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Naringenin ameliorates insulin resistance by modulating endoplasmic reticulum stress in hepatitis C virus-infected liver

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ABSTRACT

Hepatitis C virus (HCV) infection may lead to hepatic insulin resistance (IR), and endoplasmic reticulum (ER) stress has been found to induce IR. In our previous study, naringenin (NGEN) had an insulin sensitization effect on the HCV core protein (HCVCP) infected mouse livers. In the present study, we examined the effects of NGEN on HCVCP infection-induced ER stress and investigated the insulin sensitization mechanism involved. We found that XBP1s was up-regulated in the livers of HCV-infected patients, in hepatocytes with HCV infection, and in HCVCP-infected mice. HCVCP induces ER stress in the mouse liver and hepatocytes by increasing XBP1s and downstream gene expression. Pre-treatment with NGEN inhibited the ER stress and downstream gene expression both in vivo and in vitro. Similar to the HCVCP infection results, NGEN also inhibited the ER stress in tunicamycin-treated Huh-7.5.1 cells. In addition, the role of IRE1α in HCVCP-induced IR was detected, and knockdown of IRE1α abolished HCVCP-stimulated IR. Overexpression induced IR but could be abolished by NGEN. NGEN also blocked the HCVCP-induced IRE1α expression levels that were up-regulated in vivo. Our data reveal that ER stress may be associated with HCV-induced IR, and NGEN treatment inhibited ER stress activity and increased insulin sensitivity by decreasing the expression of IRE1α.

1. Introduction

Hepatitis C virus (HCV) infection has become a serious health issue associated with substantial morbidity and mortality, and approximately 185 million people have been infected with HCV worldwide [1]. It is estimated that 30% of chronically infected patients eventually develop progressive liver disease including cirrhosis and end stage liver disease, HCV-related liver disease represents the leading indication for liver transplantation in the USA and Europe [2]. HCV causes not only severe liver problems but also extrahepatic manifestations, such as insulin resistance (IR) and type 2 diabetes mellitus (T2DM), but the factors involved remain unclear.

The endoplasmic reticulum (ER) is an organelle for synthesis, folding, and post-translational modifications of proteins, and its homeostasis is crucial for hepatocytes. Increasing evidence indicates that virus infection often disturbs ER homeostasis and leads to an accumulation of unfolded or misfolded proteins in the lumen of the ER. This induces an ER stress response or unfolded protein response (UPR), which has profound effects on HCV virus replication and pathogenesis [3]. The UPR is distinguished by the action of three signaling proteins named IRE1α (inositol-requiring protein-1α), PERK (protein kinase RNA-like ER kinase), and ATF6 (activating transcription factor 6) [4,5]. Upon ER stress, IRE1α splices the mRNA encoding X-box binding protein 1 (XBP1) via its RNase activity, thereby generating functional spliced XBP1 (XBP1s) to activate the gene expression of a subset of UPR-associated regulators [6].

Previous studies have demonstrated that ER stress and the IRE1α/XBP1 pathway protect against hepatic lipid accumulation and regulate insulin sensitivity by regulating hepatic *de novo* lipogenesis or promoting very low-density lipoprotein (VLDL) assembly and secretion [7–9]. After XBP1 knock-out mice were fed a high fat-diet, they displayed impaired glucose tolerance and insulin sensitivity [10]. XBP1

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also decreased glucose dysfunction via funneling its effects on improving insulin sensitivity and stimulating insulin secretion [11]. In pancreatic beta-cells, IRE1α also functions in the regulation of insulin biosynthesis [12]. However, the role of ER stress in regulating HCV infection-induced insulin resistance has never been investigated.

In our previous study, naringenin (NGEN) increased insulin sensitivity in HCV core protein (HCVCP)-infected livers by up-regulating PTEN in a p53-dependent manner [13]. Recent studies found that NGEN protected H9c2 myocardial cells against hypoxia/reoxygenation (H/R) injury via attenuating ER stress-mediated apoptosis [14]. In view of the beneficial effects of NGEN on the regulation of ER stress and insulin sensitivity, we raised the hypothesis that HCVCP infection induce IR by activating ER stress, and that NGEN may be a potential ER stress inhibitor in the prevention of IR in HCVCP infection.

In the present study, we evaluated the effect of HCV infection on ER stress in liver tissues from clinical samples and a mouse model. Additionally, we examined the effect of NGEN on the HCVCP-induced ER stress pathway in vivo and in vitro. Finally, we analyzed the effect of IRE1α on HCVCP-induced IR and the role of NGEN in the process. These findings elucidated the link between HCVCP infection and ER stress in liver tissue, and present a novel mechanism through which NGEN inhibits ER stress and ameliorates insulin resistance under HCV infection conditions.

2. Materials and methods

2.1. Gene-cloud of biotechnology information (GCBI)

Gene expression data were obtained from the Gene Expression Omnibus (GSE84346, GSE84587 and GSE64605). The GCBI (Shanghai, China, https://www.gcbi.com.cn) website was used to identify DEGs between normal liver and HCV infected liver tissues. In the Differential Gene Expression Analysis module on the GCBI platform, we chose a fold expression change > 5 at cutoff values Q < 0.05 and P < 0.05 to screen out different expression genes.

