Life Sciences 225 (2019) 20–28

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Nicotinamide riboside protects against liver fibrosis induced by CCl_4 via regulating the acetylation of Smads signaling pathway

Rui Jiang[a,c], Yujia Zhou[a], Sufan Wang[a], Nengzhi Pang[a], Yuanling Huang[a], Mingtong Ye[a], Ting Wan[a], Yun Qiu[a], Lei Pei[a], Xuye Jiang[a], Yufeng Huang[b], Hainan Yang[b], Wenhua Ling[a], Xufeng Li[b,*], Zhenfeng Zhang[b,*], Lili Yang[a,**]

[a] Department of Nutrition, Guangdong Provincial Key Laboratory of Food, School of Public Health, Sun Yat-sen University, Guangzhou, Guangdong Province 510080, People's Republic of China
[b] Department of Radiology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province 510260, People's Republic of China
[c] Nutrition Clinic, The Maternal & Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi Zhuang Autonomous Region 530003, People's Republic of China

ARTICLE INFO

Keywords:
Nicotinamide riboside
Sirt1
P300
HSCs
TGF-β
Deacetylation

ABSTRACT

Aims: Increasing nicotinamide adenine dinucleotide (NAD^+) by Nicotinamide riboside (NR) provides protective benefits in multiple disorders. However, the role of NR on liver fibrosis is unclear. We performed in vivo and in vitro experiments to test the hepatic protective effects of NR against liver fibrosis and the underlying mechanisms.

Materials and methods: Mice were injected with CCl_4 to establish liver fibrosis model. NR was given by gavage to explore the hepatic protection of NR. LX-2 cells were given a TGF-β stimulation ± NR, the activation of LX-2 cells and the acetylation of Smads were analyzed. To further confirm the role of Sirt1 on the protective pathway of NR, we knockdown Sirt1 in LX-2 cells.

Key findings: We found NR could prevent liver fibrosis and reverse the existing liver fibrosis. NR inhibited the activation of LX-2 cells induced by TGF-β, activated Sirt1 and deacetylated Smad2/3. Sirt1 knockdown diminished the inhibiting effect of NR on LX-2 cells activation, and increased expressions of acetylated Smads. In conclusion, NR could prevent liver fibrosis via suppressing activation of hepatic stellate cells (HSCs). This protective effect was mediated by regulating the acetylation of Smads signaling pathway.

Significance: NR protected mice against liver fibrosis induced by CCl_4.

NR suppressed activation of hepatic stellate cells induced by TGF-β.

NR protects liver fibrosis via increasing the activity of Sirt1 and decreasing the expression of P300, resulting in the deacetylation of Smads in stellate cells.

1. Introduction

Liver fibrosis is one of the diseases with high mortality and morbidity around the world, which may process to a series of irreversible diseases, including liver cirrhosis and hepatic failure [1]. Liver fibrosis is characterized by superfluous deposition of extracellular matrix (ECM). Hepatic stellate cells (HSCs), the specific myofibroblasts and endothelial cells in liver, are the main source of ECM. Normally, the generation and degradation of ECM were kept in balance. During the persistent stimulus of hazardous factors, such as toxic chemicals, alcohol drinking, and viral infection [2], HSCs were activated and led to ECM deposition, resulting in liver fibrosis [3–5].

TGF-β is the major one of the pro-fibrogenic and inflammatory factors secreted by damaged liver tissues, which plays pivotal roles in the process of HSCs activation [6–8]. Upon binding to transforming growth factor receptor, Smad2/3 are phosphorylated. P-Smad2/3 (Phosphorylated Smad2/3) further bind to the common mediator Smad (co-Smad)-Smad4, forming the complexes, which relocate to the

* Corresponding authors at: Department of Radiology, The Second Affiliated Hospital of Guangzhou Medical University, 250 Changgang East Road, Guangzhou, Guangdong Province 510260, People's Republic of China.
** Correspondence to: L. Yang, Department of Nutrition, School of Public Health, Sun Yat-sen University, No. 74 Zhongshan Road 2, Yuexiu District, Guangzhou, Guangdong Province 510080, People's Republic of China.
E-mail addresses: lixufeng@gzhmu.edu.cn (X. Li), zhangzhf@gzhmu.edu.cn (Z. Zhang), yangll7@mail.sysu.edu.cn (L. Yang).

https://doi.org/10.1016/j.lfs.2019.03.064
Received 28 January 2019; Received in revised form 25 March 2019; Accepted 25 March 2019
Available online 27 March 2019

nucleus [9,10]. It has been suggested that transcription of target gene, such as collagen1, needs the binding of (activated) specific transcription factors to DNA promoter elements, which often require bridging coactivators, such as CBP/P300, the interaction partners of cAMP response element binding protein (CREB) [11,12]. As an acetylase, P300 regulates transcriptional activity by acetylation of Smad2/3 [13–15]. In previous studies, P300 had been identified as a significant regulatory factor in TGF-β/Smads signaling pathway, which represents a major signaling route in fibrogenic activity in multiple organizations [16,17]. Another study also demonstrated that AMPK plays a role in protecting against TGF-β-induced fibrogenic activity in HSCs by regulating P300 [18]. Thus, factors regulating the expression or acetylation effect of P300 may play an influential role in TGF-β/Smads signaling pathway, which further affects the formation of liver fibrosis.

Nicotinamide riboside (NR), a natural form of vitamin B_3 existing in milk, is a defined biosynthetic precursor of nicotinamide adenine dinucleotide (NAD^+) via Nrk-dependent pathway. Studies have demonstrated that NR has beneficial effects on metabolic disorders including hepatic inflammation and steatosis, induced by diet, aging, or other hazardous factors [19–22], which were associated with the increase of cellular NAD^+ and increase of mitochondrial function. NAD^+ is a substrate for NAD^+-consuming enzymes in cells. The histone deacetylase sirtuin1 (Sirt1), one of NAD^+-consuming enzymes, participates in multiple metabolic diseases through deacetylating target proteins [23]. Accumulating evidence have documented that Sirt1 exerts tissue protective effects in liver injury mediated by activating several signaling pathways [24–26]. It was also reported recently that Sirt1 could regulate TGF-β/Smads pathway, and regulate the expression of P300 in lung fibrosis [27]. Although our knowledge on the benefit effects of NR on liver diseases has expanded over the past few years, the role of NR in protecting against liver fibrosis is currently unclear.

In this study, we will unveil whether NR can prevent liver fibrosis and reverse the developed liver fibrosis induced by CCl_4, and whether Sirt1-P300-acetylation of Smads signaling pathway is involved in the protective effect of NR on liver.

2. Materials and methods

2.1. Animal experiments

This study was carried out in agreement with the recommendations of the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Care and Protection Committee of Sun Yat-sen University (Approval No. IACUC-2014-0305). Male C57BL/6 mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China), and were housed under a 25 °C with a 12 h day/night cycle and had ad libitum access to water and food throughout the experiment. Random allocation was taken to divide mice into four groups: control group (n = 8), CCl_4-treated group (n = 8), 8-week NR prevention group (NR-prev) (n = 8), and 4-week NR therapy group (NR-ther) (n = 8). Mice were given an intraperitoneal (IP) injection with either 0.5 μL/g b.w. CCl_4 (CCl_4 group, NR group and NR treatment group) or homologous volume of vehicle (Ctrl) twice per week for 8 weeks as described previously [28]. NR-prev group mice were given a gavage of NR of 400 mg/kg b.w./day, NR was from ChromaDex (Irvine, CA, USA) with a purity of 99.9%, Ctrl group and CCl_4 group mice were given a homologous volume (10 μL/g b.w.) of saline gavage throughout the experiment, meanwhile, NR-ther group mice were given saline for week 1–4, and received 400 mg/kg b.w./day NR treatment for week 5–8. At the end of the 4th week, three of NR-ther mice were sacrificed to test the formation of liver fibrosis. After 8 weeks, all mice were sacrificed, blood and tissue samples were collected. The sections of livers were fixed in 10% formalin, embedded in paraffin, sectioned and stained for standard hematoxylin and eosin (H&E) and sirius red. Both H&E staining and Sirius red staining were observed by an inverted optical microscope (Leica Microsystems, Buffalo Grove, IL).

