

Silybin restored CYP3A expression through the SIRT2/NF- κ B pathway in mouse
nonalcoholic fatty liver disease

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Acetylated p65 inhibits cyp3a transcription

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Abbreviations

AC-NF- κ B p65, acetylation-NF- κ B p65

BCA, bicinchoninic acid;

BSA, bovine serum albumin;

CM, conditioned medium;

CYP450, cytochrome P450;

DDI, drug-drug interaction;

DMEM, Dulbecco's modified eagle medium;

ECL, enhanced chemiluminescence;

eIF2 α , eukaryotic initiation factor 2 α ;

ELISA, enzyme-linked immunosorbent assay;

ER, endoplasmic reticulum;

FBS, fetal bovine serum;

H&E, hematoxylin and eosin;

HFD, high-fat diet;

IL-1 β , interleukin-1 β ;

IRE, inositol-requiring transmembrane kinase/endoribonuclease;

I κ B α , inhibitor of NF- κ B α ;

NAFLD, nonalcoholic fatty liver disease;

NAM, nicotinamide;

NAMPT, nicotinamide phosphoribosyltransferase;

NF- κ B, nuclear factor kappa-B;

NLRP3, NOD-like receptor family protein 3;

NMN, nicotinamide mononucleotide;

P450, cytochrome P450;

PA, palmitate;

PARP1, poly-ADP ribosyl polymerase;

PBS, phosphate-buffered solution;

PCR, polymerase chain reaction;

PDH, glucose-6-phosphate dehydrogenase;

PVDF, polyvinylidene fluoride;

PXR, pregnane X receptor;

SB, silybin;

SERCA, sarco/endoplasmic reticulum calcium ATPase;

SIP, surface immunogenic protein;

SIRT, sirtuin;

TBST, Tris-buffered saline containing 0.5% Tween-20;

TG, thapsigargin;

TNF- α , tumor necrosis factor- α ;

Abstract

Silybin is widely used as a hepatoprotective agent in various liver disease therapies and has been previously identified as a CYP3A inhibitor. However, little is known about the effect of silybin on CYP3A and the regulatory mechanism during high-fat-diet (HFD)-induced liver inflammation. In our study, we found that silybin restored CYP3A expression and activity that were decreased by HFD and conditioned medium (CM) from palmitate (PA)-treated Kupffer cells. Moreover, silybin suppressed liver inflammation in HFD-fed mice and inhibited NF- κ B translocation into the nucleus through elevation of SIRT2 expression and promotion of p65 deacetylation. This effect was confirmed by overexpression of SIRT2, which suppressed p65 nuclear translocation and restored CYP3A transcription affected by CM. The hepatic NAD⁺ concentration markedly decreased in HFD-fed mice and CM-treated hepatocytes/HepG2 cells but increased after silybin treatment. Supplementing NMN as an NAD⁺ donor inhibited p65 acetylation, decreased p65 nuclear translocation, and restored *cyp3a* transcription in both HepG2 cells and mouse hepatocytes. These results suggest that silybin regulates metabolic enzymes during liver inflammation by a mechanism related to the increase in NAD⁺ and SIRT2 levels. In addition, silybin enhanced the intracellular NAD⁺ concentration by decreasing PARP1 expression. In summary, silybin increased NAD⁺ concentration, promoted SIRT2 expression and lowered p65 acetylation both *in vivo* and *in vitro*, which supported the recovery of CYP3A expression. These findings indicate that the NAD⁺/SIRT2 pathway plays an important role in CYP3A regulation during NAFLD.

Keywords: NAFLD; silybin; SIRT2; NF- κ B; CYP3A

Significance Statement

This research revealed the differential regulation of CYP3A by silybin under physiological and fatty liver pathological conditions. In the treatment of NAFLD, silybin restored, not inhibited, CYP3A expression and activity through the NAD⁺/SIRT2 pathway in accordance with its anti-inflammatory effect.

Introduction

Cytochrome P450 (CYP, CYP450 or P450) is a multifunctional enzyme superfamily, and cytochrome P450 is the most important element of oxidative metabolism since most P450 enzymes have monooxygenase activity. The substrates of P450s vary from exogenous to endogenous compounds, including drugs, hormones, lipids and others (Lewis, 2003; Lewis, 2004). The variation in P450s in liver diseases could lead to the impaired metabolism of a number of drugs. Changes in some cytochrome P450 enzymes have been found in the livers of steatosis patients and fatty liver models of experimental animals (Elbekai et al., 2004; Donato et al., 2006; Cobbina and Akhlaghi, 2017; Wang et al., 2020). These findings suggest an association between increased lipid deposition and impaired CYP enzyme activity. In addition, patients with NAFLD often have complications such as type 2 diabetes, obesity, cholecystitis, arteriosclerosis, and hypertension and could undergo multiple long-term treatments at the same time (Edginton and Willmann, 2008; Dostalek et al., 2011). Therefore, it is important to avoid drug-drug interactions and side effects caused by metabolic enzyme variations.

CYP3A is one of the main subenzymes of the P450 family in the liver, and it is transcriptionally regulated by nuclear receptors, such as pregnane X receptor (PXR) (Goodwin et al., 2002; Kliewer et al., 2002; Martínez-Jiménez et al., 2007). PXR is a ligand-dependent nuclear receptor that regulates the transcription of CYP3A4 by forming a complex that binds to DNA sequences. Under chronic liver injury, PXR inhibits liver inflammation through suppression of the NF- κ B pathway (Li et al., 2012). Inflammatory stimulation activates NF- κ B, and the NF- κ B subunit p65 directly interacts with the DNA-binding domain of RXR α and the DNA-binding, hinge and ligand-binding domains of PXR, preventing the latter from binding to the consensus DNA sequence, thereby inhibiting transactivation of PXR/RXR α , which in turn affects the transcription and expression of CYP3A4 (Gu et al., 2006; Zhou et al., 2006).

Sirtuin 2 (SIRT2) is an NAD⁺-dependent deacetylase mainly localized in the cytoplasm that can also shuttle into the nucleus and is involved in many physiological and pathological processes, such as inflammation, oxidative stress, carcinogenesis and cell cycle regulation (Lee et al., 2014; Singh et al., 2018; Wang et al., 2019). The relationship between SIRT2 and inflammation has been elucidated by numerous studies (Mendes et al., 2017; Kitada et al., 2019; He et al., 2020). Hottiger *et al.* reported that SIRT2 directly inhibits and deacetylates the Lys-310 site of the NF-κB subunit p65, resulting in decreased expression of NF-κB-regulated inflammatory genes and playing an important role in inflammation (Rothgiesser et al., 2010). In the liver, nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in the biosynthesis process of NAD⁺ and NAMPT-mediated NAD⁺ biosynthesis in metabolic organs is severely compromised by a high-fat diet (HFD) (Yoshino et al., 2011; Dall et al., 2018). Parent poly-ADP ribosyl polymerase-1 (PARP1) is a nuclear protein of the PARP enzyme family that acts as a DNA gap sensor enzyme by consuming NAD⁺ and ATP (Gibson et al., 2016; Ryu et al., 2018). Both PARPs and sirtuins cleave NAD⁺ into nicotinamide (NAM) and ADP-ribose, resulting in the irreversible consumption of NAD⁺. The decrease in NAD⁺ levels caused by a HFD further affects NAD⁺-dependent SIRT2 deacetylation.

Silybin is the main active compound of *Silybum marianum*, or milk thistle, used in different liver disorders, such as chronic liver diseases, cirrhosis and hepatocellular carcinomas, because of its antioxidant and anti-inflammatory effects (Federico et al., 2017; Abenavoli et al., 2018). Although silybin has been recognized as an *in vitro* inhibitor of CYP3A4 and 2C9 (Sridar et al., 2004), how it affects CYP3A *in vivo* in liver disorders and what the underlying mechanism is remain unclear. Our previous study demonstrated that the anti-inflammatory effect of silybin is based on restoring NAD⁺ levels in hepatocytes to maintain SIRT2 activity and then inhibiting NLRP3 inflammasome assembly to reduce the release of IL-1β (Zhang et

al., 2018). Furthermore, cytokines are important transcriptional regulators of P450s during liver inflammation (Morgan, 1997; Renton, 2004; Febvre-James et al., 2018). Whether silybin regulates CYP3A through the NAD⁺/SIRT2/NF-κB pathway in fatty liver disorder needs further investigation.

We propose the hypothesis that the differential regulation of CYP3A4 by silybin under normal physiological and pathological states may not be mediated by a direct effect on the nuclear receptor of metabolic enzyme regulators but may be due to its anti-inflammatory effect. In the present study, we evaluated the effects of silybin on the NAD⁺/SIRT2/NF-κB pathway and determined how it restored CYP3A activity in the livers of HFD-fed mice.