2.2. Patient tissue samples for gene expression studies

This study was approved by the institutional review board of the Committee on Clinical Investigation at the Second Affiliated Hospital of Anhui Medical University and is in accordance with Health Insurance Portability and Accountability Act regulations. Written informed consent was obtained from all subjects prior to participation. We obtained liver biopsies from the Second Affiliated Hospital of Anhui Medical University taken from control patients without HCV infection and cases with HCV-infected liver tissues. We reclassified two samples resulting in 11 HCV uninfected non-neoplastic (controls), 19 HCV + non-neoplastic (infected) samples.

2.3. Materials

HCV CMV-FLAG-Core-R (pMO29) (HCV sub-genotype 1b) was a gift from Melanie Ott (Addgene plasmid # 24480) [15], flag-HsIRE1α-pBabePuro was a gift from David Ron (Addgene plasmid # 54337) [16], pGL4-ERSE1-luc2P-Hygro was a gift from Seiichi Oyadomari (Addgene plasmid # 101789), adenovirus knockdown IRE1α (shIRE1α) and the negative control was obtained from Hanbio Biotechnology Co., Ltd., Shanghai, China. Naringenin (#S2394) was obtained from Selleck Chemical (Houston, TX, USA), tunicamycin (#B7417) was purchased from Apexbio (Shanghai, China). The antibodies used in the western blotting are as follows: β-actin (Santa Cruz, SC-47778), p-IRE1α (Ser724) (Abcam, ab48187), IRE1α (Abcam, ab37073), XBP-1s(CST, #27901), XBP1t (Abcam, ab37152), GRP78/BiP (Abcam, ab21685), Akt (CST, #4685), Phospho-Akt (Thr308)(CST, #13038). The secondary antibodies used are as following; anti-rabbit (proteintech, SA00001-2), anti-mouse (Proteintech, SA00001-1). Human IRE1α shRNA expression vector (sc-40705-SH) and the negative control were purchased from Santa Cruz.

2.4. Animal model and treatment

The Institutional Animal Care and Use Committee of Anhui Medical University approved this study, and the study conformed to the procedures described in the Guide for the Care and Use of Laboratory Animals of the Anhui Medical University. Male C57BL/6J mice (6–8 weeks old), weight 20–25 g, were purchased from the Shanghai Slac Laboratory Animal Co, Ltd. (Shanghai, China). The animals were housed in microisolator cages in a room at 20 °C ± 2 °C, with a relative humidity of 55% ± 15%, and a 12/12 h light/dark cycle. Care of the animals and all experimental procedures were conducted in accordance with the institutional guidelines for animal research. To testify the time for ER stress activation for HCVCP infection, mice were injected with HCVCP or GFP adenovirus, after 1, 2 and 3 weeks of infection, mice were sacrificed and harvest the liver tissues. For the NGEN anti-ER stress effect analysis, mice were divided into four groups according to their body weight randomly: GFP group, HCVCP group, HCVCP + 25 mg/kg NGEN group and HCVCP + 50 mg/kg NGEN group. Mice were infected with HCVCP for 2weeks and NGEN treatment was built as previously described [13]. At the end of the experiment, mice were fasted before sacrifice, blood and tissues were collected.

2.5. Cell culture and transfection

Human hepatoma cell line Huh-7.5.1 cells were obtained from ATCC and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; #12800017, Invitrogen Life Technologies, Basel, Switzerland) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Invitrogen Life Technologies). Huh-7.5.1 cells were transfected with expression vectors in 6-well plates for RT-qPCR and immunoblotting. After overnight culture, cells were incubated for 5 h in serum-free DMEM containing DNA lipo2000 (Thermo Fisher) complex and then grown in DMEM supplemented with 10% FBS for 48 h.

2.6. Luciferase reporter assays

Uninfected and HCV-infected Huh-7.5.1 cells were seeded into 24-well plate and transfected with ERSE response luciferase reporters by using lipo2000 according to the manufacturer's protocol for luciferase assay. 24 h after transfection, Cells were treated with 1 μM NGEN for another 24 h. The Cells were harvested and cellular lysates were analyzed for luciferase reporter assay kit (Promega, Madison, WI). Relative luciferase activity (RLA) was normalized by dividing the firefly luciferase value by the *Renilla* luciferase value. The ERSE reporter construct monitored activity of the endoplasmic reticulum (ER) stress pathway. The ERSE is a motif that mediates the transcriptional response to ER stress.

2.7. RT-qPCR

Total RNA was extracted from tissues or cells using RNAiso Plus (Takara Bio Inc., Dalian, China), DNaseI-treated RNA was used for firststrand cDNA synthesis using MMLV reverse transcriptase (Promega Corporation, Madison, WI, USA) and oligo$(dT)_{15}$ according to the manufacturer's protocol and 1 μL cDNA samples were used for conventional PCR amplifications. RT-qPCR was performed using SYBR Premix Master Mix (Thermo Scientific Inc., Waltham, MA, USA). Primer sequences used in the experiments were as follows:

Mouse-XBP1s forward 5′- CTGAGTCCGAATCAGGTGCAG -3′, reverse 5′- GTCCATGGGAAGATGTTCTGG -3′; mouse-XBP1t forward 5′- TGGCCGGGTCTGCTGAGTCCG -3′, reverse 5′- GTCCATGGGAAGATGT TCTGG -3′; mouse-ERdj4 forward 5′- ATAAAAGCCCTGATGCTGAAGC

-3′, reverse 5′- GCCATTGGTAAAAGCACTGTGT -3′; Mouse-EDEM forward 5′- CGCGGAGACCCTTCCAATC-3′, reverse 5′- CCAATGTATCCAA GGCATCAACC-3′; mouse-PDI forward 5′- CATGCTAGTCGAGTTCTT CGC-3′, reverse 5′- CAGTGCAATCCACCTTTGCTAA-3′; mouse-36B4 forward 5′- TGAGATTCGGGATATGCTGTTGG-3′, reverse 5′- CGGGTC CTAGACCAGTGTTCT-3′.

