (APN) on NAFLD using *Ldlr*$^{-/-}$ mice fed with a high fat diet (HFD). APN is a natural flavonoid and the anti-inflammation of the APN has well been established [21, 22]. However, the effect of APN on NAFLD and the mechanism remained elusive. The present study demonstrates that APN has a protective effect on the development of NAFLD, and the mechanism could be involved in inhibition of NLRP3/NF-κB signaling pathway.

APN is one of the most widespread flavonoids in vegetables (parsley, celery, onions), fruits (oranges), herbs (chamomile, thyme, oregano, basil) and plant-based beverages (tea, beer and wine) [23]. A huge number of studies have reported the antioxidant and anti-inflammatory properties of APN [24, 25]. In addition, anti-atherogenic [26], anti-apoptotic [27] and the protective effects in NAFLD, cardiac hypertrophy and hypertension [28] have been reported. In our study, we found APN treatment reduced body weight and liver weight, and this anti-obesity effect is consistent with previous reports [17, 19]. And, plasma triglyceride decreased significantly after APN administration on HFD, which is more likely to reflect a decrease in hepatic lipid accumulation. The protective effect of NAFLD of APN was shown in HE and Oil red O staining of liver. Histological analysis revealed significantly less hepatic lipid deposition in APN supplementation mice when compared with control mice. Furthermore, an important finding of our study is that APN protected NAFLD through inhibition of NLRP3/NF-κB signaling pathway.

The role of inflammasome activation in NAFLD has received significant attention in recent years [29, 30]. The NLRP3 inflammasome is primarily activated by inflammatory stimuli. The initiating step involves signal in which inflammatory stimuli were recognized by toll-like receptors, leading to activation of NF-κB. And, activation of NF-κB up-regulates inactive NLRP3. Then activation of NLRP3 triggers the transformation of procaspase-1 to caspase-1, as well as the production and secretion of mature IL-1β and IL-18 [31, 32]. Our result demonstrated that HFD enhanced the mRNA expression level of

inflammation genes (F4/80, MCP-1, TNF-α,IL-6NLRP3, NF-κB, TGF-β1, IL-1β and IL-18) expression. In vivo experiments, we found that APN significantly down-regulated the inflammation gene expression and protein levels. And in vitro experiments, our result showed that APN attenuate the lipid deposition in OA-stimulated HepG2 cells, in which NLRP3/ NF-κB signaling was inactivated significantly. In this study, we not only found that APN improved dyslipidemia, but also found that APN alleviated inflammation and lipid accumulation by inhibiting NLRP3/ NF-κB signaling pathway.

Conclusions

In conclusion, we have demonstrated that the anti-NAFLD effect of APN was dependent on NLRP3/ NF-κB signaling pathway. These findings indicated that APN has therapeutic potentials in the prevention of NAFLD. Therefore, APN supplementation may be considered as potential prevention strategy for NAFLD.

Abbreviations

APN: Apigenin, NAFLD: non-alcohol fatty liver disease, HFD: high fat diet, Ldlr: Low density lipoprotein receptor, OA: oleic acid, TC: total cholesterol (TC), TG: triglyceride, TNF-α: tumor necrosis factor-α, IL: interleukin,

Declarations

Acknowledgment

Not applicable

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Availability of data and materials

All data generated of analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Author's contributions

ZL designed the study, LL, SZ and JTZ conducted the experiments, LL did sample analysis and data analysis, ZL wrote the manuscript, SJL revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Animal Care Ethics Committee of the Henan Provincial People's Hospital.

Consent for publication

Not applicable

Competing interest

The authors have no conflicts of interest to disclose.