Materials and Methods

Materials

Silybin was obtained from Zeland (Nanjing, China). nicotinamide mononucleotide (NMN), thapsigargin (TG) and DPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Palmitate (PA) was obtained from Sinoreagent Chemical Reagent Company (Shanghai, China). Primary antibodies targeting the following proteins were used in the present study: inositol-requiring transmembrane kinase/endoribonuclease (IRE)-1α (ab37073, Abcam, Cambridge, MA, USA), phosphorylated p-IRE-1α (ab48187, Abcam, Cambridge, MA, USA). Anti-eukaryotic initiation factor 2A (eIF2α) (I45) polyclonal antibody ([BS3651, Bioworld, Technology, St. Louis Park, MN, USA](#)), anti-eIF2α (phospho-S51) polyclonal antibody ([BS4787, Bioworld, Technology, St. Louis Park, MN, USA](#)), and anti-GAPDH monoclonal antibody ([AP0063, Bioworld, Technology, St. Louis Park, MN, USA](#)) were also used. IL-1β, TNF-α and TGF-β1 ELISA kits were purchased from ExCell Bio (Jiangsu, China).

Animals and treatment

Male C57BL/6 mice (Changzhou Cavans Experimental Animal Co., Ltd.) at the age of 6 weeks were allowed to acclimatize to a 12-h/12-h light/dark cycle at a constant temperature (22 ± 2 °C) and had free access to food and water. Mice were fed a standard HFD (Nanjing Qinglongshan Experimental Animal Center) for 12 weeks. Silybin (40 or 80 mg/kg/day) and carboxymethylcellulose sodium (CMC-Na) were administered intragastrically for the last 4 weeks. All experiments were performed in compliance with the guidelines of the Animal Experimentation Board of China Pharmaceutical University on animal usage.

Primary hepatocyte isolation and culture

Primary hepatocytes were prepared as previously described (Edwards et al., 2013) with slight modifications. Briefly, mice were anesthetized by intraperitoneal injection of chloral hydrate, sprayed with alcohol and placed in a biological safety cabinet. The liver was perfused with type IV collagenase at a flow rate of 5 ml/min until the liver collapsed. The entire liver was harvested and placed in a dish, and the liver capsule was gently punctured to release the liver cells. After filtration through a 100- μ m cell sieve, the supernatant was centrifuged at 4 °C and 50 \times g for 2 min, after which the pellet was resuspended in an appropriate amount of serum-free DMEM and then centrifuged twice at 4 °C and 50 \times g for 2 min. The final pellet was resuspended in 1-3 ml of DMEM containing 10% fetal bovine serum (FBS), and the cells were counted. The cells were cultured in an incubator at 37 °C for 4 h, and then the obtained adherent cells were primary mouse hepatocytes.

Kupffer cell isolation and culture

Primary hepatocytes were prepared as previously described (Bourgognon et al., 2015) with slight modifications. The initial steps were the same as the extraction and separation of primary mouse hepatocyte cells, and after centrifugation three times, the supernatant was centrifuged at 4 °C and 1350 \times g for 15 min to discard the supernatant. After resuspension in 3 ml serum-free DMEM, 15 ml of 50% SIP was added. Then, the supernatant was discarded by

centrifugation at 4 °C and 1350 ×g for 15 min. The obtained cells were resuspended in 1 ml of DMEM containing 10% FBS, counted, and plated. After incubation at 37 °C for 30 min, the obtained adherent cells were mouse liver Kupffer cells.

Preparation of conditioned medium (CM) from Kupffer cells

The supernatant of Kupffer cells cultured overnight was discarded, DMEM with or without PA (10 mM) was gently added to the Kupffer cell culture flask, and the cells were incubated for 24 h. Then, the levels of relevant inflammatory factors in the supernatant were determined. Similar to the animal experiments, the supernatant was diluted with serum-free DMEM for use.

HepG2 cell culture

HepG2 cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) with 10% FBS(Gibco, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in the presence of 5% CO₂ in a culture incubator. The obtained cells were seeded in 6-well plates, pretreated with silybin (50 µM) or NMN (1 mM), and then stimulated with PA (100 µM) or the supernatant of Kupffer cells.

ELISA

IL-1β, TNF-α and TGF-β1 levels were measured with ELISA kits according to the manufacturer's instructions.

Real-time quantitative PCR

All qPCRs were performed in triplicate. PCR was carried out in a final volume of 20 µl in the presence of 1×SYBR Green/ROX qPCR Master Mix (Bio-Rad, CA, USA). The primers for *cyp3a11* were as follows: forward, 5'- CCGAGTGGATTTTCTTCAGC-3'; reverse, 5'- GAGCCTCATCGATCTCATCC -3'. The primers for *cyp3a4* were as follows: forward, 5'- CAATGGACTGCATAAATAACCG -3'; reverse, 5'- GAGCCAAATCTACCTCCTCACA - 3'. The primers for β-actin (mouse) were as follows: forward, 5'-

ACCACACCTTCTACAATGAG -3'; reverse, 5'- ACGACCAGAGGCATACAG -3'. The primers for β -actin (human) were as follows: forward, 5'- GCGTGACATTAAGGAGAAG -3'; reverse, 5'- GAAGGAAGGCTGGAAGAG -3'.

Western blot analysis

The protein samples were subjected to protein separation by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel). After electrophoretic separation, the proteins were transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk/TBST, the membrane was reacted with primary antibodies (1:1000 for anti-cytochrome P450 3A4, NF- κ B-p65 (K303) polyclonal, acetyl-NF- κ B (Lys310), SIRT2 polyclonal, NAMPT polyclonal and PARP1 polyclonal antibodies), and then the primary antibody was removed. Next, the membrane was reacted with the corresponding secondary antibody (1:7500). Protein bands were visualized using the Bio-Rad ChemiDoc XRS+ Chemiluminescence Imaging System after the addition of enhanced chemiluminescence reagent (ECL).

CYP3A activity assay

Liver microsomes were isolated with a previously described method (Kaul and Novak, 1987). The protein concentration of the microsomes was determined using a BCA protein assay kit (Beyotime Technology, Shanghai, China), and the microsome samples were stored at -80 °C until use. The incubation system contained 75 μ l of microsomes (the final concentration was 0.5 mg/ml), 5 μ l of different concentrations of substrate and 20 μ l of NRS (2 mM NADPH, 10 mM G-6-p, 1 U/ml PDH, and 10 mM MgCl₂). After incubation, the samples were quickly placed on ice, and the reaction was stopped by adding 300 μ l of methanol to the internal standard. The enzyme activity of CYP3A was characterized by midazolam 4-hydroxylation and testosterone 6 β -hydroxylation with liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis performed as previously described by our lab (Peng et al., 2015). Data were processed with the Michaelis-Menten model.

NAD⁺ measurement

Hepatocyte NAD⁺ levels were determined with a LC-MS/MS method previously established by our laboratory (Liu et al., 2016).

Immunofluorescence

Cells were seeded in a confocal petri dish and treated with drugs for 24 h. Then, the cells were washed 3 times with precooled PBS for 5 min each. An appropriate amount of 4% paraformaldehyde was added, and the cells were fixed for 20 min. After blocking with 10% BSA in PBS for 2 h, the cells were washed 3 times with ice-cold PBS for 5 min each time. Then, the cells were incubated with primary antibodies at 4 °C overnight. The primary antibody was discarded, and the cells were washed with PBS 3 times for 5 min each. DyLight 488- or DyLight 594-conjugated secondary antibody was added to the cells and incubated at room temperature for 2 h in the dark. The cells were then incubated with an appropriate amount of DAPI in the dark for 10 min before being observed by laser confocal microscopy (Olympus, Tokyo, Japan).

HepG2 cell transfection

Fresh medium containing lentiviral particles was added and mixed gently. After 12-16 h of infection in the cell culture incubator, the culture medium containing the lentiviral particles was aspirated, and culture medium containing 5% inactivated FBS was added to the cells to continue the culture. After 72 h of infection, complete medium containing puromycin was prepared according to the optimum drug screening concentration obtained in a previous experiment, and culture was continued for 72 h, observing the cells every day. After 3 days of drug screening, the infected cells screened by puromycin were digested and identified by Q-PCR or Western blotting to obtain SIRT2-overexpressing HepG2 cells.

Statistical analysis

The data are presented as the means \pm SEM of measurements obtained from five or eight

batches of cells or six mice in each group per experiment. The statistical analysis among groups was performed using one-way ANOVA followed by Tukey's post hoc test. The significance of differences between two groups was determined by Student's t-test (unpaired, two-tailed). Values of $P < 0.05$ were considered statistically significant.

Results

Silybin attenuated inflammation and pathological changes in the livers of HFD-fed mice

The livers of mice fed a HFD changed significantly based on histopathological results. H&E staining showed obvious intracellular vacuolar defects, steatosis, and inflammatory infiltration in the livers of HFD-fed mice (Fig. 1A). Masson staining revealed significant fibrosis (Fig. 1B), and oil red O staining indicated the accumulation of numerous lipid droplets (Fig. 1C) in the livers of HFD-fed mice. Immunohistochemical staining of F4/80, which is a specific marker of Kupffer cells, suggested that large amounts of Kupffer cells were generated in the livers of HFD-fed mice (Fig. 1D). Most of these pathological changes were significantly improved by silybin treatment. Cytokine secretion data also suggested inflammation and fibrosis in the livers of HFD-fed mice, as evidenced by elevated IL-1 β , IL-6, TNF- α and TGF- β 1 levels. Silybin (40 or 80 mg/kg) administration effectively reduced the levels of these inflammatory factors to relieve liver inflammation and liver fibrosis (Fig. 2A-D). Considering the inflammatory state of the liver, we investigated the response of Kupffer cells, the liver-resident macrophages, to PA stimulation. Kupffer cells secreted a number of inflammatory factors into the supernatant after PA treatment (Fig. 2E), which was consistent with the liver inflammatory state. The supernatant obtained from PA-treated Kupffer cells was used in subsequent cell experiment as CM.