Homo-XBP1s forward 5′- CTGAGTCCGAATCAGGTGCAG -3′, reverse 5′- ATCCATGGGGAGATGTTCTGG -3′; homo-XBP1t forward 5′- GACGGGACCCCTAAAGTTCTG -3′, reverse 5′- CTTCTTTCGATCTCTGG CAGTC -3′; homo-EDEM forward 5′- CGGACGAGTACGAGAAGCG -3′, reverse 5′- CGTAGCCAAAGACGAACATGC -3′; homo-PDI forward 5′- GGTGCTGCGGAAAAGCAAC -3′, reverse 5′- ACCTGATCTCGGAACCTT CTG -3′; homo-ERdj4 forward 5′- CTGCCCTTGGGCTCACTAAT -3′, reverse 5′- CACTCTGCAACGCAAGTCAC -3′; homo-GAPDH forward 5′- TGTGGGCATCAATGGATTTGG -3′, reverse 5′- ACACCATGTATTCCGG GTCAAT -3′;

The expression levels of mRNA were normalized to 36B4 or GAPDH. The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 40 s.

2.8. Western blot

Total lysates from tissues or cells were obtained by lysing in RIPA buffer with protease inhibitors cocktail (#HY-K0010, MedChem Express, Shanghai, China). Protein concentration was measured by the BCA assay (Bio-Rad, Hercules, CA, USA). Proteins were extracted and separated in 10% Tris glycine/SDS-polyacrylamide gels and transferred to PVDF membranes (#IPFL00010, Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk and incubated with specific antibodies overnight at 4℃. β-actin (Proteintech, #66009-1-Ig) was used as the endogenous control. Primary antibodies were used at the dilution of 1:1000. Anti-Phospho Akt (Thr308) (#13038), anti-Akt (#4685) and anti-XBP1s (#27901) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-p-IRE1α (Ser724) (ab48187), anti-IRE1α (Ser724) (ab37073), anti-XBP1t (ab37152) and anti-GRP78/BiP (ab21685) were obtained from Abcam (Cambrdige, MA, USA). Blots were then incubated with relevant secondary antibodies, HRP-conjugated Goat Anti-Rabbit IgG (SA00001-2) and HRP-conjugated Goat Anti-mouse IgG (SA00001-1) were purchased from Proteintech (Wuhan, China) for 1 h. Bands were detected with the enhanced chemiluminescence detection systeme (P10200, New Cell & Molecular Biotech Co., Ltd) and Bio-Rad ChemiDocTM MP imaging system. Relative abundance was measured with Image J software.

2.9. Statistical analysis

The data were expressed as the means ± standard error of mean (SEM) and analyzed using the SPSS 20.0 statistical software (SPSS Inc., USA). The comparisons between groups were analyzed using ANOVA followed by least-significant difference post hoc analysis, and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. ER stress was activated in HCV-infected livers

To unravel the biological significance of ER stress on liver disease progression in clinical situations, we analyzed the human GEO database (GSE84346, GSE84587 and GSE64605) available in the public domain. The GSE84346 database contains 22 HCV infected patients and 6 negative control tissues without treatment, and XBP1s mRNA levels were significantly elevated in HCV infected livers (Fig. 1A). GSE84587 included 2 HCV-infected primary human hepatocytes and 2 negative control, as indicated in Fig. 1B, and HCV infection significantly upregulated the XBP1s mRNA levels. Long-term culture in human serum produced growth-arrested, hepatocyte-like cells whose gene profile significantly overlapped with that of primary human hepatocytes.

Long-term culture of Huh-7.5.1 cells in human serum was performed, and then, the cells were infected with high-risk Q^{70}/M^{91} and control R^{70}/L^{91} followed by gene-expression profiling and bioinformatics analysis. As shown in Fig. 1C, Q^{70}/M^{91} and R^{70}/L^{91} both showed higher XBP1s mRNA levels than non-infected cells, and Q^{70}/M^{91} increased the XBP1s mRNA levels more significantly than R^{70}/L^{91}. It has been shown that patients with HCV infection exhibit ER stress in the liver. We found that XBP1s mRNA and protein levels were significantly up-regulated in HCV patients as compared to healthy patients (Fig. 1D). We next asked whether XBP1s was upregulated in animals by other pathological conditions that induce ER stress.

