Ascorbic acid attenuates cell stress by activating the fibroblast growth factor 21/fibroblast growth factor receptor 2/adiponectin pathway in HepG2 cells

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Abstract. Increasing prevalence of obesity-induced non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) has been reported. Ascorbic acid (AA), also known as vitamin C, an excellent antioxidant, has been shown to exert beneficial effects on NAFLD; however, the underlying mechanisms are yet to be fully elucidated. In the present study, the role of AA on cell stress in tumor necrosis factor α (TNFα)-treated HepG2 cells was investigated. Our findings revealed that exposure to AA effectively ameliorated TNFα-induced cell stresses, including hypoxia, inflammation and endoplasmic reticulum (ER) stress by reducing the expression of *Hif1α* and its target genes (glucose transporter 1), pro-inflammatory genes (monocyte chemoattractant 1) and ER stress-related genes (glucose-regulated protein, 78 kDa). AA also decreased the protein level of HIF1α. Additionally, AA significantly increased the secretion of total adiponectin and high molecular weight (HMW) adiponectin. Mechanistically, AA was determined to increase the expression of fibroblast growth factor 21 (FGF21) and its receptor, fibroblast growth factor receptor 2 (FGFR2). Knockdown of FGFR2 not only decreased the levels of total adiponectin and HMW adiponectin, but almost abolished the beneficial effects of AA in ameliorating cell stress. Collectively, the findings of our study demonstrated that AA may attenuate hepatocyte stress induced by TNFα via activation of the FGF21/FGFR2/adiponectin pathway. This could a novel mechanism of action of AA, and its potential for the treatment of NAFLD/NASH.

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Introduction

Obesity, a common public health concern worldwide, is associated with the development of chronic metabolic diseases, including non-alcoholic fatty liver disease (NAFLD), which is characterized by the excessive accumulation of triglycerides (TAG) in the liver without inflammation (1). Studies have reported that NAFLD in patients may progress to non-alcoholic steatohepatitis (NASH), liver sclerosis and even hepatocellular carcinoma (2,3) . Of these patients, obese individuals are more likely to suffer from NAFLD/NASH as increases in body weight have been shown to be positively associated with the accumulation of fat content in the liver (4). Amelioration of cellular stress in NAFLD/NASH models revealed an improvement in the levels of biomarkers related to cellular stress, such as markers of hypoxia, inflammation and endoplasmic reticulum (ER) stress, has been reported (5).

Adiponectin has been demonstrated to exert marked insulin-sensitizing and anti-inflammatory effects (6). Reduced circulating adiponectin levels (hypoadiponectinemia) have been linked to the etiology of obesity and obesity-related diseases (7). Adiponectin can be used to combat various types of liver damage, including liver disease induced by carbon tetrachloride, fructose, a high-fat diet, high cholesterol levels and ethanol (8-11). More importantly, a previous study demonstrated that a decrease in adiponectin levels is an independent risk factor of developing NAFLD (12). In recent years, researchers have found that high molecular weight (HMW) adiponectin is the predominant active form of adiponectin in the liver, and is closely associated with obesity and obesity-associated disorders (13-15). Fibroblast growth factor 21 (FGF21), a member of the FGF family, is secreted mainly from tissues with high metabolic activity, such as the liver and adipose tissue (16). FGF21 treatment has been reported to attenuate or eliminate the progression to NASH in animals (17). Furthermore, FGF21 knockdown has been shown to be associated with markedly more severe hepatic steatosis and inflammation, which can progress to severe NASH in mouse models of NAFLD (18,19). Increasing evidence suggests

that FGF21 may be developed as a novel agent candidate for the treatment of NAFLD/NASH (20,21). Studies have shown that, partially dependent on peroxisome proliferator-activated receptor-γ activity, FGF21 can stimulate the transcription and secretion of adiponectin in adipocytes (22,23). The activation of the FGF21/adiponectin pathway has been reported to inhibit liver TAG accumulation, thereby reversing hepatic steatosis and improving NAFLD/NASH (23,24).