Silybin restored the expression and activity of CYP3A decreased by HFD and CM

We investigated the transcription, protein expression and activity of CYP3A in the livers of HFD-fed mice and CM-stimulated hepatocytes. The results showed that the expression and

activity of CYP3A11 in mouse livers were significantly decreased after exposure to a HFD. In addition, the activity of CYP3A11 was slightly inhibited by high-dose silybin (80 mg/kg) treatment in normal diet-fed mice, which was consistent with previous literature (Wang et al., 2015). However, silybin was effective in restoring the expression and activity level of CYP3A11 in HFD-fed mice (Fig. 3A-D). Similar results were obtained *in vitro*. The expression and activity of CYP3A4 and CYP3A11 was inhibited in CM-treated HepG2 cells and mouse primary hepatocytes, respectively, and these effects were reversed by silybin (Fig. 4A-F). Silybin partly restored PXR mRNA and protein expression in CM-treated mouse primary hepatocytes as well (Fig. 4G,H).

Silybin inhibited NF- κ B translocation through promotion of p65 deacetylation

To clarify how CM affects the transcription of *cyp3a*, we further investigated the nuclear translocation of the NF- κ B p65 subunit. We found that the ratio of acetylated p65 to total p65 was enhanced by HFD and CM treatment (Fig. 5A-C). As shown in Fig. 5D, p65 accumulated in the nucleus in CM-treated HepG2 cells to suppress PXR transcriptional activity. Silybin almost completely eliminated the nuclear translocation of p65 induced by CM. Our previous study had demonstrated that silybin was quite effective in preventing NLRP3 inflammasome assembly through the SIRT2/NAD⁺ pathway during fatty liver disease. In addition, SIRT2 plays an important inhibitory role in inflammation by deacetylating p65 to decrease the expression of NF- κ B-regulated inflammatory genes (Rothgiesser et al., 2019). We hypothesized that this anti-inflammatory pathway was also of great importance in CYP3A regulation during HFD-induced hepatitis because of the transcriptional regulation of PXR by p65. Thus, we determined the expression of SIRT2 and the intracellular NAD⁺ level (Fig. 6A-F). Silybin increased NAD⁺ concentration, elevated SIRT2 expression and decreased p65 acetylation both *in vivo* and *in vitro*, which supported the recovery of PXR transcriptional activity and CYP3A expression.

Overexpression of SIRT2 suppressed p65 nuclear translocation and restored CYP3A transcription

To further verify the role of SIRT2 in CYP3A regulation, we constructed SIRT2-overexpressing HepG2 cells using a lentiviral transfection method. Overexpression of SIRT2 largely decreased the acetylation of p65 and the ratio of acetylated p65 to total p65 with or without CM (Fig. 7A). CM stimulation greatly promoted the translocation of NF- κ B p65 into the nucleus, which was reversed by SIRT2 transfection (Fig. 7B). The regulatory effect of SIRT2 on CYP3A was also observed. NF- κ B p65 translocation caused by CM reduced the mRNA level of *cyp3a*; however, *cyp3a* transcription was restored by SIRT2 overexpression, similar to silybin (Fig. 7C).

NAD⁺ supplementation regulated CYP3A transcription

Since SIRT2 is an NAD⁺-dependent deacetylase, we tested the NAD⁺ level in the context of HFD-induced fatty liver disease. The NAD⁺ concentration markedly decreased in the livers of HFD-fed mice and CM-treated hepatocytes/HepG2 cells. Silybin performed well in restoring NAD⁺ levels (Fig. 6A-C). Then, we investigated the role of NAD⁺ in *cyp3a* regulation by adding NMN as an NAD⁺ donor. The enhancement of intracellular NAD⁺ levels was quite effective in reversing CM-induced decrease in SIRT2 expression (Fig. 8A, B), p65 acetylation (Fig. 8C, D), p65 nuclear translocation (Fig. 8E), and *cyp3a* transcription in both HepG2 cells and mouse hepatocytes (Fig. 8F-I), suggesting that the role of silybin in regulating metabolic enzymes during liver inflammation was achieved by increasing NAD⁺ and SIRT2 levels.

Silybin restored intracellular NAD⁺ levels through NAMPT and PARP1

We further studied the NAD⁺ synthetase NAMPT and the NAD⁺-depleting enzyme PARP1, which are related to NAD⁺ levels, in primary hepatocytes and HepG2 cells by Western blotting (Fig. 9). The results indicated that CM caused a decrease in NAD⁺ levels by downregulating NAMPT expression and enhancing PARP1 expression. Silybin slightly but

not significantly increased NAMPT expression, but it obviously reduced PARP1 expression. The down regulated PARP1 expression might be the reason for the restoration of intracellular NAD⁺ levels.

Discussion

This study revealed the differential regulation of CYP3A by silybin under physiological and pathological conditions. Silybin has been recognized as an enzyme inhibitor in both *in vivo* and *in vitro* studies (Zuber et al., 2002; Fuhr et al., 2007; Wang et al., 2015) and is not an inducer of CYP3A4 in primary human hepatocytes (Kosina et al., 2005). However, all these studies were based on healthy animals or healthy hepatocytes, and silybin is a well-known hepatoprotective agent for the treatment of various liver diseases. The influence of silybin on P450s under liver injury is quite different from that under normal conditions because of its hepatoprotective effects. Its effect on enzyme activity is the net effect of hepatoprotection and enzyme inhibition. According to our data, both HFD and PA treatment led to a decrease in CYP3A expression and activity, and silybin did not inhibit but instead restored CYP3A expression and activity. Therefore, we further explored the underlying mechanism. In our previous research, silybin showed a strong anti-inflammatory effect by inhibiting NLRP3 inflammasome assembly, and this effect depended on the NAD⁺/SIRT2 pathway (Zhang et al., 2018). Thus, we investigated whether the regulatory effect of silybin on CYP3A was related to this pathway.

Kupffer cells are resident macrophages of the liver and comprise approximately 15% of liver cells. In the early stage of NAFLD, Kupffer cells expand and secrete cytokines such as IL-1 β and TNF α . These cytokines, as inflammatory signals, exert many downstream effects, including their impact on P450s (Morgan, 2001; Duarte et al., 2015; Tacke, 2017). Previous research investigated how cytokines regulate P450s through NF- κ B under LPS-induced inflammation (Morgan et al., 2002; Wree et al., 2014). Recently, the NLRP3 inflammasome has been recognized as an important factor in the development of inflammation, and it drives

liver diseases, such as NAFLD, hepatitis, and liver fibrosis, by activating caspase-1 and releasing cytokines (Wree et al., 2014; Mridha et al., 2017). Based on our former studies, silybin prevents NLRP3 inflammasome activation through the NAD⁺/SIRT2 pathway to downregulate AC- α -tubulin expression (Zhang et al., 2018). This evidence suggests that the effect of silybin on CYP3A might be related to its anti-inflammasome activity. Thus, we further investigated how the NAD⁺/SIRT2 pathway influences CYP3A transcription.

Alterations in NAD⁺ homeostasis have been found in many diseases, including metabolic syndrome, cancer, and even cardiovascular disease (Matasic et al., 2018; Katsyuba et al., 2020). NAD⁺ and NADH are in rapid cycling, and the total pool size of NAD(H) depends on the relative rates of synthesis and degradation. SIRT2 is an NAD⁺-dependent deacetylase and the only sirtuin protein predominantly located in the cytoplasm and also in the mitochondria and nucleus (Wang et al., 2019). A considerable number of SIRT2 substrates and SIRT2-related proteins have been identified, which play important roles in physiological and pathophysiological conditions (Wang et al., 2019). NF- κ B is one of the proteins regulated by SIRT2. The posttranslational modification of the p65 subunit determines the outcome of NF- κ B-mediated transcription of various cellular genes. Phosphorylation and acetylation are the most common posttranslational modifications of the p65 subunit. Acetylated p65 is prone to disassociate from I κ B α and change its subcellular localization (Chen et al., 2001; Greene and Chen, 2004). Our study showed that a HFD caused a decrease in NAD⁺ levels and SIRT2 expression in hepatocytes, resulting in elevated levels of NF- κ B p65 subunit acetylation in the nucleus. Its translocation into the nucleus ultimately affects the transcription of CYP3A. In the nucleus, NF- κ B can inhibit CYP3A through its repressive effect on PXR (Na et al., 1999; Gu et al., 2006; Zordoky and El-Kadi, 2009). This study helped us understand how silybin restored cyp3a transcription during liver injury while inhibiting CYP3A activity under physiological conditions.