2.2. Cell culture and treatment

LX-2 cells, an immortalized human hepatic stellate cell line, were kindly donated from Dr. Scott Friedman's lab in Mount Sinai School of Medicine. LX-2 cells were cultured in Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences, Logan, UT, USA) with 2% fetal bovine serum and 1% penicillin and streptomycin at 37 °C with a 5% CO_2 atmosphere. Cells were divided into 4 groups: control group (Ctrl, cells were incubated with basal culture medium), TGF-β treatment group (TGF-β, cells were incubated with 4 ng/mL TGF-β in basal culture medium), 0.5 mmol/L NR supplementary group (0.5NR, cells were incubated with 4 ng/mL TGF-β and 0.5 mmol/L NR in basal culture medium), 1.0 mmol/L NR supplementary group (1.0NR, cells were incubated with 4 ng/mL TGF-β and 1.0 mmol/L NR in basal culture medium).

2.3. siRNA transfection

LX-2 cells were transfected with Sirt1-siRNA (Santa Cruz, Dallas, TX, USA) or Scramble siRNA (Santa Cruz, Dallas, TX, USA) for 24 h, and then were incubated with 4 ng/mL TGF-β in basal culture medium or 4 ng/mL TGF-β with 1.0 mmol/L NR in basal culture medium for 96 h.

2.4. Treatment of LX-2 cells with NAMPT inhibitor

LX-2 cells were treated by vehicle or 1.0 nmol/L FK866 (APO866, Daporinad, Selleck) or 10 nmol/L FK866, with or without TGF-β (4 ng/mL) or NR (1.0 mmol/L) for 96 h.

2.5. Measurements of alanine transaminase (ALT)

ALT was measured in mice serum by ALT assay kit (Jiancheng Biotech, Nanjing, Jiangsu, China) following the manufacture's instruction.

2.6. Quantification of NAD^+ family and ATP, ADP, AMP levels in liver

Liver sections were quick frozen with liquid nitrogen, and were grinded immediately. The grinding sample were added with cold 80% (v/v) methanol in H_2O and homogenized. After centrifugation, supernatant were collected, filtrated using C18-SPE column (Agela Technologies, Wilmington, DE, USA), and vacuum dried. After resuspension with 80% (v/v) methanol in H_2O, the levels of NAD^+, $NADP^+$, NAM, NMN, ATP, ADP and AMP in liver samples were measured with a HyperCard column (Thermo Fisher Scientific, Waltham, MA, USA) with UPLC-QTOF System (Infinity/6538, Agilent Technologies, Santa Clara, CA, USA). Specific mobile phases in this study are as follows: (A) 7.5 mmol/L ammonium acetate with 0.05% (v/v) ammonium hydroxide; (B) 0.05% (v/v) ammonium hydroxide in acetonitrile as described previously [29].

2.7. Protein extraction and immunoblotting

Proteins of liver tissue and cells were extracted using RIPA lysis buffer (Beyotime, Shanghai, China) with 1% PMSF. The lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant were aspirated and protein concentration was measured by BCA protein assay kit (Beyotime Biotechnology, Shanghai, China), and then 30-50 mg proteins were used for immunoblotting. Proteins were separated using 8%–12% SDS-PAGE and then transferred to appropriate polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for an hour and then incubated with primary antibodies for overnight, antibodies are shown in the Table S1. After be incubated with HRP-conjugated secondary antibodies, the membranes were used

for film exposure with ECL Reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Immunoprecipitation and co-immunoprecipitation

Total cell lysates were prepared as described previously. After incubated with IgG antibody and suspended (25% v/v) agarose conjugate (Santa Cruz, Dallas, TX, USA) for 30 min at 4 °C, cell lysates (approximately 1000 μg of total cellular protein) were centrifuged at 3000 rpm for 30s at 4 °C. Supernatant were transferred to incubate superfluous primary antibodies for 2 h and incubated with agarose conjugate for overnight. Pellets were collected after centrifuging at 300 rpm for 30s at 4 °C, washed with PBS, and then resuspended in 2× electrophoresis sample buffer (Santa Cruz, Dallas, TX, USA). Twenty microliters of boiled sample were used for immunoblotting.

2.9. RNA extraction and quantitative Real-Time PCR

Total RNA was extracted from liver tissues or cells according to the manufacturer's protocol of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using cDNA Synthesis kit (TaKaRa, Tokyo, Japan). Real-Time PCR samples were prepared with SYBR Green Supermix kit (Takara, Tokyo, Japan), and Real-Time PCR was performed on a Vii7 system (ABI, Carlsbad, CA, USA). The $2^{-\Delta\Delta Ct}$ threshold cycle method were used to analyze the data. Primer sequences are shown in the Table S2.

2.10. Statistical analysis

Statistical analysis was performed using SPSS 20.0 Software (SPSS, Chicago, IL, USA). The significance of differences between two groups was determined by unpaired two-tailed Student *t*-test. We compared multiple groups with a one-way analysis of variance (ANOVA) with a Dunnett's test. Results are presented as individual data points or mean ± standard error of the mean (SEM). $P < 0.05$ was considered as statistically significant. Graph bars were obtained using Graph Pad Prism 5.0 software (Graph Pad Prism, San Diego, CA, USA).

3. Results

3.1. NR protects and reverses the development of fibrosis in CCl_4 injected mice

In this study, serum ALT, an indicator of hepatocyte damage, was significantly elevated by CCl_4, whereas NR could reduce the serum ALT level both in the 4-week therapeutic intervention and the 8-week NR preventive treatment (Fig. 1B). CCl_4 is a strong inducer of hepatic fibrosis. Sirius red staining revealed that CCl_4 injected mice exhibited a high level of collagen deposition as compared with control mice, and collagen deposition was alleviated in mice livers with 8-week NR preventive treatment (Fig. 1C–D). As for NR therapeutic treated group, three mice in the group were sacrificed at the end of stage 1 (CCl_4 injecting without NR treatment for 4 weeks). The paraffin sections of livers were used for testing whether the fibrosis were developed. As is shown, after treated with CCl_4 for 4 weeks, collagen deposition were observed in the mice livers, which indicated that fibrotic changes occurred in mice livers after 4-week CCl_4 stimulation (supplementary material Fig. S1A). Nevertheless, after 4-week NR therapeutic treatment (continued treatment with CCl_4), collagen deposition was reduced in mice livers of NR therapy group (Fig. 1C). CCl_4 also induced inflammatory cells infiltration into livers, while both 8-week and 4-week NR treatment attenuated this inflammatory response in mice (Fig. 1C). An important marker of HSCs activation, smooth muscle actin (α-SMA), was increased by CCl_4 stimulation and decreased by NR preventive and therapeutic treatment (Fig. 1E, F). These data were also confirmed by the fibrosis gene expressions, such as acta2 (α-SMA), col1a1 (collagen1) (Fig. 1G, J). Interestingly, these protective effects were accompanied with the levels of TGF-β in mice livers, which were upregulated by CCl_4, and downregulated by NR both in preventive and therapeutic treatment, in both mRNA and protein levels (Fig. 1H, I).