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Tables

Table 1 Primer list for quantitative real-time PCR

Gene Name	Forward primer (5'-3')	Reverse Primer (5'-3')
F4/80	TTTCCTCGCCTGCTTCTTC	CCCCGTCTGTATTCAACC
TNFα	CTGTGAAGGGAATGAATGTT	CAGGGAAGAATCTGGAAAGGTC
MCP1	TCCCAATGAGTAGGCTGGA	AAGTGCTTGAGGTGGTTGT
IL-1β	AGGCTCCGAGATGAACAA	AAGGCATTAGAAACAGTCC
TGF-β1	GGCGGTGCTCGCTTTGTA	TCCCGAATGTCTGACGTAT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA
NLRP3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
NF-κB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG
GAPDH	TCCTTGGAGGCCATGTGGGCCAT	TGATGACATCAAGAAGGTGGTGAAG

Figures

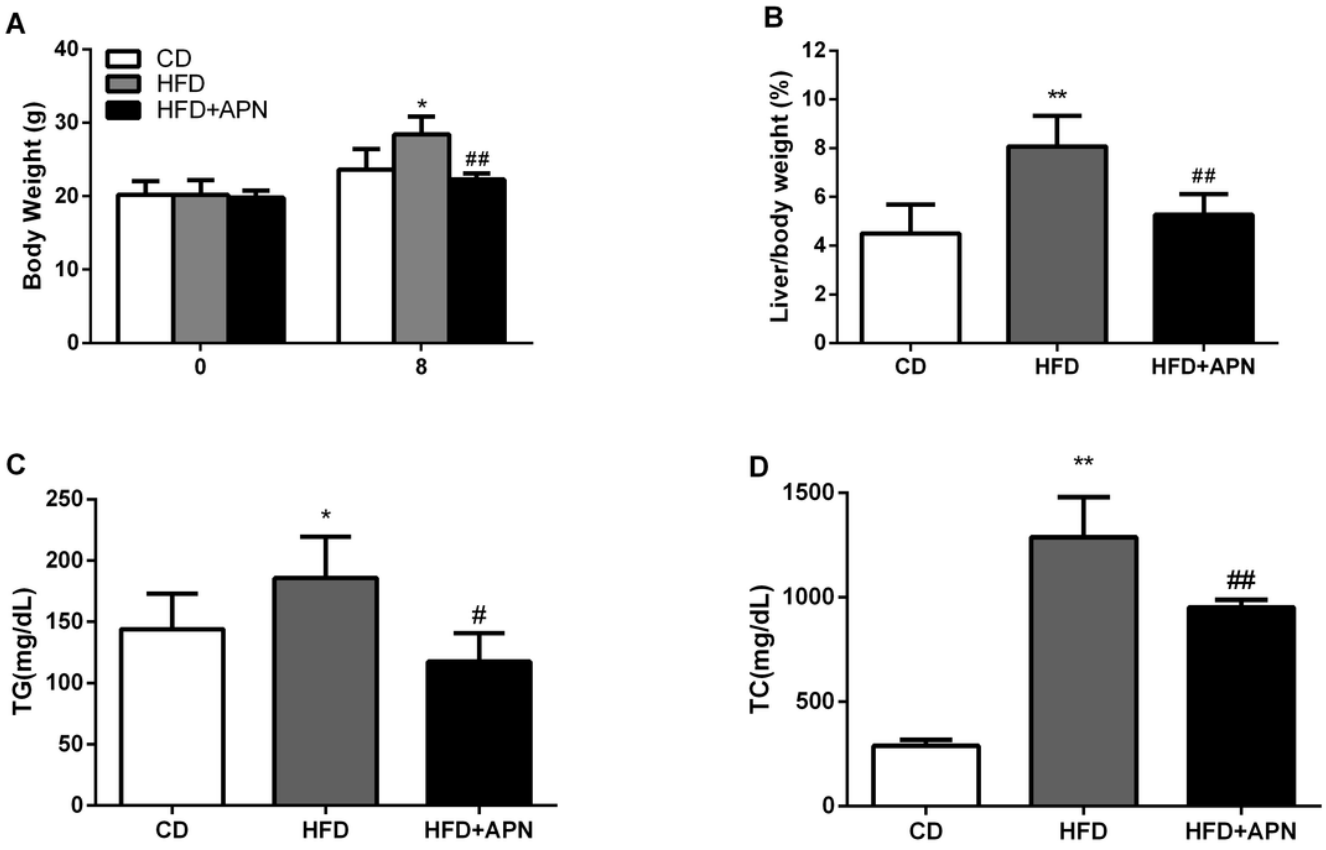

Figure 1

Effect of Apigenin (APN) treatment on body weight, liver weight and plasma lipid levels of Ldlr-/- mice fed with HFD. Body weight (A), ratio of liver weight to body weight (B), plasma TG、C、and TC (D) levels. Data are presented as mean ±SEM, n=8, *P<0.05, **P<0.01 for HFD mice vs. CD mice. #P<0.05, ##P<0.01 for HFD mice vs. HFD+APN mice.