Ascorbic acid (AA), also known as vitamin C, is mainly found in a number of vegetables and fruits. As an excellent antioxidant, AA has a wide range of benefits, namely health-promoting and disease-preventing, and therapeutic properties (25). Studies have shown that AA, along with other antioxidants, such as vitamin E, can effectively inhibit oxidative stress, thereby reversing NAFLD (26,27). Apart from its antioxidative activity, AA can protect cells from stress via non-antioxidative pathways (28,29); however, these potential effects of AA alone on NAFLD/NASH and its mechanisms of action have not yet been well characterized. Additionally, AA has been reported to promote the secretion of HMW adiponectin from human adipocytes (30). To the best of our knowledge, whether AA can increase the expression of HMW adiponectin in hepatocytes remains unknown.

In spite of accumulating evidence, which typically describes the beneficial effects of AA on NAFLD/NASH *in vivo* or *in vitro*, further investigation is required to identify the specific roles of AA in obesity-associated factors-induced cell stress in hepatocytes. Thus, the aim of the present study was to determine the effects of AA on obesity-associated cell stress induced by tumor necrosis factor α (TNFα) in HepG2 cells and further explore the possible underlying mechanisms.

Materials and methods

Materials. TNFα (cat. no. T6674) and AA (cat. no. A4403) were purchased from Sigma-Aldrich (Merck KGaA). Antibodies against FGF21 (cat. no. ab171941), hypoxia inducible factor 1α (HIF1α; cat. no. ab2185) and fibroblast growth factor receptor 2 (FGFR2; cat. no. ab109372) were obtained from Abcam; phosphorylated 5'AMP-activated protein kinase (p-AMPK; cat. no. 2531) and AMPK (cat. no. 2532) antibodies were from Cell Signaling Technology, Inc.; β-actin (cat. no. sc-47778), the horseradish peroxidase-conjugated secondary antibody goat-anti-rabbit (cat. no. sc-2004) and goat-anti-mouse (cat. no. sc-2005) IgG antibodies were obtained from Santa Cruz Biotechnology, Inc. All other reagents were from Sigma-Aldrich (Merck KGaA) unless otherwise stated.

Cells and cell culture. HepG2 cells, a human hepatoma-derived cell line, were obtained from the American Type Culture Collection (HB-8065) and cultured routinely in a humidified atmosphere containing 5% CO_2 at 37˚C with Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (30 mg/ml). Cells were seeded into plates 24 h prior to the treatments at ~80% confluence. Cells were co-cultured with or without AA (0, 100, 200, 500, and 1,000 μM) and 10 ng/ml TNFα for 24 h in a humidified atmosphere containing 5% CO_2 at 37˚C. According to the findings from this experiment, 100 μM AA selected for further experimentation as this concentration effectively inhibited increases in the expression of cell stress-related genes induced by TNFα (data not shown). The cell groups were as follows: Control group, cultured in DMEM; TNFα group, treated with TNFα (10 ng/ml); and TNFα + AA group, cells co-cultured with TNFα (10 ng/ml) and AA (100 μM). For each assay, at least three independent experiments were conducted.

Reverse transcription-quantitative PCR (RT-qPCR). All primers were designed using Primer Express 3.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.; Table I). RT reactions were conducted using an RT kit (cat. no. RR036A; Takara Bio, Inc.) and SYBR®-Green PCR Super mix (cat. no. A25741; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The conditions of PCR used Applied Biosystems ViiA™ 7 Dx qPCR System in this study were as follows: Initial denaturation: 95˚C, 5 min; 40 cycles of denaturation (95˚C, 30 sec), annealing (58˚C, 30 sec), and elongation (72˚C, 60 sec). The fluorescent signals were detected during the extension phase, Quantification cycle (Cq) values of the sample were calculated. The expression of *Gapdh* was assessed as a housekeeping gene. The relative expression of the gene of interest was analyzed using the $2^{-\Delta\Delta Cq}$ method (31). All the experiments were repeated three times.