3.2. NAD^+ content and energy homeostasis were corrected by NR treatment in mice

As a confirmed biosynthetic precursor of NAD^+, NR was reported to have the ability to boost NAD^+ pool in vivo. Thus, we explored whether NR could increase NAD^+ levels in livers of CCl_4 injected mice. Hepatic NAD^+ levels were significantly downregulated by CCl_4, which were replenished by NR under 4-week therapy or 8-week preventive intervention (Fig. 2A). Although change in CCl_4 injected mice was not statistically significant compared to control, $NADP^+$ levels were significantly upregulated in both 4-week therapeutically and 8-week NR preventively treated mice (Fig. 2B). In line with the changes of NAD^+ and $NADP^+$ levels, Nam and NMN were slightly decreased (no statistical significance) in CCl_4 injected mice compared with control, which were upregulated in mice livers of 4-week therapeutic treatment with NR, in spite of the insignificant elevation in 8-week NR preventive treatment group (Fig. 2C–D). NAD^+ is a co-enzyme for redox reaction. We further investigated whether NAD^+ content changes could influent energetic status in mice livers. As a result, CCl_4 decreased hepatic ATP contents significantly, and NR supplementation, to some extent, could reverse the loss of ATP contents, but the differences showed no significance between NR preventively/therapeutically treated mice and CCl_4 injected mice (Fig. 2E). There were no significant changes of AMP levels in our study, however, the ratio of hepatic ATP/AMP were significantly decreased in CCl_4 treated mice, which were slightly increased in both 4-week and 8-week NR treated mice compared with CCl_4 treated mice, but without reaching statistical significance (Fig. 2F–G).

3.3. NR supplementation increased Sirt1 protein expression and suppressed P300 expression in vivo

Not only playing a role in redox reaction, NAD^+ also impacts metabolism by activating NAD^+-consuming enzymes. We further observed protein expression of Sirt1, and found that Sirt1 was decreased slightly in CCl_4 injected mice livers (whereas the difference was not statistically significant), and was upregulated in 8-week NR treated mice livers (Fig. 3A–B).

To explore the mechanism of NR on protecting against liver fibrosis in mice, we next investigated TGF-β/Smads pathway, as increased TGF-β expression had been detected in the livers of CCl_4 injected mice. We first investigated mRNA expressions of TGF-β receptors in mice. We found that mRNA expression of tgfbr2 (TGF-β receptor 2) was increased by CCl_4, which was reduced by NR preventive treatment, and the decreased effect of NR was also found in 8-week therapeutic treated mice, although the difference was not significant between CCl_4 group and NR-ther group (Fig. 3C). Western blot revealed that CCl_4 didn't increase protein expressions of P-Smad2 and P-Smad3, and NR couldn't attenuate phosphorylation of Smad2 and Smad3 in mice livers. Nevertheless, the expression of Smad4 was suppressed by 4-week NR treatment although its expression was not significantly upregulated by CCl_4 (Fig. 3D–G).

Recent studies suggest a potential crosstalk between Sirt1 and P300. Since Sirt1 is a vital histone deacetylase, we further investigated whether the transcriptional co-activator P300 protein was impacted by NR treatment. Interestingly, P300 expression in mice livers was higher in CCl_4 injected mice, while the level of which was suppressed by both preventive and therapeutic NR treatment (Fig. 3H–J).

We further investigated the activity of Sirt1 in mice livers. We found that the acetylation of Smad2/3 was increased in mice livers of CCl4 group compared with Ctrl mice, while it was decreased by NR both in groups of preventive and therapeutic treatment (Fig. 3K, L).

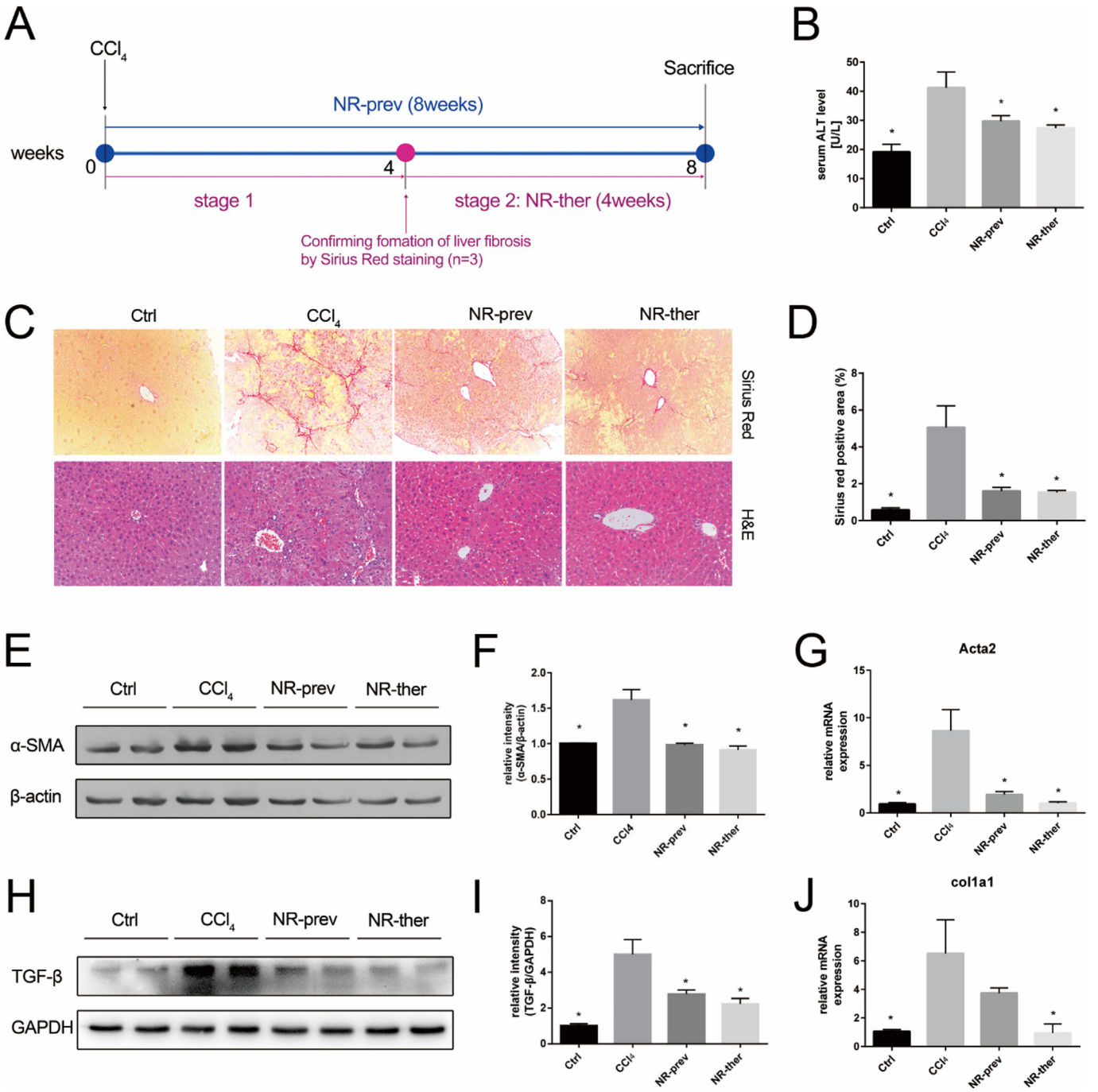


Fig. 1. NR protected against liver injuries induced by chronic CCl_4 exposure. Mice were given either CCl_4 or vehicle intraperitoneally twice a week, with or without NR supplementation for 8 weeks. (A) Schematic protocols of the four experimental groups. (B) Levels of serum ALT. (C) Sirius red staining and H&E staining of liver. (D) Quantification of Sirius red staining from n = 4–8 livers/group. (E–F) Immunoblotting and quantification of α-SMA in mice liver tissues. (G) Relative mRNA expression of Acta2 (α-SMA) in mice liver tissue. (H, I) Immunoblotting, quantification of TGF-β. (J) Relative mRNA expression of col1a1 (collagen1) in mice liver tissue. n = 4–8 in each group. One-way ANOVA with a post-Dennett's test were used for all statistical analyses. *$P < 0.05$ compared to the CCl_4 group.