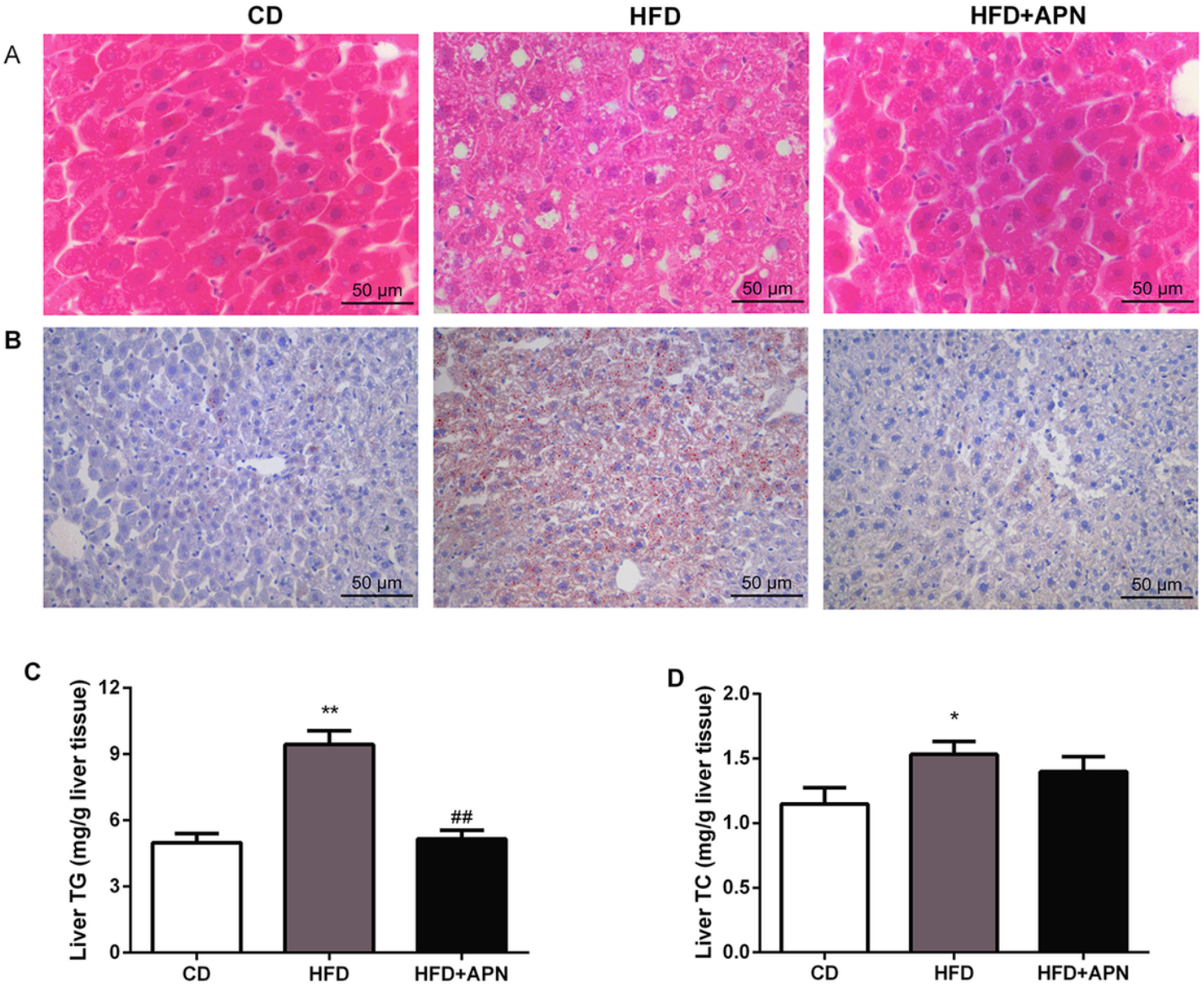

Figure 2

Effect of Apigenin (APN) treatment on hepatic lipid accumulation in Ldlr-/- mice fed with HFD. Representative histology of H&E (A)and Oil Red O staining (B), TG (C) and TC (D) in mice livers. Data are presented as mean ±SEM, n=8, *P□0.05, **P□0.01 for HFD mice vs. CD mice. ##P□0.01 for HFD mice vs. HFD+APN mice.