3.2. NGEN inhibits HCVCP-infection induced ER stress in vivo

Next, we sought to identify the effects of NGEN on HCV-induced ER stress in the liver and hepatocytes. Treatment of mice with HCVCP infection gradually and substantially elevated *XBP1s* and *ERdj4* mRNA levels in the liver (Fig. 2A), and we also used primary hepatocyte to study the effect of NGEN on HCVCP induced ER stress. A time-course study also verified the ER stress induction of Xbp1s and ERdj4 mRNA in hepatocyte (Fig. 2B): Xbp1s and ERdj4 mRNA continuously increased after HCVCP infection and reached a maximum at 24 h. A similar increase was observed 24 h after tunicamycin (Tm) treatment (Fig. 2C). The ability of NGEN to decrease GRP78/BiP expression was verified in HCVCP-infected mouse liver tissues (Fig. 2D). To validate the function of NGEN on ER stress, we examined the *XBP1s*, *ERdj4*, *EDME* and *DPI* mRNA levels using RT-qPCR. As expected, treatment with NGEN abrogated the ability of HCVCP to induce ER stress (Fig. 2E). Our results demonstrate the ability of NGEN to inhibit HCVCP-induced ER stress in vivo.

3.3. NGEN inhibited ER stress in Huh-7.5.1 cells

The finding that XBP1 splicing activation is required for NGEN induction prompted us to search for an upstream UPR regulatory pathway. Because the splicing of XBP1 mRNA to its active form depends on IRE1 endonuclease activity, we determined the role of NGEN on IRE1α-induced ER stress. As expected, HCVCP infection significantly up-regulated the phosphorylation of IRE1α and spliced XBP1s expression levels in Huh-7.5.1 cells (Fig. 3A), and 0.5 μM or 1 μM NGEN treatment for 24 h diminished the increases in *XBP1s* and *ERdj4* mRNA levels (Fig. 3B). The ERSE reporter construct monitors the activity of theER stress pathway, and the ERSE is a motif that mediates the transcriptional response to ER stress [17].

To determine how HCVCP induces the ER stress pathway, we assessed the activity of ERSE reporters in un-infected or Huh-7.5.1-infected cells treated with different concentrations of NGEN. Compared with green fluorescent protein (GFP)-infected Huh-7.5.1 cells, HCVCP infection increased ERSE reporter activity, and NGEN treatment decreased HCV-induced ERSE signaling (Fig. 3C). NGEN treatment also diminished the increases in P-IRE1α, XBP1s protein levels, and *XBP1s and ERdj4* mRNA levels by Tm treatment (Fig. 3D and E). Collectively, our results indicate that NGEN inhibited HCVCP-induced ER stress *in vitro* partly by the endonuclease activity of IRE1.

3.4. Overexpressed IRE1α induced insulin resistance, and NGEN treatment decreased IRE1α expression

To evaluate the role of IRE1α on HCV-induced IR, the IRE1α expression vector and shRNA were transfected into Huh-7.5.1 cells, and IRE1α mRNA and protein levels were analyzed using RT-qPCR and western blot (Fig. 4A). Huh-7.5.1 cells were transfected with the IRE1α expression vector and shRNA, and then infected with HCVCP adenovirus and treated with or without 1 μM NGEN for 24 h. At the end of the treatment, 10 nM insulin was added for an additional 5 min.