3.4. HSCs were activated by TGF-β treatment and NR attenuated the activation of HSCs through deacetylating Smads

As we have found that CCl4 could induce liver fibrogenic activation, in which HSCs activation in response to TGF-β plays a pivotal role, we carried out the experiments with LX-2 cells to investigate whether NR can inhibit stellate cell activation and how NR influences TGF-β/Smads pathway. We first explored whether NR supplementation inhibits LX-2 activation at different time points after cells were treated with TGF-β. In our study, protein expression of α-SMA in LX-2 cells was augmented after treated with TGF-β for 48 h. At the time point of 72 h after treated with TGF-β, both 0.5 mmol/L and 1.0 mmol/L NR exhibited its inhibiting effect on LX-2 activation, and the even more inhibiting effect was found after 96 h' TGF-β treatment (Fig. 4A–B). These data were confirmed by col1a1 mRNA expression in LX-2 after cells were treated with TGF-β with or without NR (Fig. 4C).

To investigate the mechanisms of NR on inhibition of the activation of LX-2 cells induced by TGF-β, the expressions of Smad2/3 and P-Smad2/3 were detected. Western blot analysis revealed that when cells were treated for 120 min, Smad2 was activated by TGF-β, and was

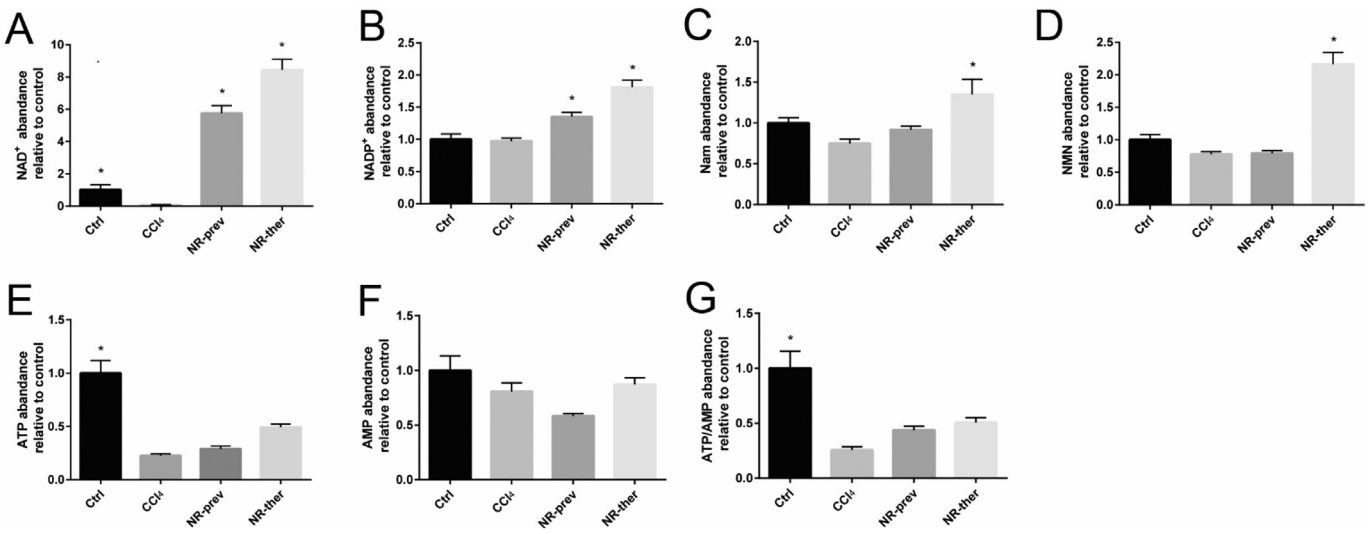


Fig. 2. NR increased NAD + levels and regulated energy metabolism in vivo. Mice were given either CCl_4 or vehicle intraperitoneally injection twice a week, with or without NR supplementation for 8 weeks. (A–F) Levels of (A) NAD^+, (B) $NADP^+$, (C) Nam, (D) NMN, (E) ATP, (F) AMP in mice liver tissues. (G) Ratio of ATP/AMP in mice tissues. n = 4–8 in each group. One-way ANOVA with a post-Dennett's test were used for all statistical analyses. $^*P < 0.05$ compared to the CCl_4 group.

suppressed by NR supplementation. In addition, the expression of P-Smad3 was suppressed by NR at the time point of 30 min after administration. The inhibitive effects of NR on the phosphorylation of Smad2 and Smad3 were temporary, and couldn't be detected after treatment for 120 mins or 30 mins respectively (Supplementary material Fig. S2A–B).

Next we investigated P300 mRNA expression in LX-2 cells at the 96 h time point, we found that NR slightly inhibited P300 expression without reaching statistical significance between NR treated groups and TGF-β treated group (Fig. 4D). Since the binding of P300 to Smad2/3 initiates the acetylation of Smad2/3, and further initiates downstream gene transcription, we explored the binding of P300 and Smad2/3 after cells were treated for 48 h. We found that TGF-β enhanced the binding of P-Smad3 to P300, while NR significantly inhibited P-Smad3/Smad3 binding to P300 (Fig. 4E–F). In in vitro experiment, we also focused on the expression and function of deacetylase Sirt1. Although we didn't find a higher Sirt1 protein expression in NR treated cells (Fig. 4A–B), the deacetylated function (activity) of Sirt1 was enhanced by NR treatment. We found that acetylation of Smads in LX-2 cells treated with TGF-β was increased, and NR supplementation could attenuate the acetylation of Smads after cells were treated with NR for 48 h, which indicated either the activation of Sirt1 or inhibition of the function of P300, or both may play key roles in the deactivation of HSC (Fig. 4G–H).

Further, we applied FK866, an NAMPT inhibitor to inhibit NAD + production, and explored whether FK866 could block the effect of NR on the activation of LX-2 cells. We found that 1 nmol/L FK866 did not block the protective effect of NR on TGF-β induced activation on LX-2 cells. While at 10 nmol/L of FK866, NR lost its ability in protection against the activation of LX-2 cells. (Supplementary material Fig. S3A–B).

3.5. Sirt1 inhibited TGF-β induced HSCs activation

To examine the role of Sirt1 in the inhibition of HSCs activation by NR, we carried out the transfection of Sirt1 siRNA to LX-2 cells. The expression of Sirt1 protein in LX-2 cells was significantly suppressed in Sirt1 siRNA transfected groups (Fig. 5A–B). In scramble siRNA treatment group, NR treatment decreased the expression of α-SMA after cells were treated with TGF-β and NR for 96 h. However, with Sirt1 knockdown, NR lost its ability in decreasing the expression of α-SMA, indicating that the inhibition on HSCs activation by NR is mostly Sirt1 dependent (Fig. 5A, C). Furthermore, we found that Sirt1 gene knockdown caused significantly increased expressions of acetylated Smad2/Smad3. Compared to cells transfected with scramble siRNA, acetylated Smad2/Smad3 were significantly enhanced in Sirt1 knockdown cells treated with TGF-β plus NR for 48 h (Fig. 5D–F).