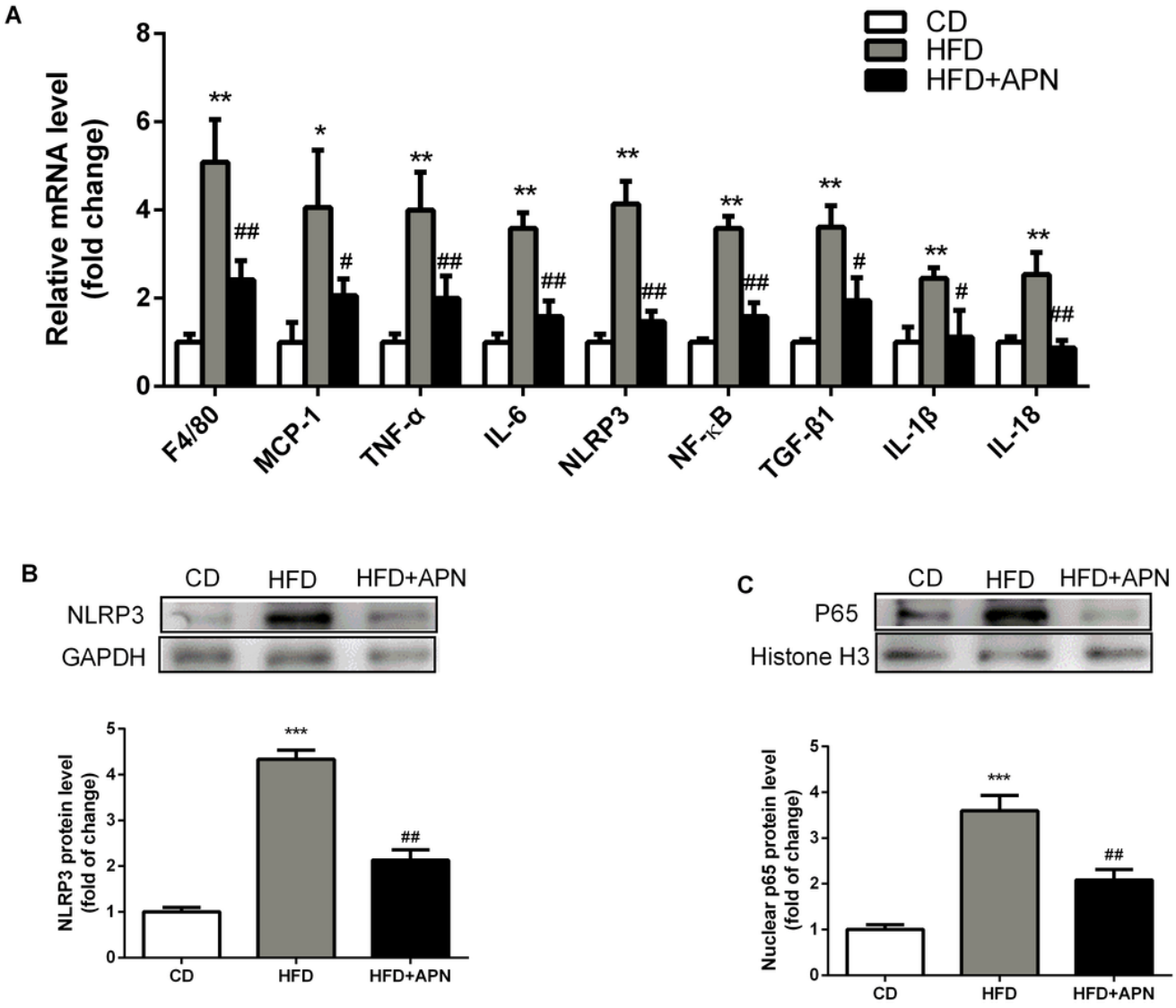

Figure 3

Apigenin attenuated inflammation in Ldlr-/- mice fed with HFD. A. The mRNA expression level of inflammatory genes in the livers of different group mice (n=6 per group). B and C. Protein level and quantitative analysis of NLRP3 and NF-κB in different group mice. Data are presented as the mean ±SEM, *P<0.05, **P<0.01 for HFD mice vs. CD mice. #P<0.05, ##P<0.01 for HFD mice vs. HFD+APN mice.