P450s comprise a superfamily of metabolizing enzymes with activity toward endogenous and xenobiotic substrates. Considering the similarity of the main P450 subenzymes, we chose

CYP3A as a representative enzyme in our study because it comprises up to 60% of the total hepatic CYP content and is responsible for the metabolism of more than 30% of clinically used drugs (Danielson, 2002; Guengerich, 2019). When it comes to liver diseases, the big drop of metabolic enzymes may cause dose adjustment in medical treatments. Compared to the HFD-fed mice group, silybin treatment almost recovered CYP3A expression, which means dose adjustment is no longer required. If not, drug-drug interactions (DDIs) may be induced. As a commonly used clinical drug, silybin showed synergistic effects on protecting the liver and regulating hepatic enzymes in liver diseases, which is different from its inhibition of CYP3A under normal physiological status. In this study, we elucidated the different regulatory effects of silybin on CYP3A under physiological conditions and liver injury pathological conditions. Silybin shows advantages in liver protection not only because of its pharmacological effects but also because of its pharmacokinetic regulation. Based on the NAD⁺/SIRT2/NF- κ B pathway, silybin both inhibits inflammation and restores the CYP3A expression decreased in NAFLD. This finding helps us to better understand the pharmacological action of silybin and to use it more rationally.

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Contributed new reagents or analytic tools: Ran Zhang and Rui Wang.

Performed data analysis: Ran Zhang and Rui Wang.

Wrote or contributed to the writing of the manuscript: Yuan Xie, Dan Xu, Ran Zhang, Rui Wang, Jiye Aa and Guangji Wang.

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Conflict of interests

The authors declare that there is no conflict of interest.

Legends

Fig. 1. Silybin improved liver steatosis, inflammation, and fibrosis. Mice fed a HFD for 12 weeks were orally administered silybin (40 or 80 mg/kg) in the last 4 weeks. (A-C) Representative photomicrographs of H&E, Masson and oil red O staining of mouse livers; (D) liver F4/80 immunohistochemical staining.

Fig. 2. Silybin reduced inflammatory cytokine secretion in the livers of HFD-fed mice. (A-D) The levels of IL-1 β , IL-6, TNF- α and TGF- β 1 in the liver were detected with ELISA kits (n = 6). Data are expressed as the means \pm SEM from 3 independent experiments. ##P \leq 0.01, ###P \leq 0.001 vs. blank; **P \leq 0.01, ***P \leq 0.001 vs. model. (E) The levels of IL-1 β , IL-6 and TNF- α in the supernatant of Kupffer cells were detected with ELISA kits (n=3). #P \leq 0.05, ###P \leq 0.001 vs. blank.

Fig. 3. Effect of silybin on the expression and activity of CYP3A in the livers of HFD-fed mice. (A) The relative mRNA levels of CYP3A11 in mouse livers were detected by Q-PCR. (B) The protein expression levels of CYP3A were detected by Western blotting. (C, D) The levels of 1'-hydroxymidazolam and 6 β -hydroxytestosterone were detected by HPLC/MS/MS. #P \leq 0.05, ###P \leq 0.001 vs. blank, *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001 vs. model.

Fig. 4. Effect of silybin on the expression and activity of CYP3A in CM-stimulated HepG2 cells and hepatocytes. (A, B) The relative mRNA levels of CYP3A4 and CYP3A11 in cells were detected by Q-PCR. (C, D) The protein expression levels of CYP3A were detected by Western blotting. (E, F) The 1'-hydroxymidazolam levels were detected by HPLC/MS/MS. #P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001 vs. blank, *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001 vs. model.

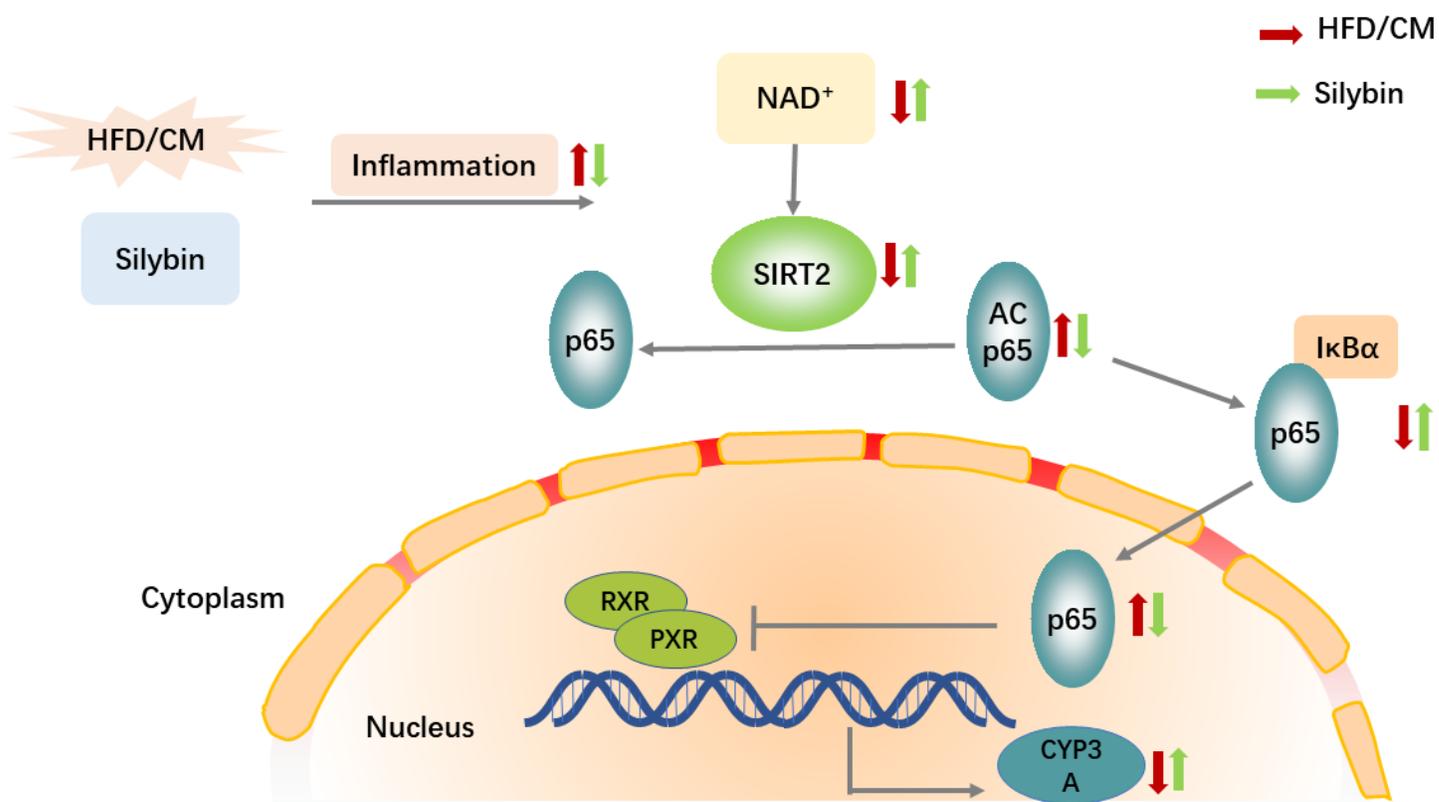
Fig. 5. Silybin decreased NF- κ B p65 acetylation and the nuclear translocation of p65. (A-C) The acetylation level of p65 in mouse primary hepatocytes and HepG2 cells was detected by Western blotting. (D) The nuclear translocation of p65 in HepG2 cells was observed by laser confocal microscopy. ###P \leq 0.01 vs. blank, *P \leq 0.05, **P \leq 0.01 vs. model.