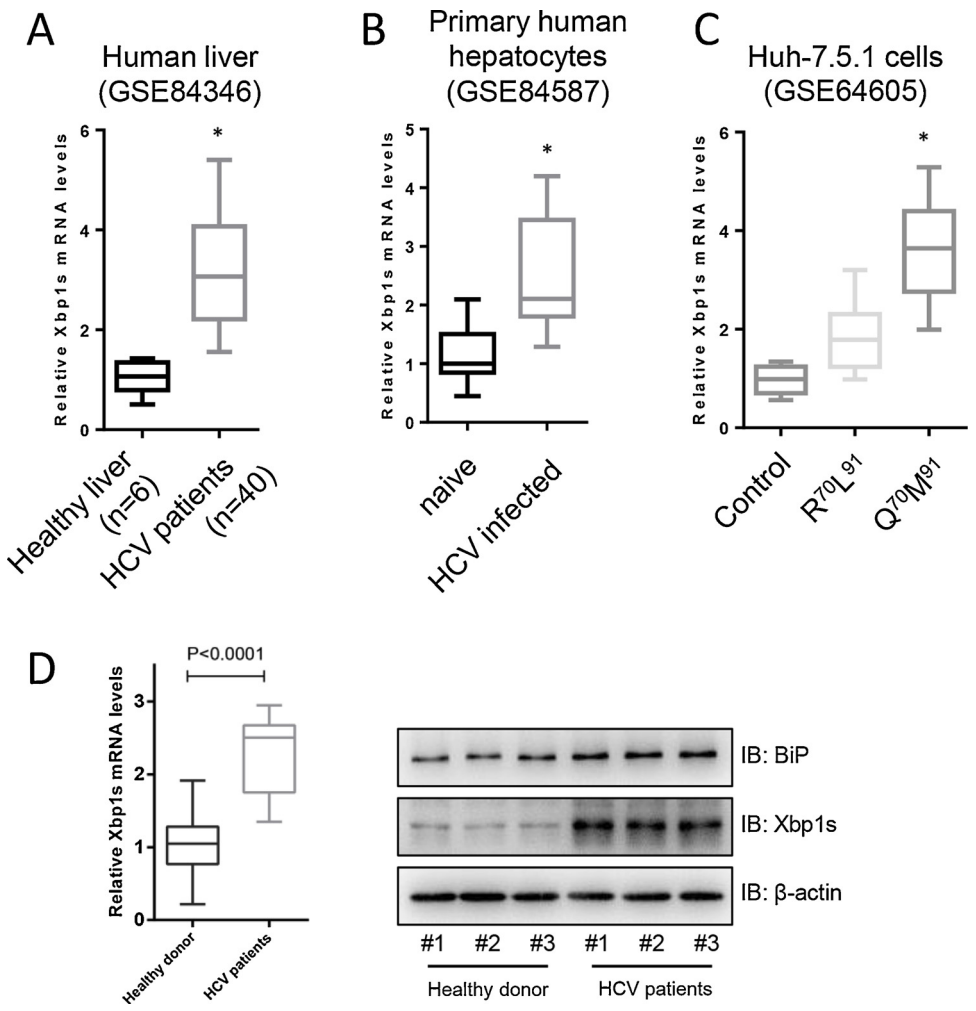

Fig. 1. ER stress was activated in HCV infected livers. (A–C) XBP1s mRNA expression levels in patients with HCV infection. For A, hepatic XBP1s mRNA levels were analyzed in healthy individuals or patients with HCV (GS84364). For B, XBP1s mRNA levels were analyzed in primary human hepatocytes infected with or without HCV (GSE84587). For C, XBP1s mRNA levels were analyzed in Huh-7.5.1 cells infected with different type of HCV virus (GSE64605). (D) XBP1s mRNA expression in HCV patients. Quantitative reverse transcription-PCR (qRT-PCR) assays for XBP1s or immunoblotted with BiP and XBP1s were done on HCV patient liver samples. Data represent the mean ± S.E., Statistical significance of the differences between healthy individuals and patients with HCV infection. *p < 0.05.

As shown in Fig. 4B, in the insulin-stimulated state, Akt phosphorylation at Thr308 was enhanced after IRE1α knockdown, while HCVCP infection induced a significant decrease, which could be rescued by co-transfection with shIRE1α. Overexpression of IRE1α and infection with HCVCP significantly decreased the phosphorylation, but pretreatment with NGEN increased the phosphorylation, with a subsequent increase in insulin signaling activity. The HCVCP-infected cells exhibited lower phosphorylation levels than shIRE1α group. Finally, we detected the IRE1α mRNA and protein levels in HCVCP-infected mice. As shown in Fig. 4C and D, HCVCP infection caused a significant increase in IRE1α expression levels, and pre-treatment with NGEN decreased the increase induced by HCVCP infection.

4. Discussion

HCV core processing and maturation occurs in the ER, which plays a major role in secretory and membrane proteins synthesis and maturation. Accumulation of misfolded or aggregated proteins in the ER may activate ER stress, which is an adaptive cellular response [18]. Chronic HCV infection leads to ER stress, which can play a role in the development of hepatic insulin resistance. Although the association between hepatic HCV infection and insulin resistance is unclear, it has been reported that HCV infection leads to hepatic ER stress and inflammation, which can induce the development of insulin resistance. In the previous study, NGEN increased insulin sensitivity in HCVCP-infected liver by up-regulating phosphatase and tensin homolog (PTEN) a in p53-dependent manner [13]. Here, we found that the insulin sensitization effect of NGEN may partially occur by inhibition of HCVCP-induced ER stress.

Previous studies have shown that the development of hepatic insulin resistance in a state of chronic HCV infection is characterized by both lipid accumulation and inflammation in the liver [19,20]. However, the detailed underlying molecular mechanisms are not yet completely understood. Recently, several studies have indicated that ER stress is a central feature of insulin resistance. Obesity-caused ER stress leads to the suppression of insulin receptor signaling through IRE1α-dependent activation of JNK and subsequent serine phosphorylation of IRS-1 [21]. GRP78, a chaperone protein, binds newly synthesized proteins for subsequent folding and negatively regulates ER transmembrane proteins such as IRE1α, PERK, and ATF6α [22]. GRP78 expression was decreased in the livers of diabetic *db/db* mice compared with diabetic *db/+* mice, and treatment of HepG2 cells with oleic acid reduced the expression of GRP78 [23]. Mice fed high fat diets had significantly higher PERK and CHOP protein expression and phosphorylation of eIF2α concurrent with suppressed GRP78, while the serum insulin levels and hepatic lipid deposition were increased [24]. These results indicate that obesity induces deleterious UPR signaling and contributes to insulin resistance in the liver, and is mediated by the activation of IRE1α and suppression of the expression of chaperone proteins and endoplasmic reticulum-associated degradation (ERAD). HCV infection induced ER stress, and considering the functional role of ER stress in insulin resistance, we guess that whether NGEN treatment increase insulin sensitivity via ER stress pathway.

Here, by the analyses of clinical GEO databases, it was determined that HCV infection promotes the ER stress pathway-associated XBP1s gene induction both in HCV-infected liver tissues, human hepatocytes,