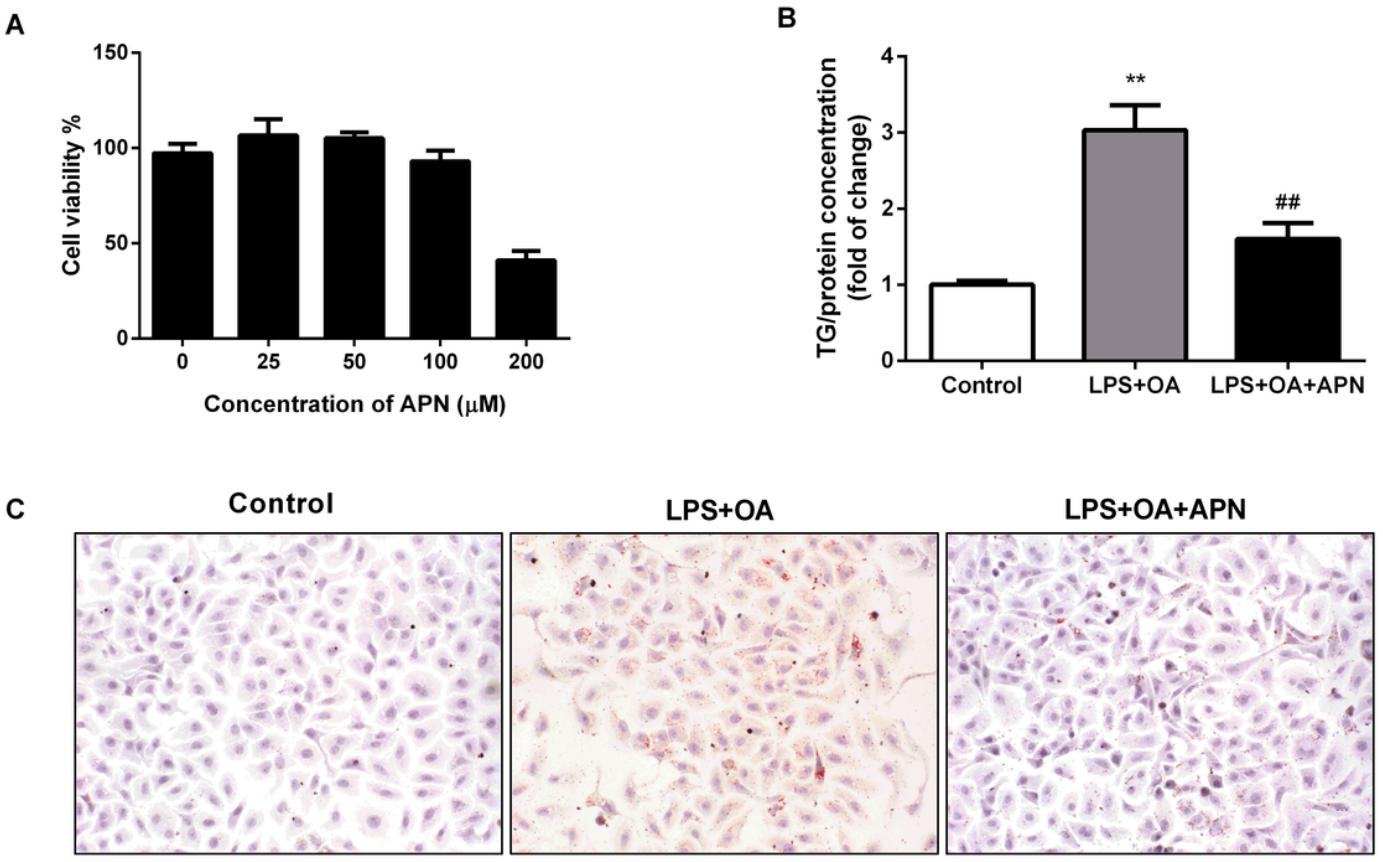

Figure 4

Apigenin ameliorates hepatocellular lipid accumulation in HepG2 cells stimulated by LPS and OA. A. Cytotoxicity of apigenin in HepG2 cells. B. TG contents in HepG2 cells stimulated by LPS and OA. C. Representative histology of Oil Red O staining of HepG2 cells. Data are presented as mean ±SEM. **P 0.01 for LPS+OA vs. Control. ##P 0.01 for LPS+OA vs. LPS+OA+APN.