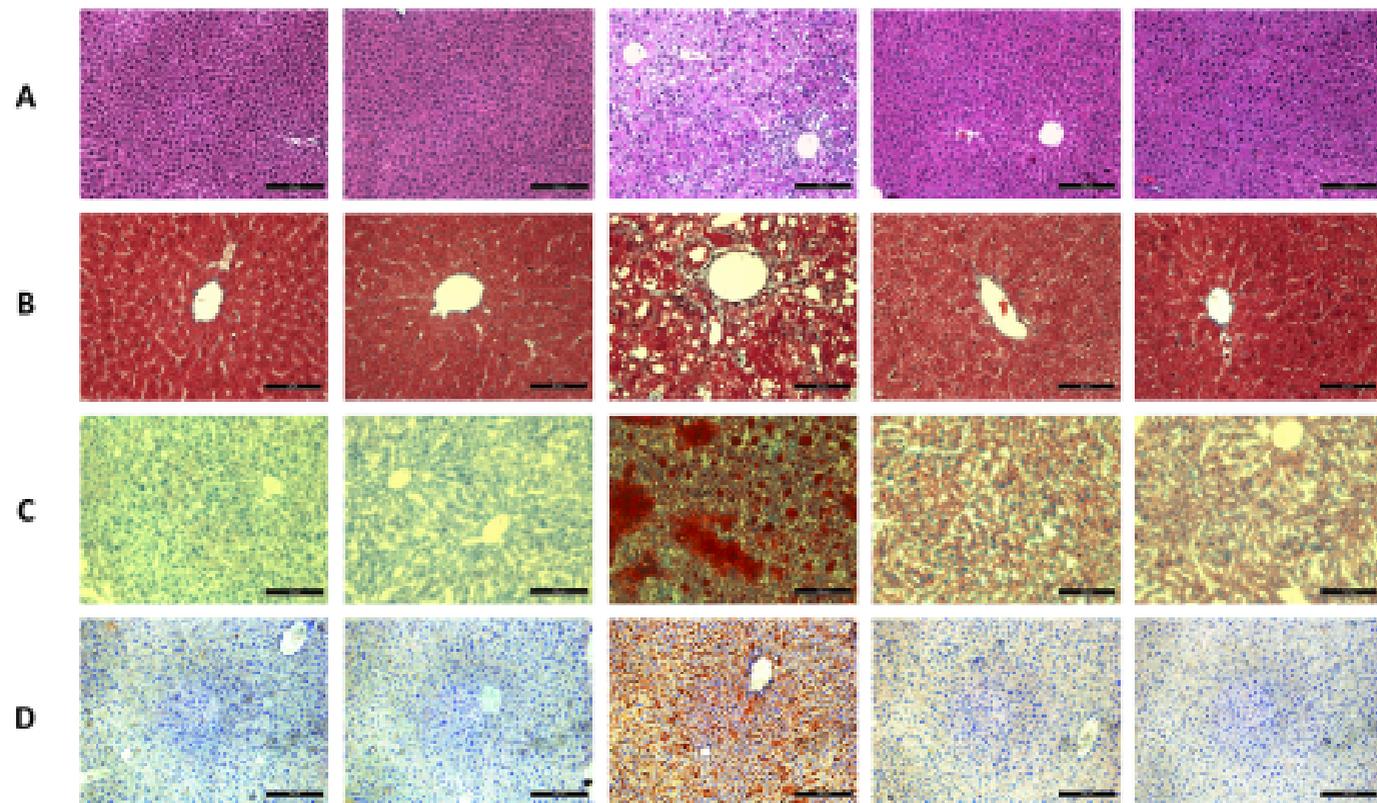
Fig. 6. Silybin restored the levels of NAD⁺ and SIRT2 expression. (A-C) The relative levels of NAD⁺ in mouse livers, primary hepatocytes and HepG2 cells were detected by LC-MS/MS. (D-F) SIRT2 expression in mouse livers, primary hepatocytes or HepG2 cells was detected by Western blotting. #P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001 vs. blank, *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001 vs. model.

Fig. 7. SIRT2 deacetylated AC-NF- κ B p65. SIRT2-overexpressing HepG2 cells were constructed by lentiviral transfection and then stimulated with CM for 24 h. (A) The expression levels of acetylated p65 in HepG2 cells were detected by Western blotting. (B) The nuclear translocation of p65 in HepG2 cells was observed by laser confocal microscopy. (C) SIRT2 overexpression enhanced cyp3a4 transcription in HepG2 cells. #P \leq 0.05, ##P \leq 0.01 vs. blank, *P \leq 0.05 vs. model.

Fig. 8. Supplementation of NAD⁺ with its donor NMN attenuated p65 acetylation and restored cyp3a4 transcription. (A-D) SIRT2, NF- κ B p65 and acetylated NF- κ B p65 protein levels in hepatocytes and HepG2 cells were detected by Western blotting. (E) NMN inhibited p65 translocation into the nucleus in HepG2 cells. (F, G) The mRNA levels of cyp3a4 in primary hepatocytes and HepG2 cells were detected by Q-PCR. (H, I) NAD⁺ levels were significantly enhanced by adding NMN to mouse primary hepatocytes and HepG2 cells. #P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001 vs. blank, *P \leq 0.05, **P \leq 0.01, vs. model.

Fig. 9. Silybin restored NAD⁺ levels through regulation of NAMPT and PARP1 expression. The protein levels of NAMPT and PARP1 in primary hepatocytes and HepG2 cells were detected by Western blotting. #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001 vs. blank, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 vs. model.





HFD
Silybin(mg/kg)

-
-

-
80

+
-

+
40

+
80

Figure 1

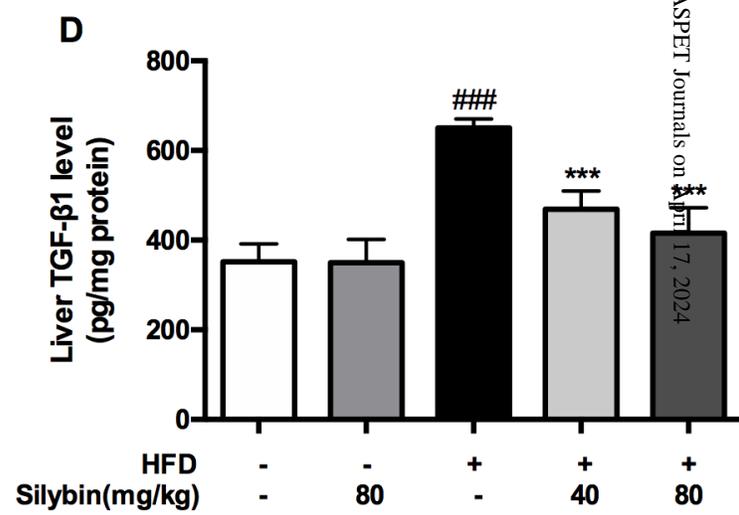
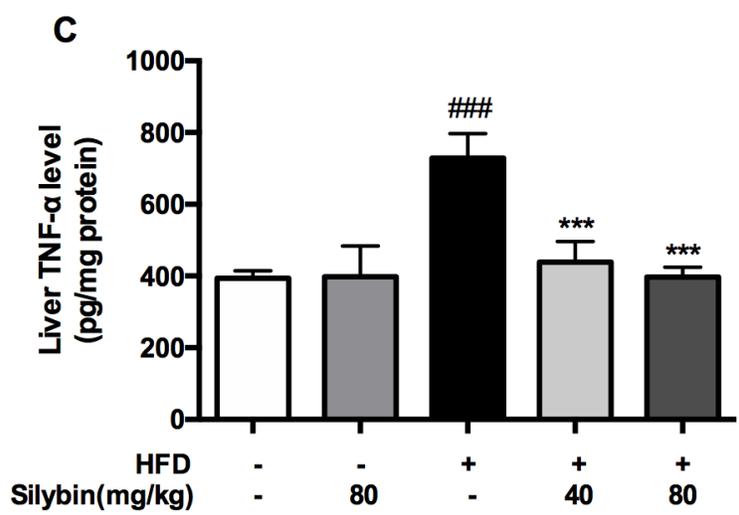
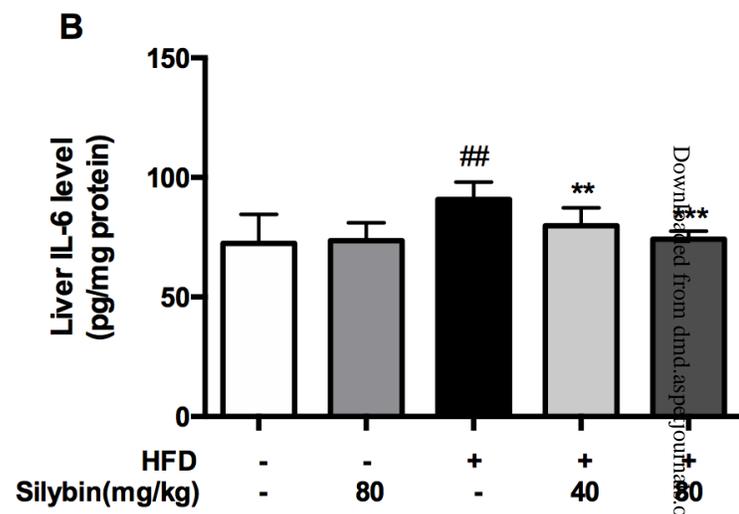
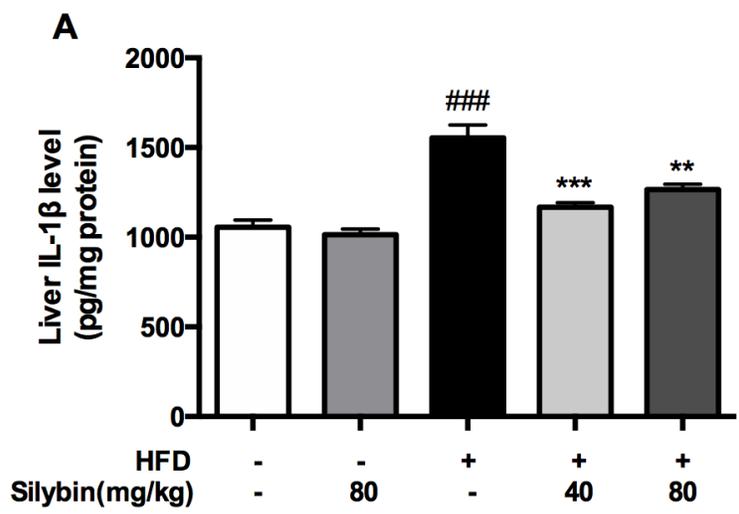