4. Discussion

As is well-known, liver fibrosis is the last reversible stage in chronic hepatopathy. Preventing liver fibrosis and reversing the early developed fibrosis are of significance in reducing incidence of advanced liver diseases. Previous researches mainly concentrated on the role of NR on liver steatosis and inflammation. In current study, we focused on the effects of NR on liver fibrosis and HSCs activation. We found that NR could ameliorate liver fibrosis in CCl_4-treated mice, and could suppress the activation of HSCs. These effects probably result from replenishing intracellular NAD^+ and activating Sirt1, regulating TGF-β/Smads signaling pathway by deacetylating Smad2/3 and decreasing the binding of Smads to P300. As far as we know, this is the first study systematically demonstrating both the preventive and therapeutic effects of NR on liver fibrosis, and exploring the underlining mechanisms on acetylation of Smads by NR.

An array of evidence have proven that enhancing intracellular NAD^+ levels could effectively protect or reverse multifarious metabolic disorders, mediated by improving oxidative respiration or activating the NAD^+-dependent enzymes [30–33]. Supplying biosynthetic precursors of NAD^+, such as NA (nicotinic acid) or NR, is an effective method to replenish NAD^+ [34]. NA has been applied to clinic, however, it was observed that high dosage of NA could induce flushing [35]. NR is a form of vitamin B3, which is also an emerging precursor of NAD^+ by salvage pathway. It was reported that NR alleviated hepatic metaflammation and steatosis [22]. In line with these data and furthermore, our findings demonstrated that NR also showed the ability to replenish NAD^+ levels in livers and inhibit liver fibrosis, which was illustrated by a less collagen deposition, suppressed expressions of α-SMA and collagen 1 in NR administered mice livers.

Activation of stellate cell appears to be a key step in the development of liver fibrosis. CCl_4 is a commonly used inducer of liver fibrosis. It was reported that CCl_4 could increase the level of TGF-β in mice livers [36]. TGF-β has been characterized as a crucial mediator of stellate cell activation in liver fibrosis. As is known, TGF-β signal was transported into cells by TGF-β receptors on cell membranes [37]. In our data,

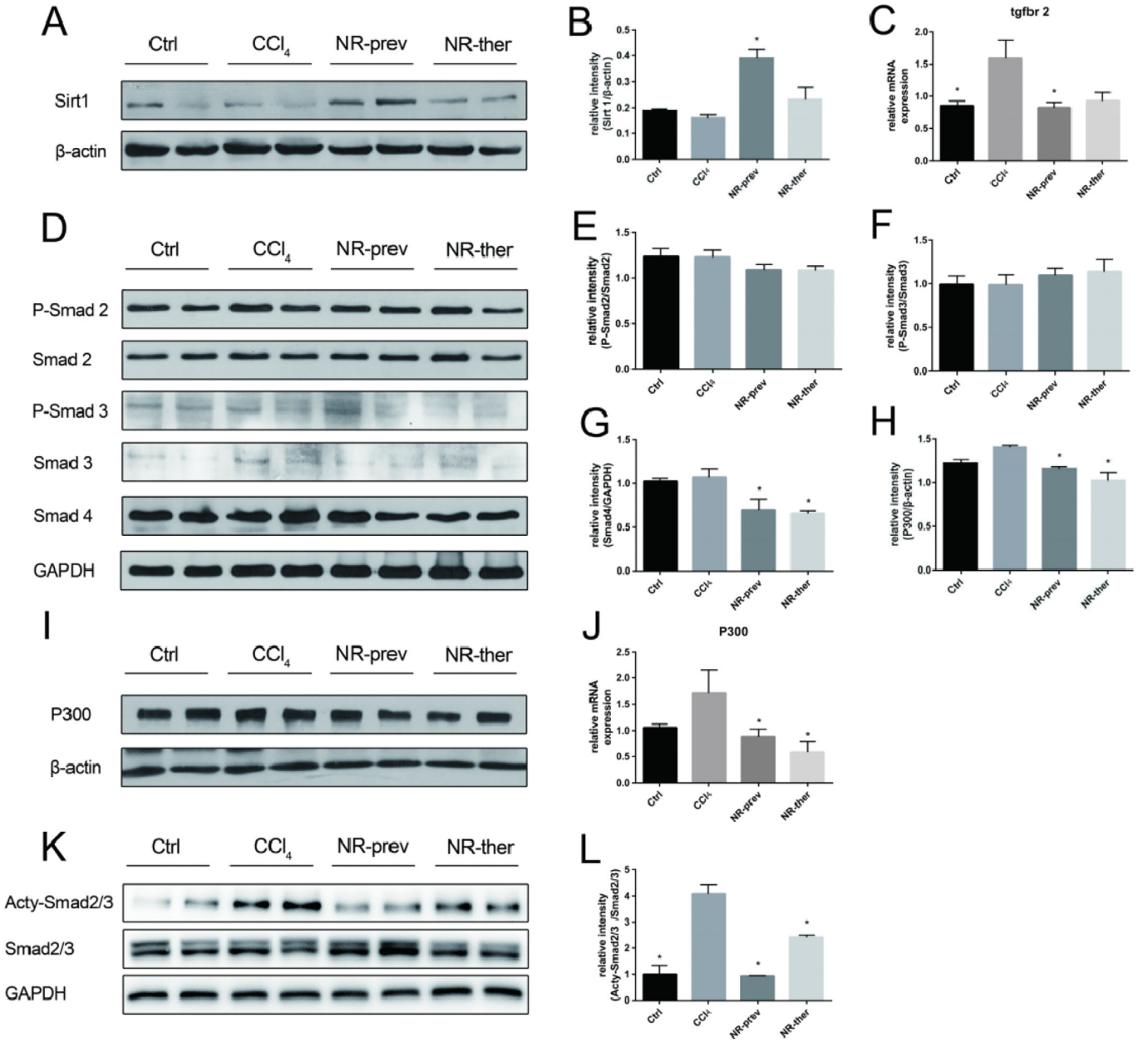
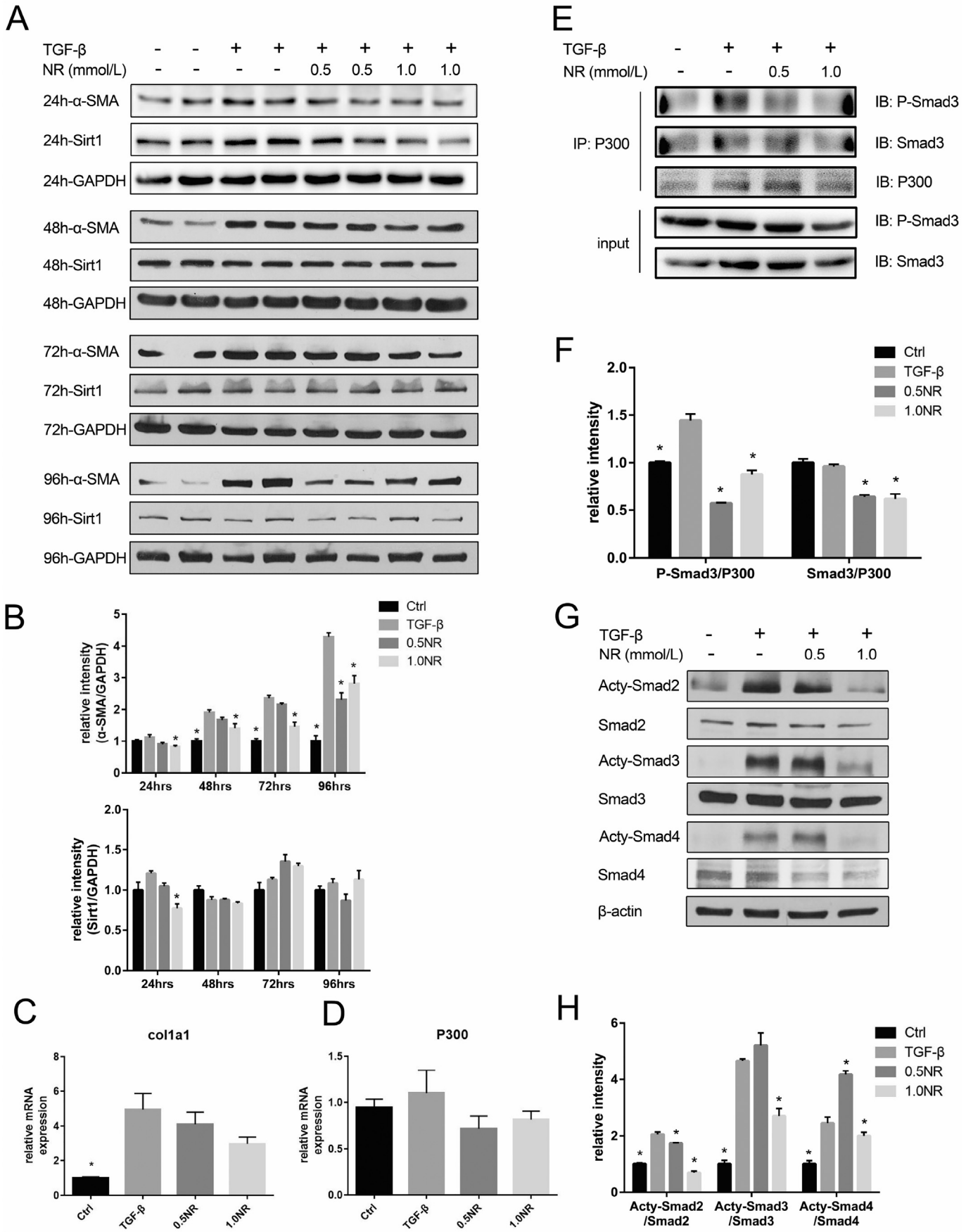