Western blot analysis. Cells were harvested and homogenized in lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) at 4˚C. The homogenates were centrifuged at 12,000 x g at 4˚C for 15 min. Protein was quantified using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). Protein samples (20 μg) were separated on SDS-PAGE, transferred to PVDF membranes, and blocked with 5% of BSA in TBS-T buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.15% Tween-20) at room temperature for 90 min. Following washing with TBST, the membranes were incubated with primary antibodies at 4˚C overnight, the primary antibodies were as follows: Anti-β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology Inc.); anti-HIF1α (1:200; cat. no. ab2185; Abcam); anti-FGFR2 (1:1,000; cat. no. ab109372; Abcam); anti-FGF21 (1:1,000; cat. no. ab171941; Abcam); anti-p-AMPK (1:1,000; cat. no. 2531; Cell Signaling Technology, Inc.), anti-AMPK (1:1,000; cat. no. 2532; Cell Signaling Technology, Inc.), followed by incubation with appropriate secondary antibodies for 1 h at room temperature, the primary antibodies were as follows: Goat anti-rabbit IgG antibodies (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG antibodies (1:5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.). Finally, they were detected using an enhanced chemiluminescence system (EMD Millipore). The band intensities were quantified by using ImageJ software version 1.8.0 (National Institutes of Health, Bethesda, MD, USA). The calculated ratio of the intensity of the target protein to that of β-actin corresponded to the expression level of the protein.

ELISA. HepG2 cells were collected under aseptic conditions and incubated ($5x10^6$ cells/well) in 6-well culture plates at 37˚C in humidified 5% CO_2. Following treatment as aforementioned, the conditioned medium from the cells was collected for determining the secretion of total adiponectin (cat. no. ab99968,

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-Actin	GCAAAGACCTGTACGCCAACA	TGCATCCTGTCGGCAATG
Vegfa	TTGCCTTGCTGCTCTACCTCCA	GATGGCAGTAGCTGCGCTGATA
Glut1	TTGCAGGCTTCTCCAACTGGAC	CAGAACCAGGAGCACAGTGAAG
Mcp1	AGAATCACCAGCAGCAAGTGTCC	TCCTGAACCCACTTCTGCTTGG
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
Grp78	CTGTCCAGGCTGGTGTGCTCT	CTTGGTAGGCACCACTGTGTTC
Sxbp1	CTGCCAGAGATCGAAAGAAGGC	CTCCTGGTTCTCAACTACAAGGC
Fgfr2	GTGCCGAATGAAGAACACGACC	GGCGTGTTGTTATCCTCACCAG
Fgf21	CTGCAGCTGAAAGCCTTGAAGC	GTATCCGTCCTCAAGAAGCAGC
Hif1α	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA

Fgf21, fibroblast growth factor 21; Fgfr2, fibroblast growth factor receptor 2; Glut1, glucose transporter 1; Grp78, glucose-regulated protein, 78 kDa; HIF1α, hypoxia inducible factor 1α; IL-6, interleukin-6; Mcp1, monocyte chemoattractant 1; Sxbp1, spliced X-box-binding protein 1; Vegfa, vascular endothelial growth factor A.

Abcam) and HMW adiponectin (cat. no. CSB-E07270h, CUSABIO) using ELISA kits. Cells were then washed twice with ice-cold PBS and lysed in lysis buffer comprising 50 mM Tris (pH 7.4), 150 mM NaCl, 10% (w/v) glycerol, 10 mM EDTA, 1 mM $MgCl_2$, 20 mM b-glycerophosphosphate, 30 mM NaF, 1% Triton X-100, 25 mg/ml leupeptin, 25 mg/ml pepstatin and 3 mg/ml aprotinin. After incubation on ice for 20 min, cell lysates were centrifuged at 12,000 x g for 30 min at 4˚C. A BCA Protein Assay was used to quantify the total protein of cells. The concentration of HIFα was measured using a commercially available ELISA kit (cat. no. ab171577, Abcam) according to the manufacturer's instructions.

RNA interference. The small interfering RNA (siRNA) sequences targeting human *Fgfr2* were designed using siRNA primer design software (Guangzhou RiboBio Co., Ltd.; Table II). We mixed the three individual targeting siRNAs (50 nM of each) at a ratio of 1:1:1 (*Fgfr2* siRNA) for transfection. HepG2 cells were seeded on 6-well plates at 37˚C for 24 h until 50-80% confluence was attained. Cells were subsequently transfected with 50 nM of the *Fgfr2* siRNA mixture or scramble (Scr) control siRNA using ribo*FECT*™ CP (Guangzhou RiboBio Co., Ltd.).