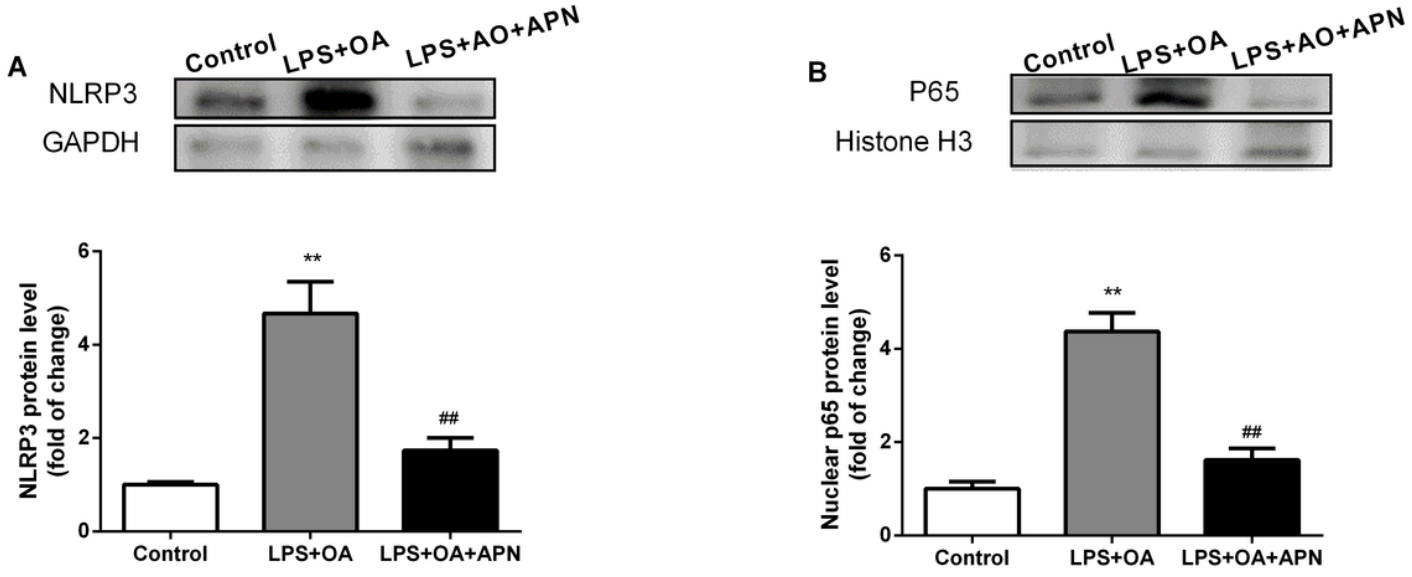

Figure 5

Apigenin inhibit NLRP3/NF-κB signaling pathway in HepG2 cells stimulated by LPS and OA. A and B. Protein level and quantitative analysis of NLRP3 and p65 in HepG2 cells stimulated by LPS and OA. Data are presented as mean ±SEM. **P0.01 for LPS+OA vs. Control. ##P0.01 for LPS+OA vs. LPS+OA+APN.