Fig. 3. NR regulated TGF-β-Smad/P300 signaling pathway in mice livers. Mice were given either CCl_4 or vehicle intraperitoneally injection twice a week, with or without NR supplementation for 8 weeks. (A) Protein expressions and (B) quantification of Sirt1 in mice liver tissues. (C) Relative mRNA expression of Tgfbr2. (D) Protein expressions and (E–G) quantification of Smad2, Smad3, Smad4 and P-Smad2, P-Smad3 levels in mice livers were measured by Immunoblotting. (H-J) P300 protein expression, quantification and relative mRNA expression in mice livers. (K, L) Immunoprecipitation (IP) was applied to assay the activity of Sirt1 in mice livers. n = 4–8 in each group. One-way ANOVA with a post-Dennett's test were used for all statistical analyses. *$P < 0.05$ compared to the CCl_4 group.

consistent with protein expression of TGF-β, tgfbr2 mRNA expression was distinctly elevated in CCl_4 administrated mice. NR treatment decreased tgfbr2 mRNA expression in mice, suggesting that the preventive and therapeutic effect of NR on liver fibrosis was in significant part mediated through influencing TGF-β signaling pathway. Although in livers, the protein expression and the phosphorylation levels of Smad2 and Smad3 were not significantly different among each group, we found the activation of Smad2/3 in the immediate hours after TGF-β stimulation in LX-2 cells, and the processes were abolished beyond 24 h, which were similar to the previous study [38]. These could be the reason why no significant variation of P-Smad2/3 was detected in in vivo studies in current research.

Not only phosphorylation of Smads regulates the TGF-β/Smads pathway and fibrosis, but also acetylation of Smads, which mediated by P300, also plays a crucial role in transcription of TGF-β target genes, such as collagen 1. Both of Sirt1 and P300 could regulate the acetylation of Smads, and further regulate the development of fibrosis. As an NAD^+-dependent enzyme, Sirt1 could be activated by boosting NAD^+ pool in vivo. Sirt1 participates in regulating various signaling pathways by the effect of deacetylation, and many lines of evidence suggested deacetylation effect of Sirt1 may play a crucial role in antagonizing liver fibrosis. It was reported that Sirt1 could regulate the transcription of PPAR by deacetylating enhancer of zeste homolog 2 (EZH2) in quiescent HSCs, which results in EZH2 inhibition or PPARγ activation, and suppresses fibrogenesis [39]. Another study suggested that Sirt1 may modulate the acetylation status of SFRS10 (official gene name, TRA2B), which also antagonizes liver fibrosis [25]. In addition, Sirt1 could antagonize or inhibit the function of P300 [40]. In lung fibrosis,



(caption on next page)

Fig. 4. NR activated Sirt1 and suppressed HSCs activation mediated by down regulating P300 expression and decreased acetylation of Smad2/Smad3. LX-2 cells were stimulated with TGF-β (4 ng/mL) in the presence or absence of NR for defined time points. (A–B) Protein expressions and quantification of α-SMA and Sirt1 were measured at the time points of 24 h, 48 h, 72 h, and 96 h after intervention. (C) After LX-2 cells were treated for 96 h, col1a1 mRNA expression was measured by q-rtPCR. (D) mRNA expression of P300 in cells. (E–F) Binding of P300 and Smad3 were measured by co-immunoprecipitation (Co-IP). (G–H) Immunoprecipitation (IP) was applied to assay the activity of Sirt1 after cells were treated for 48 h. n = 6 in each groups used for q-rtPCR, n-3 in each groups used for WB. One-way ANOVA with a post-Dennett's test were used for all statistical analyses. *$P < 0.05$ compared to the TGF-β group.

Sirt1 may act as an inhibitor of P300, which plays a vital role in transcriptional regulation of TGF-β signaling pathway [27]. In our study, downregulating the protein expressions of Acty-Smads and gene expression of P300 was also observed in liver tissues of NR treated mice, which indicates the acetylation function plays a role in the protective effect of NR on livers.

HSCs are the primary source of collagen 1, and activation of HSCs is the key step in the formation of liver fibrosis [41,42]. We further explored the mechanisms of the protective effect of NR on LX-2 cells. Previous researches have demonstrated that co-transcription factor P300 impacts the transcription and expression of target genes mediated by binding to and acetylating Smads [13,14], and Sirt1 plays its role through deacetylating target proteins. We found that, acetylated Smad2/3 and Smad4 were significantly decreased, and P-Smad3/Smad3 binding to P300 was downregulated in NR treated cells. Further study applying Sirt1 knockdown in LX-2 cells found that when Sirt1 was knocked down, NR lost its ability to inhibit the activation of LX-2 cells. In addition, we also found that acetylated Smad2/3 expression were increased in Sirt1 knockdown cells compared to cells expressing Sirt1, whether with NR treatment or not. This confirmed the solid role of Sirt1 and the deacetylation of Smads in the protection of NR on HSCs activation and liver fibrosis. Our work demonstrated a pivotal role of NR in inhibiting HSCs activation by activating Sirt1, downregulating the gene expression of P300, reducing Smads binding to P300, and inhibiting the acetylation of Smads.