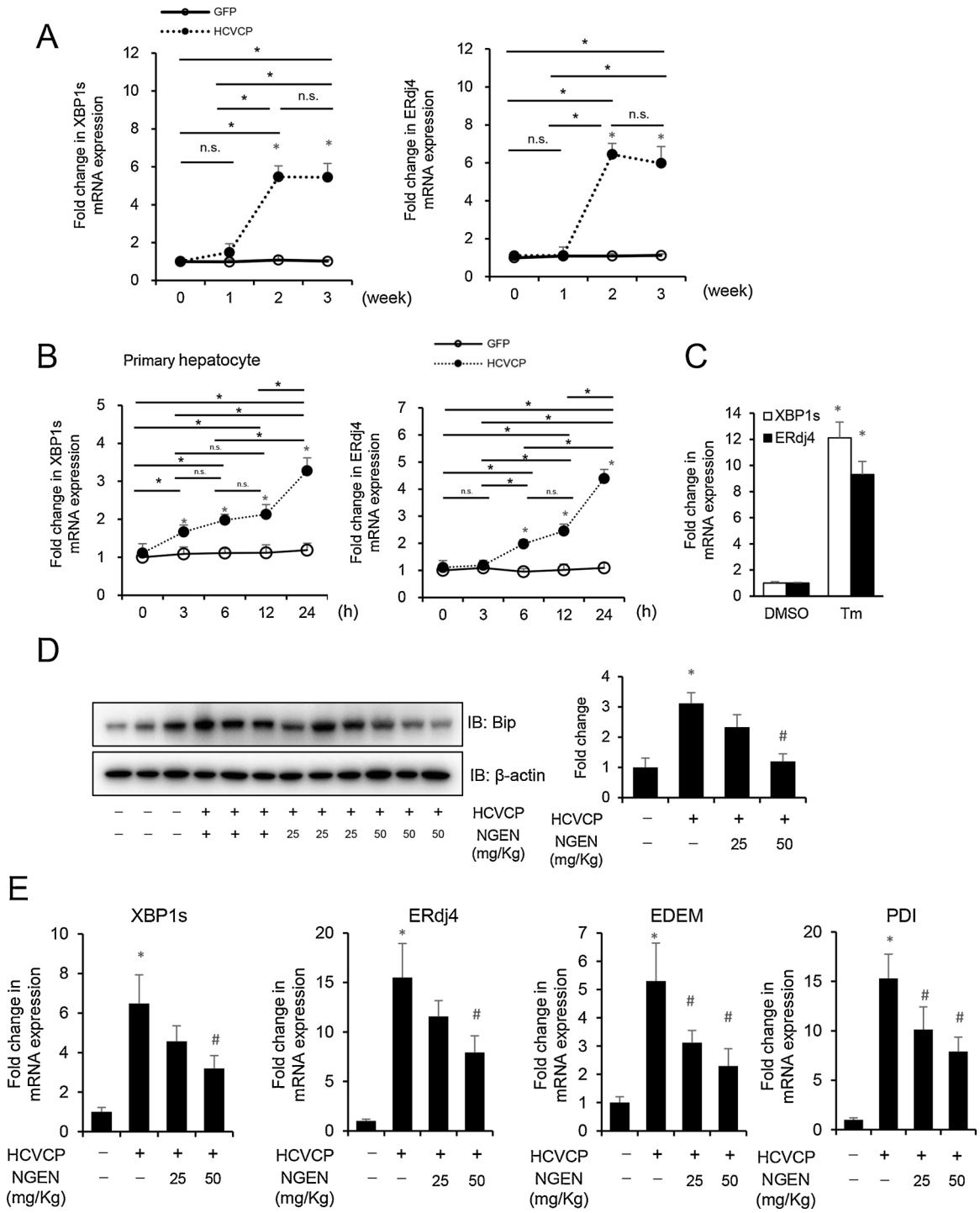

Fig. 2. NGEN treatment ameliorates HCVCP infection induced ER stress. (A) Wild type C57BL/6 mice were *i.v.* injected with GFP or HCVCP adenovirus ($n = 6$ in each group) for 0, 1, 2 and 3 weeks, (B–C) Primary hepatocyte was isolated and infected with GFP or HCVCP adenovirus for indicated time points or treated with 10 μg/mL tunicamycin for 24 h, mRNA was extracted at the indicated time points, the expression of *XBP1s* and *ERdj4* were analyzed by RT-qPCR. WT C57BL/6 mice were injected with GFP or HCVCP adenovirus for 2 weeks, pre-treated with or without 25 mg/kg or 50 mg/kg NGEN, (D) liver total lysates were immuneblotted with BiP and β-actin antibodies, (E) the mRNA levels of *XBP1s*, *ERdj4*, *EDEM* and *PDI* were detected using RT-qPCR. The data are presented as the mean ± S.E., $^{*}P < 0.05$ vs. GFP or indicated control group, $^{\#}P < 0.05$ vs. HCVCP group.

and Huh-7.5.1 cells. This outcome was supported by the RT-qPCR and western blot detection of our samples obtained from HCV-infected patients. The function of HCV infection-induced ER stress was further corroborated by the results of experiments using HCVCP adenovirus-infected mice and Huh-7.5.1 cells. HCVCP infection up-regulates the hepatic and hepatocytes *XBP1s* and *ERdj4* mRNA levels. GRP78, binds newly synthesized proteins to maintain them in a competent for subsequent folding and oligomerization state, was significantly upregulated after hepatic HCV infection, which serves as an indicator of the accumulation of unfolded polypeptides in the ER. Pre-treated with 25 or 50 mg/kg NGEN *in vivo* could decrease the protein levels. XBP1s, ERdj4, EDEM and PDI as the downstream of the IRE1α/XBP1s pathway, both were upregulated after HCVCP infection and inhibited by NGEN pretreatment *in vivo*. The *in vitro* study found that HCVCP infection induced