Figure 2

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Supernatant of Kupffer cells

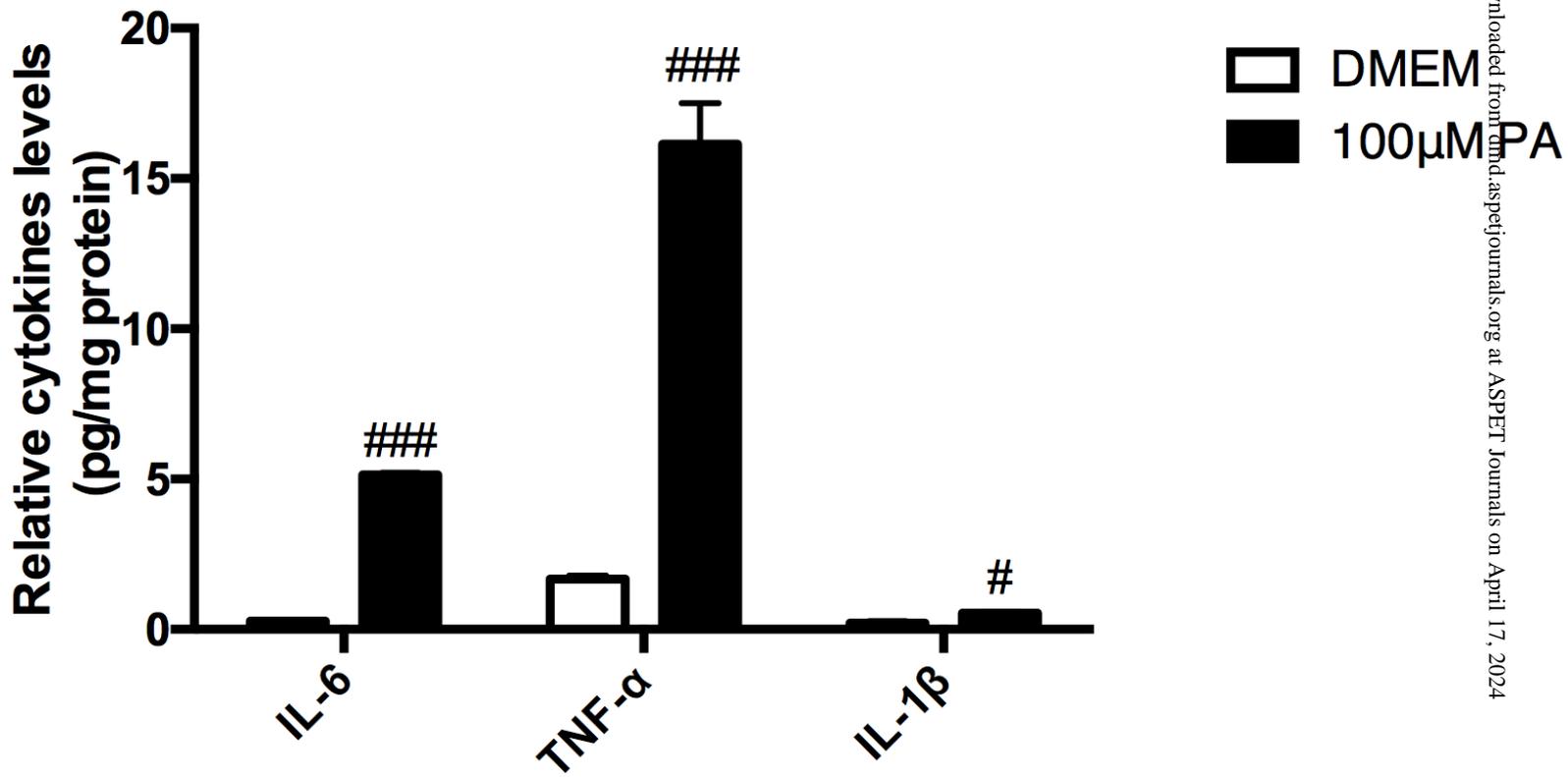


Figure 2

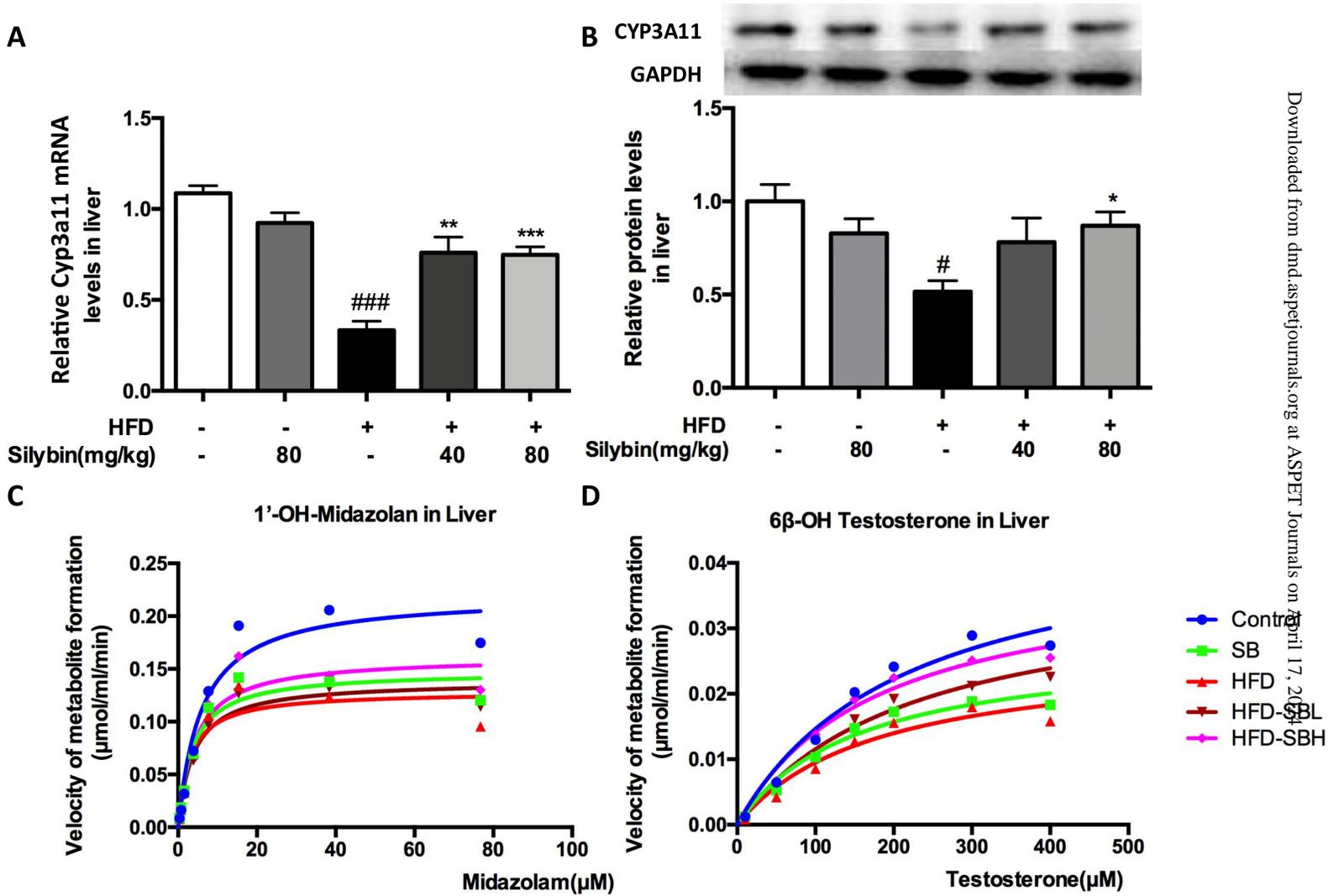


Figure 3