5. Conclusions

In conclusion, our study shed light on that NR can not only prevent but also reverse chronic CCl_4 induced liver fibrosis. These protective effects of NR are mediated by activating Sirt1, suppressing P300 expression, and attenuating the binding of P300 to Smads, which further decreases the acetylation of Smads, and finally inhibits the activation of stellate cells. Our data provide the solid evidence that NR could be a promising supplement for prevention and treatment of liver fibrosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate activated protein kinase
α-SMA	smooth muscle actin
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate

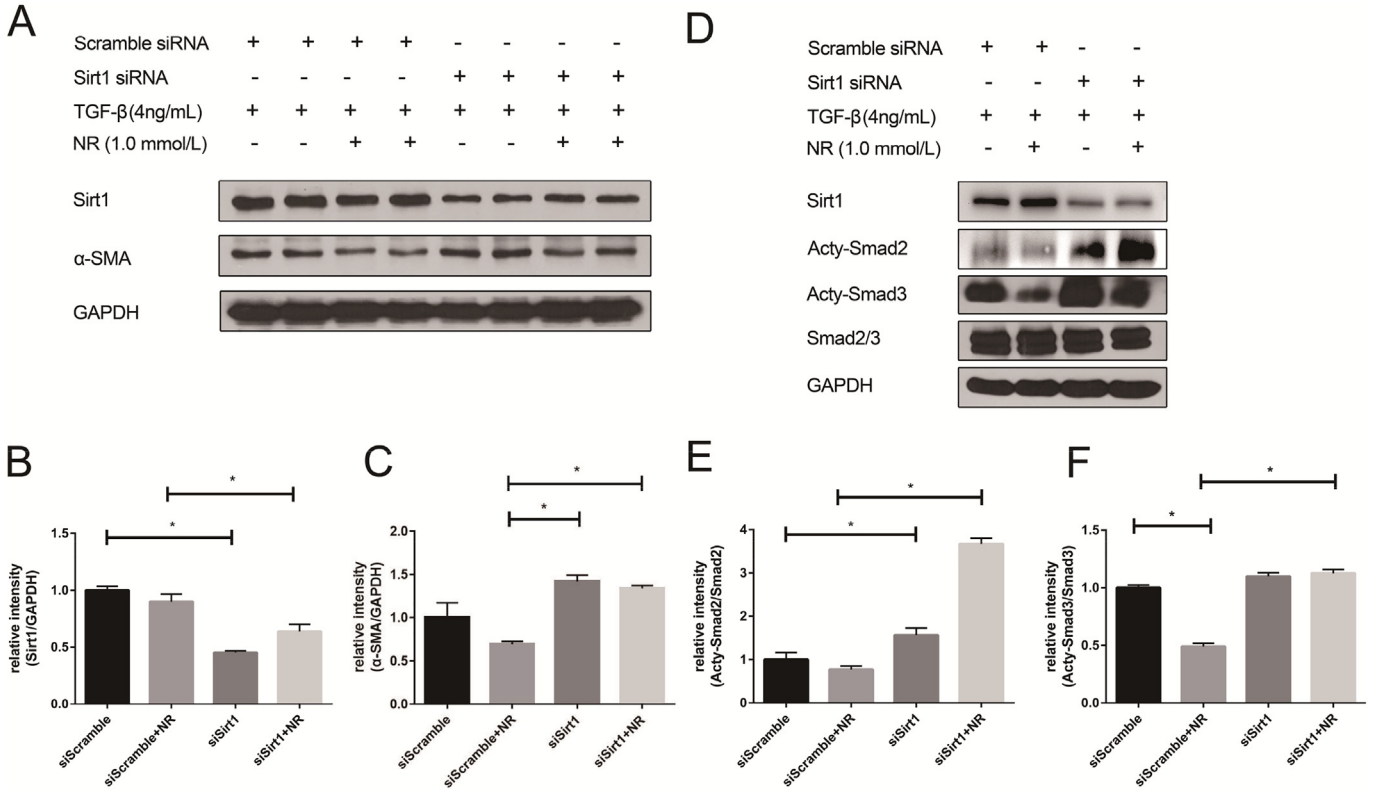


Fig. 5. NR inhibited HSCs activation via activating Sirt1. Knockdown of Sirt1, and cells were treated TGF-β (4 ng/mL) with or without NR for 96 h. (A) Immunoblotting of Sirt1 and α-SMA in Sirt1 knockdown cells. (B–C) Quantification of protein expressions of Sirt1 and α-SMA. (D) Immunoblotting of Sirt1, acetylated Smad2/3 and Smad2/3 in cells. (E–F) Quantification of protein expressions of acetylated Smad2/3. One-way ANOVA with a post-Dennett's test were used for all statistical analyses, n-3 in each groups used for WB. *$P < 0.05$ Difference between the two groups was statistically significant.

CREB	cAMP response element binding protein
ECM	extracellular matrix
HSCs	hepatic stellate cells
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NAFLD	nonalcoholic fatty liver disease
Nam	nicotinamide
NMN	nicotinamide mononucleotide
NR	nicotinamide riboside
Sirt1	sirtuin 1
TBA	total bile acid
TGF-β	transforming growth factor-β

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81573142, 81872613 to L.Y.; 81461168028 and 81672276, to Z.Z.; 81872069 to H.Y.), Tip-top Scientific and Technical Innovative Youth Talents of Guangdong special support program [2016TQ03R517], Guangzhou Science and Technology Program key projects [201803010038].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.03.064.