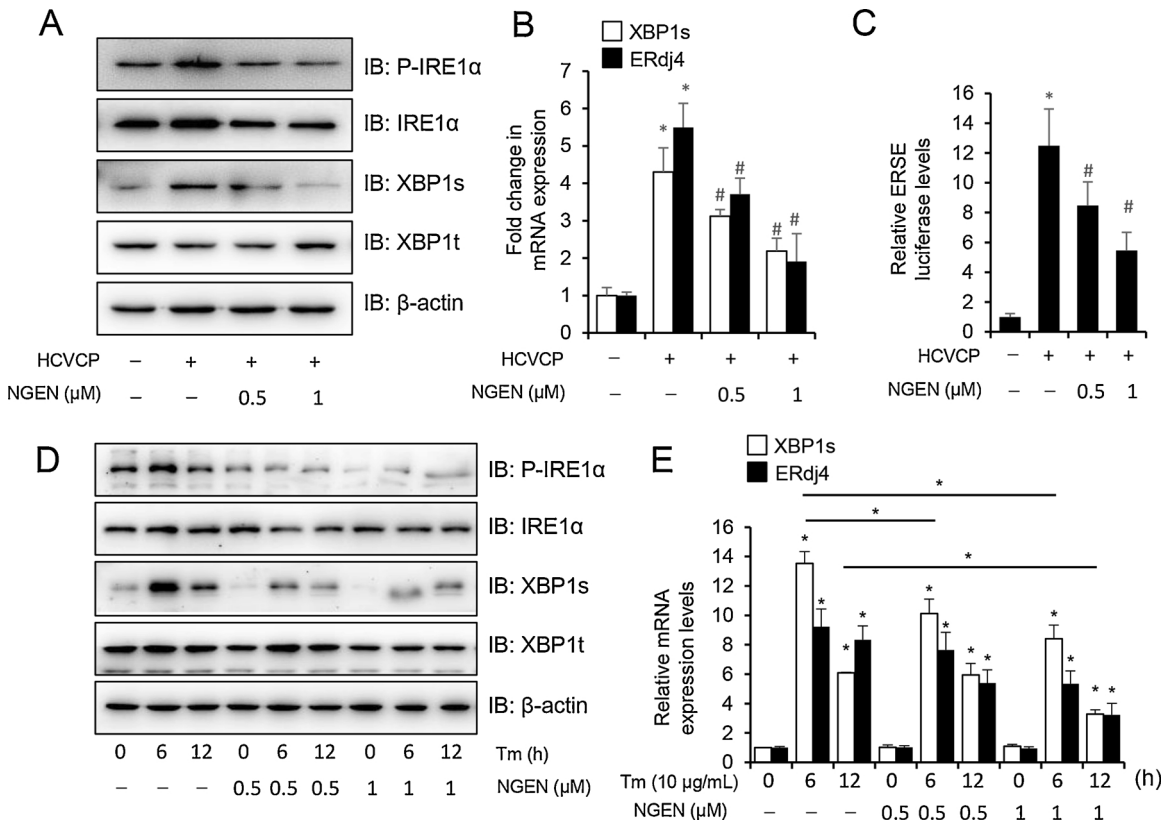

Fig. 3. NGEN inhibits ER stress in HCVCP infected Huh-7.5.1 cell. (A–B) Huh-7.5.1 cells were infected with GFP or HCVCP adneovirus, then treated with or without 0.5 or 1 μM NGEN for 48 h, western blot assay was performed to detect the phosphorylation of IRE1α and the splicing of XBP1t, RT-qPCR was used to detect the mRNA levels of *XBP1s* and *ERdj4*. (C) Huh7.5.1 cells were infected with GFP or HCVCP adneovirus. 24 h after infection, cells were transfected with plasmid pERSE-luciferase reporter and treated with NGEN for another 24 h, the pRL-TK expressing *Renilla* luciferase was co-transfected as an internal control. The activity of ER stress pathway was monitored by a dual luciferase reporter assay system at 24 h after transfection. Huh7.5.1 cells were pre-treated with 0.5 or 1 μM NGEN for 24 h and then treated with 10 μg/mL tunicamycin for 6 or 12 h, (D) total cell lysates were immunoblotted with P-IRE1α, IRE1α, XBP1s and XBP1t, (E) the mRNA levels of *XBP1s* and *ERdj4* were detected with RT-qPCR. The data are presented as the mean ± S.E. and expressed as fold-change relative to the level of cells transfected with control vector. $^{*}P < 0.05$ vs. GFP or DMSO group, $^{\#}P < 0.05$ vs. HCVCP group.

the phosphorylation of IRE1α, splicing of XBP1s, and the activation of ERSE reporter activity. Huh-7.5.1 cells were pre-treated with 0.5 or 1 μM tunicamycin for 6 h or 12 h to assay the inhibitory effect of NGEN on ER stress activation. Overall, the *in vivo* results support the notion that HCVCP infection may leads to hepatic ER stress, and pre-treat with NGEN could inhibit the effect.

It has been implicated that the UPR transducer IRE1α may act as an RNase or a scaffold through its cytosolic domain to modulate inflammatory and/or metabolic stress signaling pathways [25]. It has previously been reported that the IRE1α-XBP1 pathway was activated in the livers of non-alcoholic steatohepatitis (NASH) patients or animal models [26,27]. A recent study also showed that a high fat diet (HFD) activated IRE1α RNase activity towards both XBP1 mRNA splicing and select miRNA processing in the liver. In particular, S-nitrosylation of IRE1α, induced by obesity and associated chronic inflammation, uncouples the kinase and RNase functions of IRE1α protein, and thus suppresses IRE1α RNase activity and Xbp1 mRNA splicing in the liver [28].