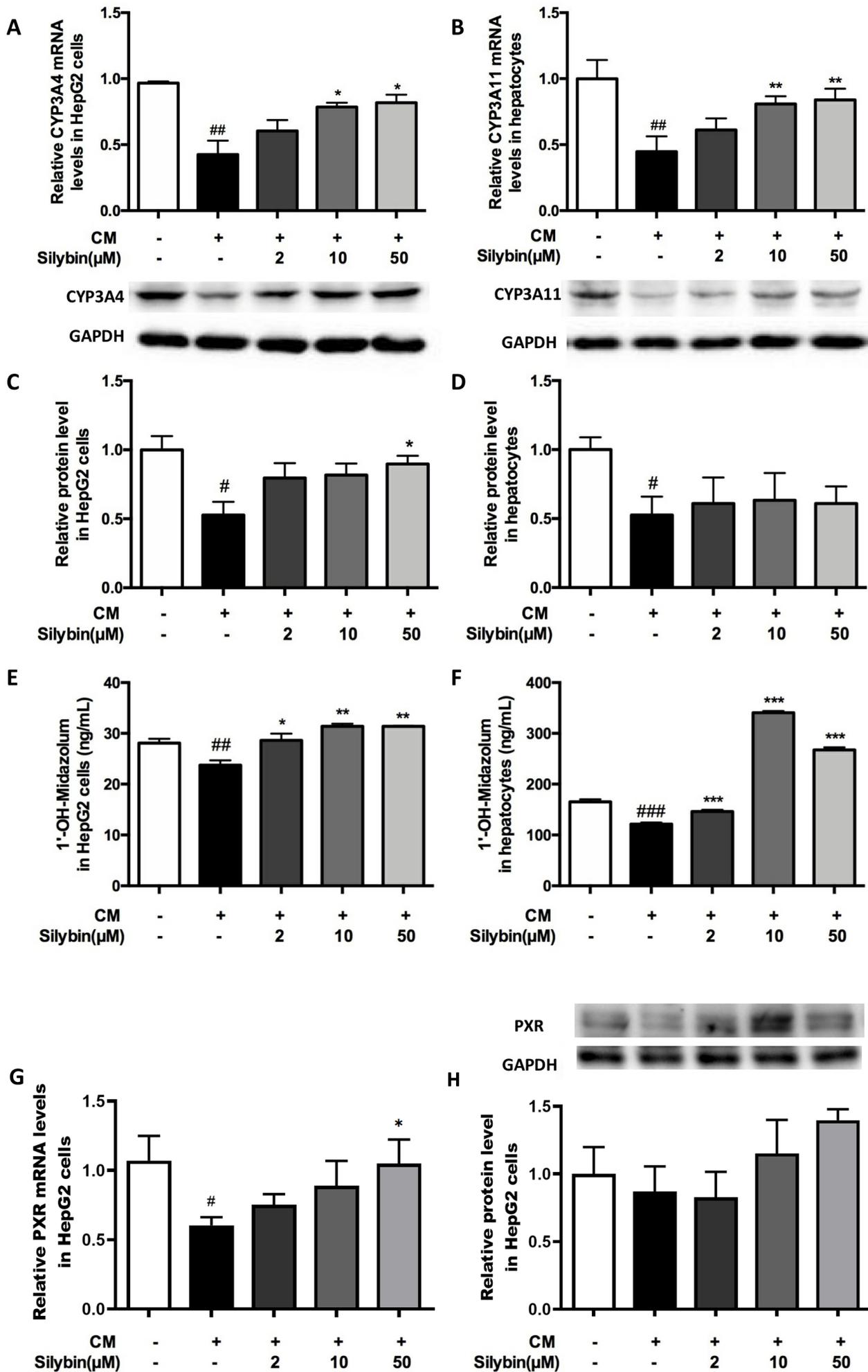


Figure 4

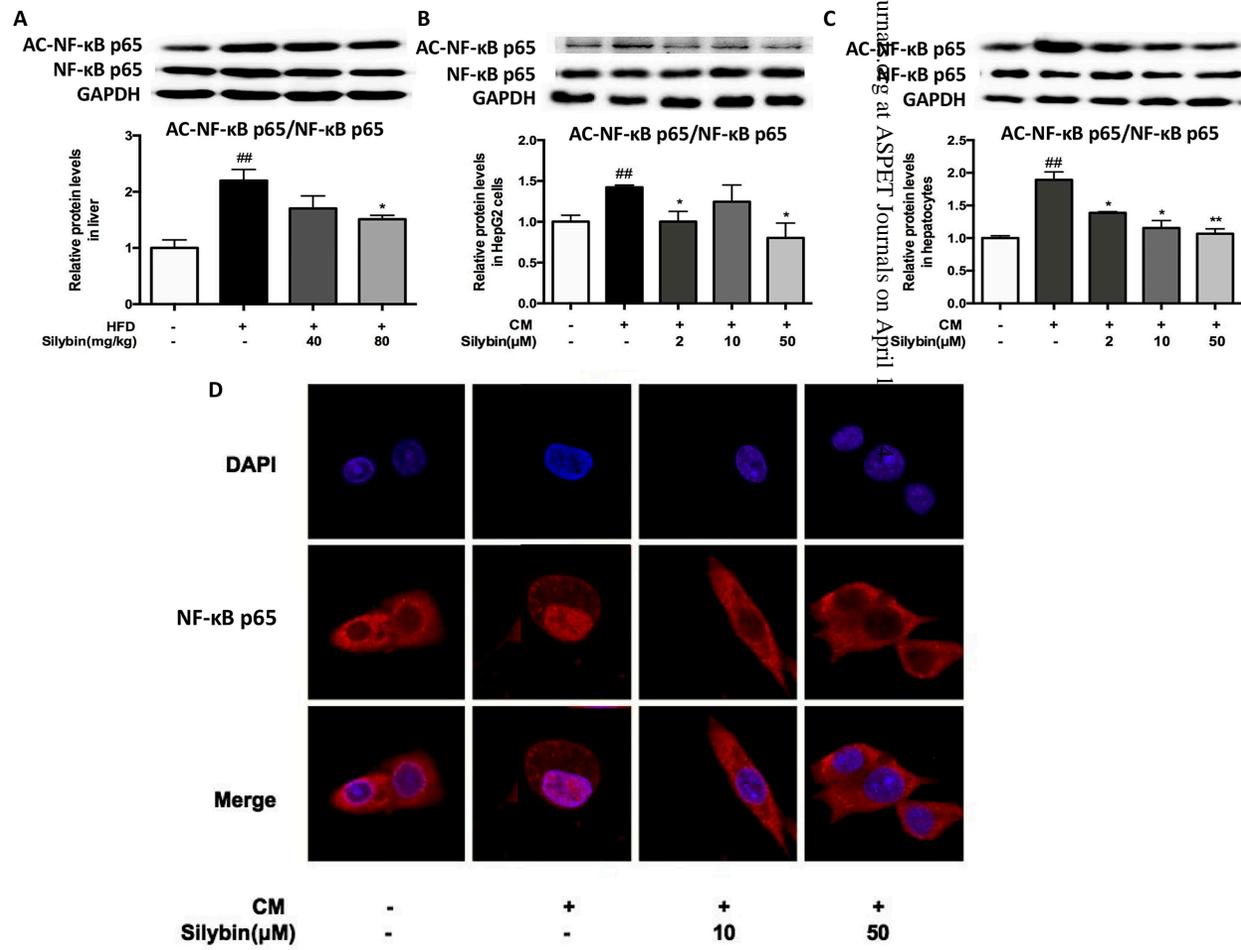


Figure 5

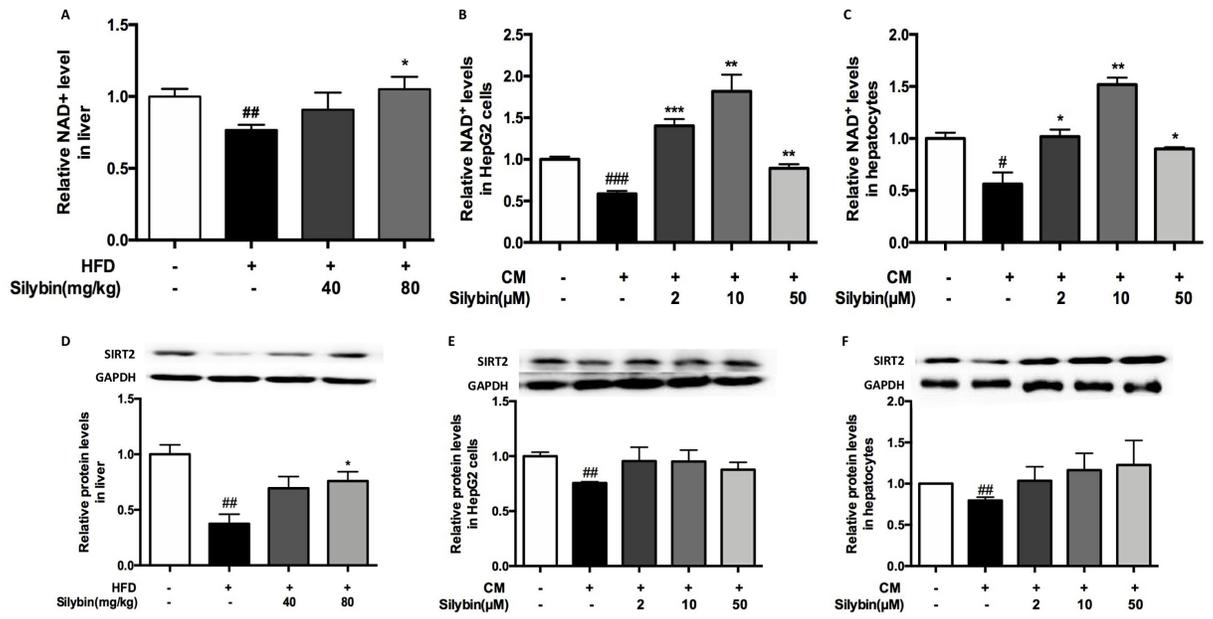


Figure 6

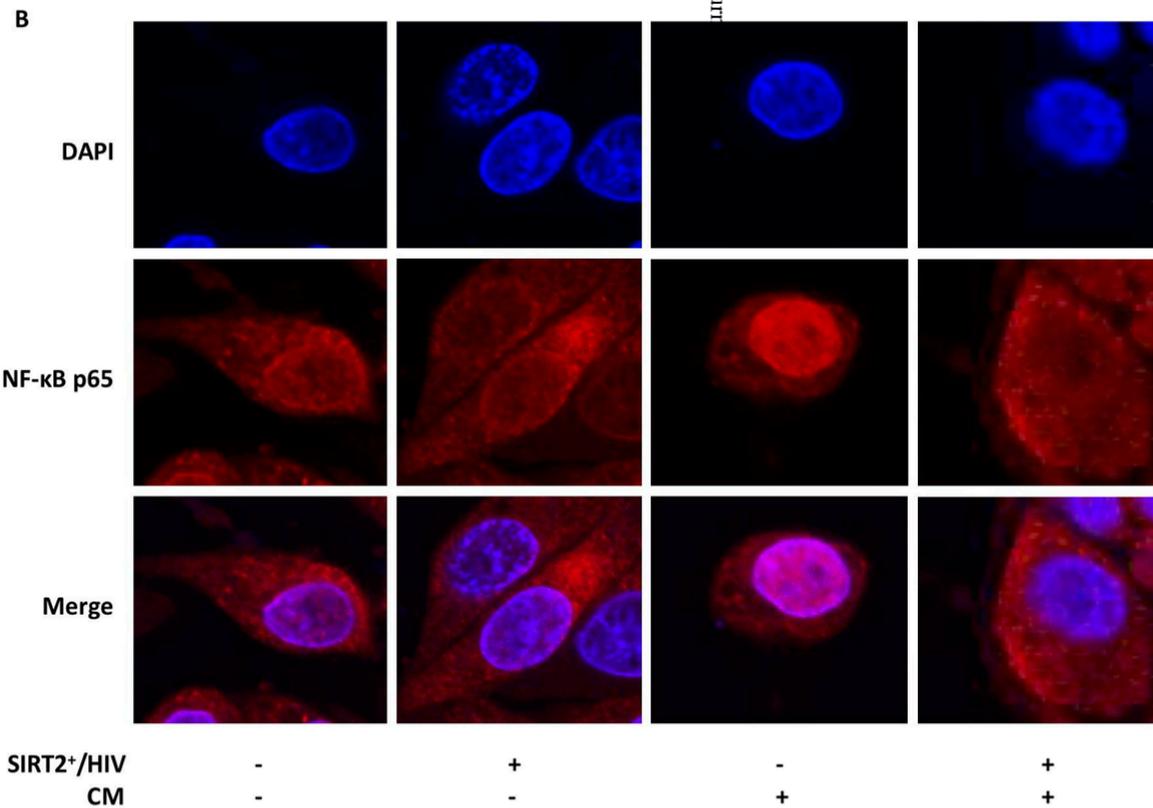