References

[1] E. Seki, R.F. Schwabe, Hepatic inflammation and fibrosis: functional links and key pathways, Hepatology 61 (2015) 1066–1079, https://doi.org/10.1002/hep.27332.
[2] D. Schuppan, Y. Popov, Hepatic fibrosis: from bench to bedside, J. Gastroenterol. Hepatol. 17 (Suppl. 3) (2002) S300–S305, https://doi.org/10.1046/j.1440-1746.17.s3.18.x.
[3] S.L. Friedman, Fibrogenic cell reversion underlies fibrosis regression in liver, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 9230–9231, https://doi.org/10.1073/pnas.1206645109.
[4] M.H. Ismail, M. Pinzani, Reversal of liver fibrosis, Saudi J. Gastroenterol. 15 (2009) 72–79, https://doi.org/10.4103/1319-3767.45072.
[5] N. Muhanna, et al., Activation of hepatic stellate cells after phagocytosis of lymphocytes: a novel pathway of fibrogenesis, Hepatology 48 (2008) 963–977, https://doi.org/10.1002/hep.22413.
[6] Y. Tahashi, et al., Differential regulation of TGF-beta signal in hepatic stellate cells between acute and chronic rat liver injury, Hepatology 35 (2002) 49–61, https://doi.org/10.1053/jhep.2002.30083.
[7] W.A. Border, N.A. Noble, Fibrosis linked to TGF-beta in yet another disease, J. Clin. Invest. 96 (1995) 655–656, https://doi.org/10.1172/JCI118107.
[8] N.M. Meindl-Beinker, S. Dooley, Transforming growth factor-beta and hepatocyte transdifferentiation in liver fibrogenesis, J. Gastroenterol. Hepatol. 23 (Suppl. 1) (2008) S122–S127, https://doi.org/10.1111/j.1440-1746.2007.05297.x.
[9] Y. Shi, A. Hata, R.S. Lo, J. Massague, N.P. Pavletich, A structural basis for mutational inactivation of the tumour suppressor Smad4, Nature 388 (1997) 87–93, https://doi.org/10.1038/40431.
[10] B.M. Chacko, et al., Structural basis of heteromeric smad protein assembly in TGF-beta signaling, Mol. Cell 15 (2004) 813–823, https://doi.org/10.1016/j.molcel.2004.07.016.
[11] R. Janknecht, N.J. Wells, T. Hunter, TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300, Genes Dev. 12 (1998) 2114–2119, https://doi.org/10.1101/gad.12.14.2114.
[12] M. Simonsson, M. Kanduri, E. Gronroos, C.H. Heldin, J. Ericsson, The DNA binding activities of Smad2 and Smad3 are regulated by coactivator-mediated acetylation, J. Biol. Chem. 281 (2006) 39870–39880, https://doi.org/10.1074/jbc.M607868200.
[13] A.W. Tu, K. Luo, Acetylation of Smad2 by the co-activator p300 regulates activin & transforming growth factor beta response, J. Biol. Chem. 282 (2007) 21187–21196, https://doi.org/10.1074/jbc.M700085200.
[14] C. Wang, M.J. Powell, V.M. Popov, R.G. Pestell, Acetylation in nuclear receptor signaling and the role of sirtuins, Mol. Endocrinol. 22 (2008) 539–545, https://doi.org/10.1210/me.2007-0379.
[15] Y. Inoue, et al., Smad3 is acetylated by p300/CBP to regulate its transactivation activity, Oncogene 26 (2007) 500–508, https://doi.org/10.1038/sj.onc.1209826.
[16] A.K. Ghosh, et al., p300 is elevated in systemic sclerosis and its expression is positively regulated by TGF-beta: epigenetic feed-forward amplification of fibrosis, J. Investig. Dermatol. 133 (2013) 1302–1310, https://doi.org/10.1038/jid.2012.479.
[17] A.K. Ghosh, W. Yuan, Y. Mori, J. Varga, Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-beta involves functional cooperation with p300/CBP transcriptional coactivators, Oncogene 19 (2000) 3546–3555, https://doi.org/10.1038/sj.onc.1203693.
[18] J.Y. Lim, M.A. Oh, W.H. Kim, H.Y. Sohn, S.I. Park, AMP-activated protein kinase inhibits TGF-beta-induced fibrogenic responses of hepatic stellate cells by targeting transcriptional coactivator p300, J. Cell. Physiol. 227 (2012) 1081–1089, https://doi.org/10.1002/jcp.22824.
[19] H.J. Lee, Y.S. Hong, W. Jun, S.J. Yang, Nicotinamide riboside ameliorates hepatic metaflammation by modulating NLRP3 inflammasome in a rodent model of type 2 diabetes, J. Med. Food 18 (2015) 1207–1213, https://doi.org/10.1089/jmf.2015.3439.
[20] C. Canto, et al., The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity, Cell Metab. 15 (2012) 838–847, https://doi.org/10.1016/j.cmet.2012.04.022.
[21] C.C. Zhou, et al., Hepatic NAD(+) deficiency as a therapeutic target for non-alcoholic fatty liver disease in ageing, Br. J. Pharmacol. 173 (2016) 2352–2368, https://doi.org/10.1111/bph.13513.
[22] K. Gariani, et al., Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice, Hepatology 63 (2016) 1190–1204, https://doi.org/10.1002/hep.28245.
[23] S.W. French, Chronic alcohol binging injures the liver and other organs by reducing NAD(+) levels required for sirtuin's deacetylase activity, Exp. Mol. Pathol. 100 (2016) 303–306, https://doi.org/10.1016/j.yexmp.2016.02.004.
[24] M. You, A. Jogasuria, C. Taylor, J. Wu, Sirtuin 1 signaling and alcoholic fatty liver disease, Hepatobiliary Surg. Nutr. 4 (2015) 88–100, https://doi.org/10.3978/j.issn.2304-3881.2014.12.06.
[25] H. Yin, et al., Deletion of SIRT1 from hepatocytes in mice disrupts lipin-1 signaling and aggravates alcoholic fatty liver, Gastroenterology 146 (2014) 801–811, https://doi.org/10.1053/j.gastro.2013.11.008.
[26] T. Ramirez, et al., Aging aggravates alcoholic liver injury and fibrosis in mice by downregulating sirtuin 1 expression, J. Hepatol. 66 (2017) 601–609, https://doi.org/10.1016/j.jhep.2016.11.004.
[27] Z. Zeng, et al., Activation and overexpression of Sirt1 attenuates lung fibrosis via P300, Biochem. Biophys. Res. Commun. 486 (2017) 1021–1026, https://doi.org/10.1016/j.bbrc.2017.03.155.
[28] X. Jiang, H. Guo, T. Shen, X. Tang, Y. Yang, W. Ling, Cyanidin-3-O-beta-glucoside purified from black rice protects mice against hepatic fibrosis induced by carbon tetrachloride via inhibiting hepatic stellate cell activation, J. Agric. Food Chem. 63 (2015) 6221–6230, https://doi.org/10.1021/acs.jafc.5b02181.
[29] S.A. Trammell, C. Brenner, Targeted, LCMS-based metabolomics for quantitative measurement of NAD(+) metabolites, Comput. Struct. Biotechnol. J. 4 (2013) e201301012, , https://doi.org/10.5936/csbj.201301012.
[30] L. Wang, et al., PPARα and Sirt1 mediate erythropoietin action in increasing metabolic activity and browning of white adipocytes to protect against obesity and metabolic disorders, Diabetes 62 (2013) 4122–4131, https://doi.org/10.2337/db13-0518.
[31] R. Cerutti, et al., NAD(+)-dependent activation of Sirt1 corrects the phenotype in a mouse model of mitochondrial disease, Cell Metab. 19 (2014) 1042–1049, https://doi.org/10.1016/j.cmet.2014.04.001.
[32] S. Imai, Dissecting systemic control of metabolism and aging in the NAD world: the importance of SIRT1 and NAMPT-mediated NAD biosynthesis, FEBS Lett. 585 (2011) 1657–1662, https://doi.org/10.1016/j.febslet.2011.04.060.
[33] R.H. Houtkooper, J. Auwerx, Exploring the therapeutic space around NAD+, J. Cell Biol. 199 (2012) 205–209, https://doi.org/10.1083/jcb.201207019.
[34] K.L. Bogan, et al., Identification of Isn1 and Sdt1 as glucose- and vitamin-regulated nicotinamide mononucleotide and nicotinic acid mononucleotide [corrected] 5′-nucleotidases responsible for production of nicotinamide riboside and nicotinic acid riboside, J. Biol. Chem. 284 (2009) 34861–34869, https://doi.org/10.1074/jbc.M109.056689.
[35] D.M. Capuzzi, J.M. Morgan, O.J. Brusco, C.M. Intenzo, Niacin dosing: relationship to benefits and adverse effects, Curr. Atheroscler. Rep. 2 (2000) 64–71, https://doi.org/10.1007/s11883-000-0096-y.
[36] N. Shrestha, L. Chand, M.K. Han, S.O. Lee, C.Y. Kim, Y.J. Jeong, Glutamine inhibits CCl4 induced liver fibrosis in mice and TGF-beta1 mediated epithelial-mesenchymal transition in mouse hepatocytes, Food Chem. Toxicol. 93 (2016) 129–137, https://doi.org/10.1016/j.fct.2016.04.024.
[37] E.H. Budi, D. Duan, R. Derynck, Transforming growth factor-beta receptors and Smads: regulatory complexity and functional versatility, Trends Cell Biol. 27 (2017) 658–672, https://doi.org/10.1016/j.tcb.2017.04.005.
[38] A. Kaimori, et al., Histone deacetylase inhibition suppresses the transforming growth factor beta1-induced epithelial-to-mesenchymal transition in hepatocytes, Hepatology 52 (2010) 1033–1045, https://doi.org/10.1002/hep.23765.
[39] M. Li, et al., SIRT1 antagonizes liver fibrosis by blocking hepatic stellate cell activation in mice, FASEB J. 32 (2017) 500–511, https://doi.org/10.1096/fj.201700612R.
[40] Y.J. Jeung, et al., Shikonin induces apoptosis of lung cancer cells via activation of FOXO3a/EGR1/SIRT1 signaling antagonized by p300, Biochim. Biophys. Acta 1863 (2016) 2584–2593, https://doi.org/10.1016/j.bbamcr.2016.07.005.
[41] I. Mederacke, et al., Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology, Nat. Commun. 4 (2013) 2823, https://doi.org/10.1038/ncomms3823.
[42] V. Hernandez-Gea, S.L. Friedman, Pathogenesis of liver fibrosis, Annu. Rev. Pathol. 6 (2011) 425–456, https://doi.org/10.1146/annurev-pathol-011110-130246.