To comprehensively understand the effect of NGEN on HCVCP-induced IR, we performed in vitro assays to determinethe insulin sensitization effect of NGEN on HCVCP-infected Huh-7.5.1 cells with IRE1α overexpression or knockdown. Compared with HCVCP infection, knockdown of IRE1α significantly increased and overexpression significantly decreased the phosphorylation levels of Akt at the ser308 site, and pre-treatment with NGEN reversed the insulin resistance effect of IRE1α. Finally, we detected the IRE1α expression levels in the livers of HCVCP-infected mice and found that HCVCP increased while NGEN treatment decreased the IRE1α levels.

These data indicate that NGEN inhibited HCVCP-induced ER stress, decreased the splicing activity of IRE1α and its downstream gene expression, and inhibited the expression of IRE1α, which finally led to the ameliorated insulin sensitivity. Our results provide novel insights for the development of NGEN as a new drug to treat HCVCP infection-induced IR and may provide a potential new strategy for the protection of hepatocytes from ER stress-associated pathological conditions.

Authors contribution

XM and YW designed the experiments. BJ, DY, GY, YC and FC performed the experiments. YH analyzed the data. PC and CY contributed reagents, materials, and analysis tools. BJ, DY, XM and YW wrote the original draft.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgments

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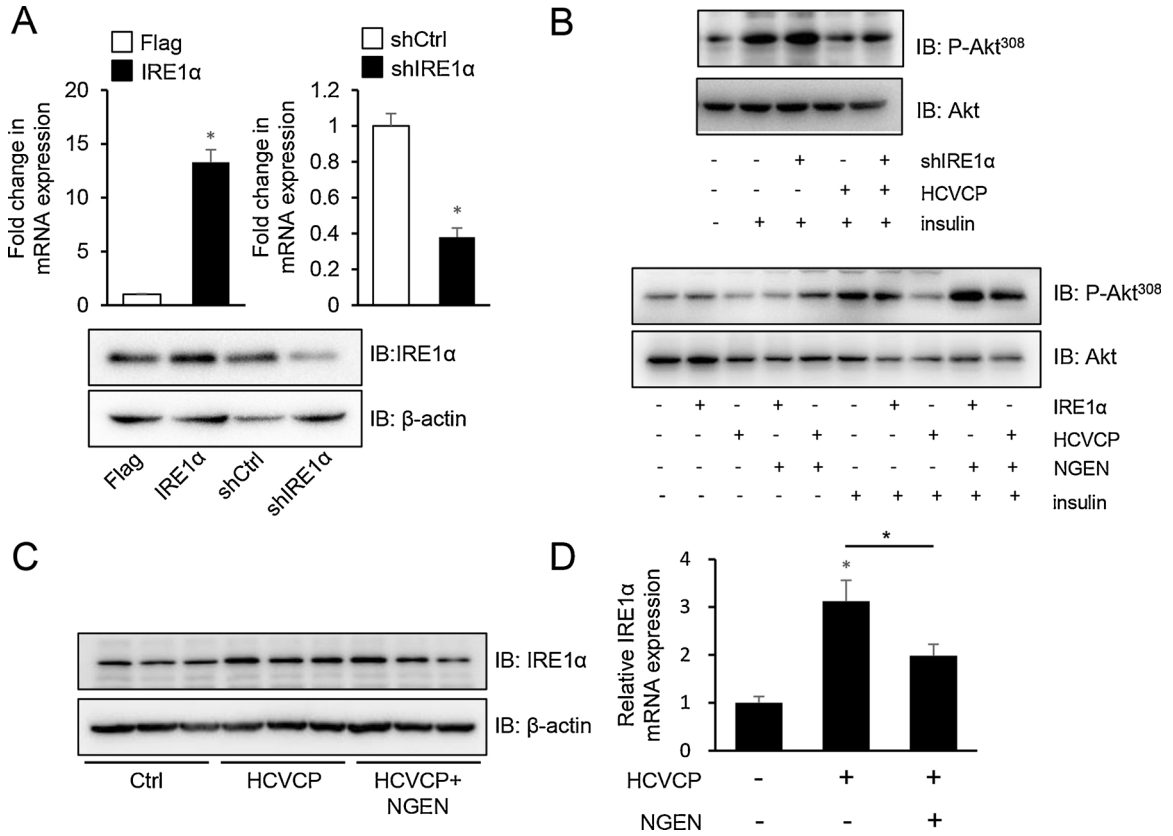

Fig. 4. NGEN ameliorates HCVCP induced IR by down-regulate the IRE1α levels. (A) Huh-7.5.1 cells were transfected with Flag, IRE1α, shCtrl and shIRE1α expression vectors, the IRE1α protein and mRNA levels were detected with western blot and RT-qPCR. (B) Huh-7.5.1 cells were transfected with expression vectors and treated with or without 1 μM NGEN for 48 h, after starved in serum free DMEM for 5 h, cells treated with or without 10 nM insulin for 5 min, western blot assay was performed to detect the phosphorylation of Akt. (D) The IRE1α protein and mRNA levels in HCVCP infected mice liver tissues were analyzed using western blot and RT-qPCR. The data are presented as the mean ± S.E. and expressed as fold-change relative to the level of cells transfected with control vector. $^{*}P < 0.05$.

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