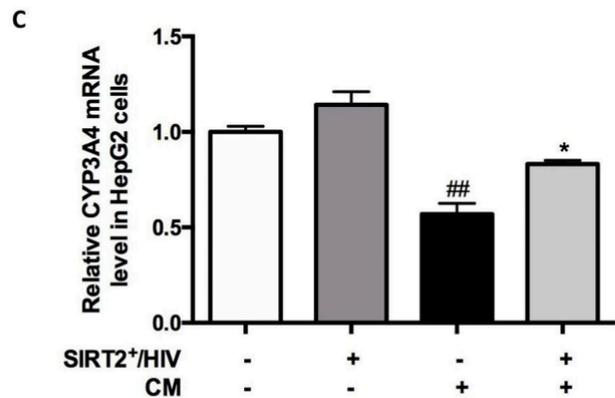
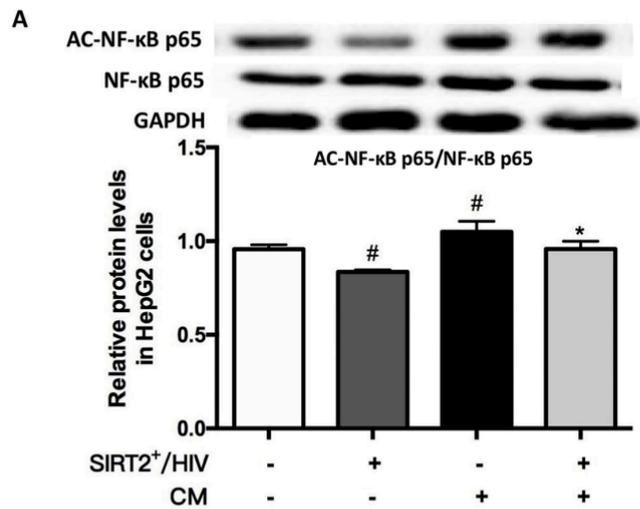


Figure 7

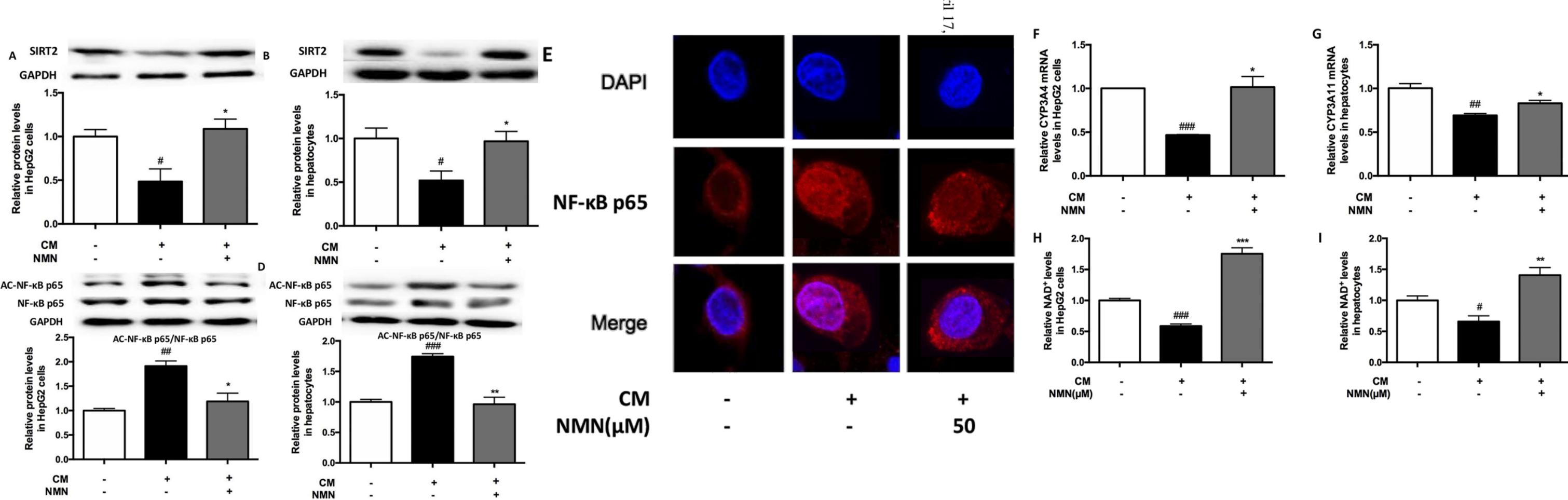


Figure 8

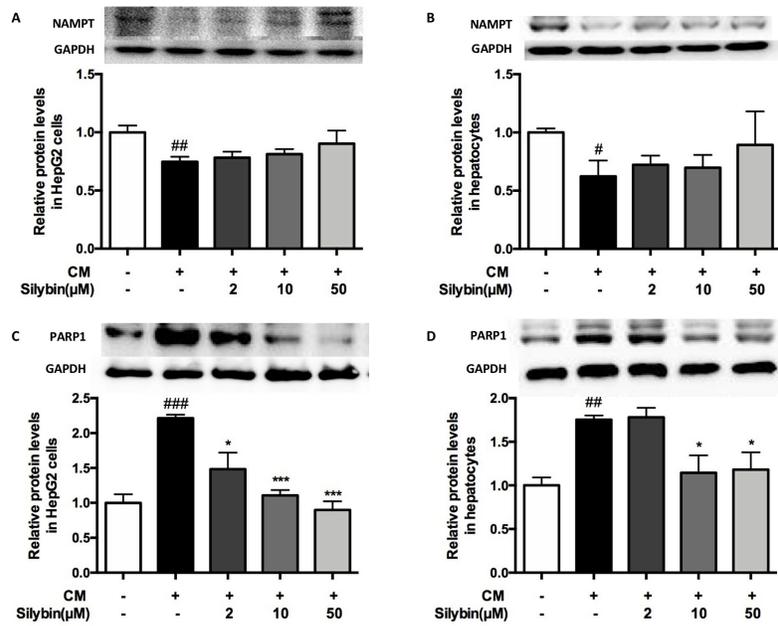


Figure 9