Statistical analysis. All experiments were performed in triplicate. The results are presented as the mean ± standard error of mean. Significant differences were determined by one-way ANOVA followed by the Least Significant Difference test using SPSS 18.0 software (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

AA reduces TNFα -induced cell stress in HepG2 cells. To establish a cellular model of obesity-related NAFLD, TNFα, an obesity-associated insult factor, was used to induce cell stress in HepG2 cells. As presented in Fig. 1, treatment with TNFα (10 ng/ml) for 24 h was determined to successfully promote cellular hypoxia, inflammation and ER stress, as determined by the significant increase in HIF1α expression compared with the control. Incubation with AA (100 μM) for 24 h significantly decreased the protein and mRNA expression levels of HIF1α in HepG2 cells compared with TNFα treatment alone (Fig. 1A-C). Additionally, AA treatment resulted in a significant decrease in the mRNA levels of glucose transporter 1 (*Glut1*), an important target gene of HIF1α (Fig. 1D) (32). In addition, AA reduced the mRNA levels of the inflammatory factor, monocyte chemoattractant 1 (*Mcp1*; Fig. 1E), and the ER stress factor, glucose-regulated protein, 78 kDa (*Grp78*; Fig. 1F). However, the mRNA expression levels of vascular endothelial growth factor A (*Vegfa*; Fig. 1D), interleukin (*Il*)*-6* (Fig. 1E) and spliced X-box-binding protein 1 (*Sxbp1*; Fig. 1F) were markedly unaffected by TNFα and AA treatment for 24 h.

AA activates the FGF21/FGFR2/adiponectin pathway. In addition, we examined the effects of AA treatment on adiponectin expression, and the secretion of total and HMW adiponectin. Compared with the control group, the secretion of HMW adiponectin, but not that of total adiponectin was inhibited by TNFα treatment. However, TNFα and AA co-treatment resulted in a significant increase in the secretion of total and HMW adiponectin compared with TNFα treatment alone (Fig. 2A and B). We also detected the expression of the upstream molecules involved in the signaling pathways of adiponectin. The addition of AA significantly increased the expression of FGFR2 and FGF21 than with TNFα alone; compared with the control, TNFα treatment notably induced the expression of FGF21 (Fig. 2C-E). Conversely, the expression levels of p-AMPK and AMPK were markedly unaffected in all treatment groups (Fig. 2D and E), suggesting that the AMPK signaling pathway may not have been activated by AA.

Fgfr2 knockdown attenuates the effects of AA on total and HMW adiponectin. In order to confirm the role of FGF21/FGFR2 in beneficial effects of AA in this study, FGFR2 expression was silenced via RNA interference. Compared with *Scr* siRNA, the

Table II. Fgfr2 siRNA primer used for transfection.

siRNA	Forward primer (5'-3')	Reverse primer (5'-3')
Fgfr2-1	GCCCUCCUUCAGUUUAGUUTT	AACUAAACUGAAGGAGGGCTT
Fgfr2-2	GGACAAAGAGAUUGAGGUUTT	AACCUCAAUCUCUUUGUCCTT
Fgfr2-3	CCAGAGGCAUGGAGUACUUTT	AAGUACUCCAUGCCUCUGGTT

Fgfr2, fibroblast growth factor receptor 2; siRNA, small interfering RNA.

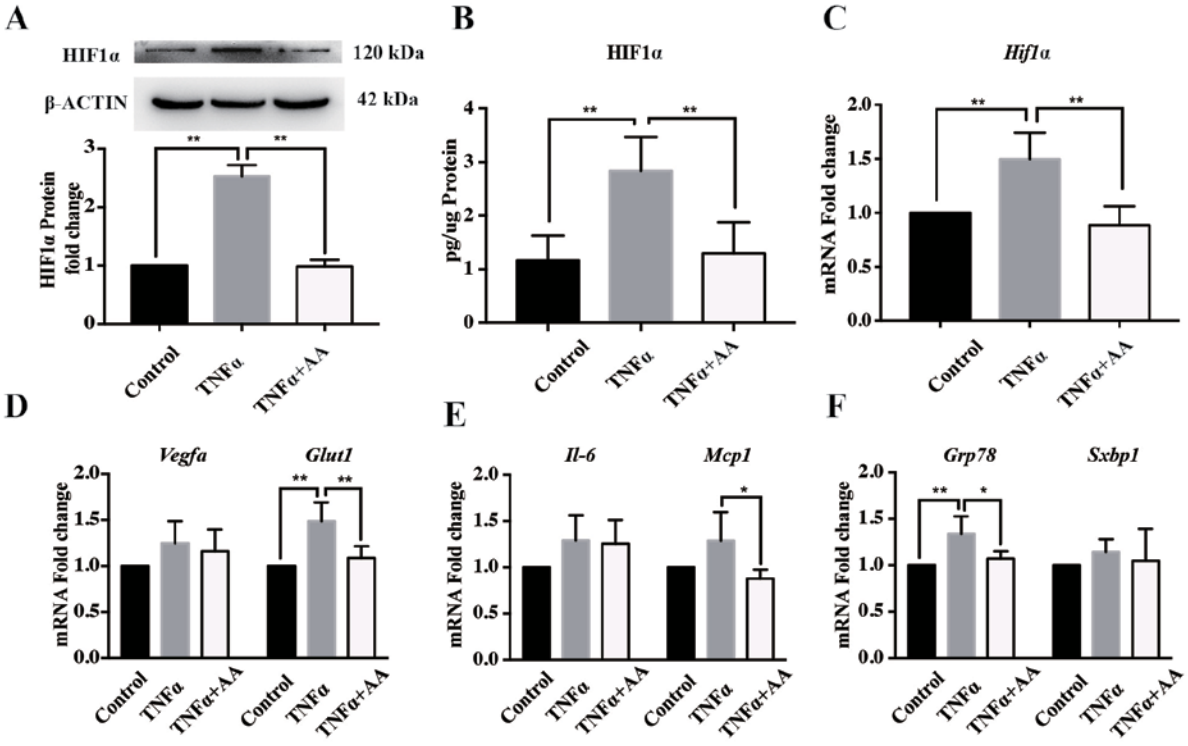

Figure 1. AA (100 μM) attenuates TNFα-induced (10 ng/ml) cell stress. (A and B) HIF1α expression in HepG2 cells was determined by western blotting and ELISA; (C) mRNA expression levels of mRNA levels in HepG2 cells were assessed cells by RT-qPCR, respectively. The mRNA expression levels of factors related to (D) hypoxia, (E) inflammation and (F) endoplasmic reticulum stress were assessed in HepG2 cells by RT-qPCR. Data are expressed as fold changes compared with the control group. All data are presented as the mean ± standard error of the mean. *P<0.05; **P<0.01. AA, ascorbic acid; Glut1, glucose transporter 1; Grp78, glucose-regulated protein, 78 kDa; HIF1α, hypoxia inducible factor 1α; IL-6, interleukin-6; Mcp1, monocyte chemoattractant 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Sxbp1, spliced X-box-binding protein 1; TNFα, tumor necrosis factor α; Vegfa, vascular endothelial growth factor A.

transfection of siRNA targeting *Fgfr*2 successfully inhibited the expression of FGFR2 (P<0.001; Fig. 3A and B). Compared with *Scr* siRNA, *Fgfr2* knockdown significantly suppressed the increase in the secretion of both total adiponectin and HMW adiponectin induced by AA (Fig. 3C and D).

Fgfr2 knockdown abolishes the effects of AA on cell stress. We finally assessed the effects of *Fgfr2* knockdown on cellular stress in HepG2 cells. In the *Scr* siRNA-transfected group, AA treatment appeared to reverse the increase in all cell stress markers at the mRNA and protein levels due to TNFα, including HIF1α (Fig. 4A and B) and its target genes, *Glut* and *Vegfa* (Fig. 4C and D), and the gene expression of inflammatory-related factors (*Mcp1* and *Il-6*; Fig. 4E and F) and ER stress-related factors (*Grp78* and *Sxbp1*; Fig. 4G and H); significant differences were observed between the *Scr*-siRNA and *Fgfr2*-siRNA groups following co-treatment. Furthermore, the role of AA in improving NAFLD/NASH proposed in the present study is summarized as a schematic diagram in Fig. 4I. AA was suggested to suppress obesity-associated insult via the inhibition of TNFα-induced cellular hypoxia, inflammation and ER stress. These benefits of AA are likely to be mediated by the activation of the FGF21/FGFR2/adiponectin signaling pathway in hepatocytes.

Discussion

Long-term exposure to obesity-associated insults induced by TNFα and saturated fatty acids causes lipid accumulation in the liver, which leads to fatty liver disease, known as NAFLD, which may progress to NASH (33). In the present study, TNFα-treated HepG2 cells were employed to examine

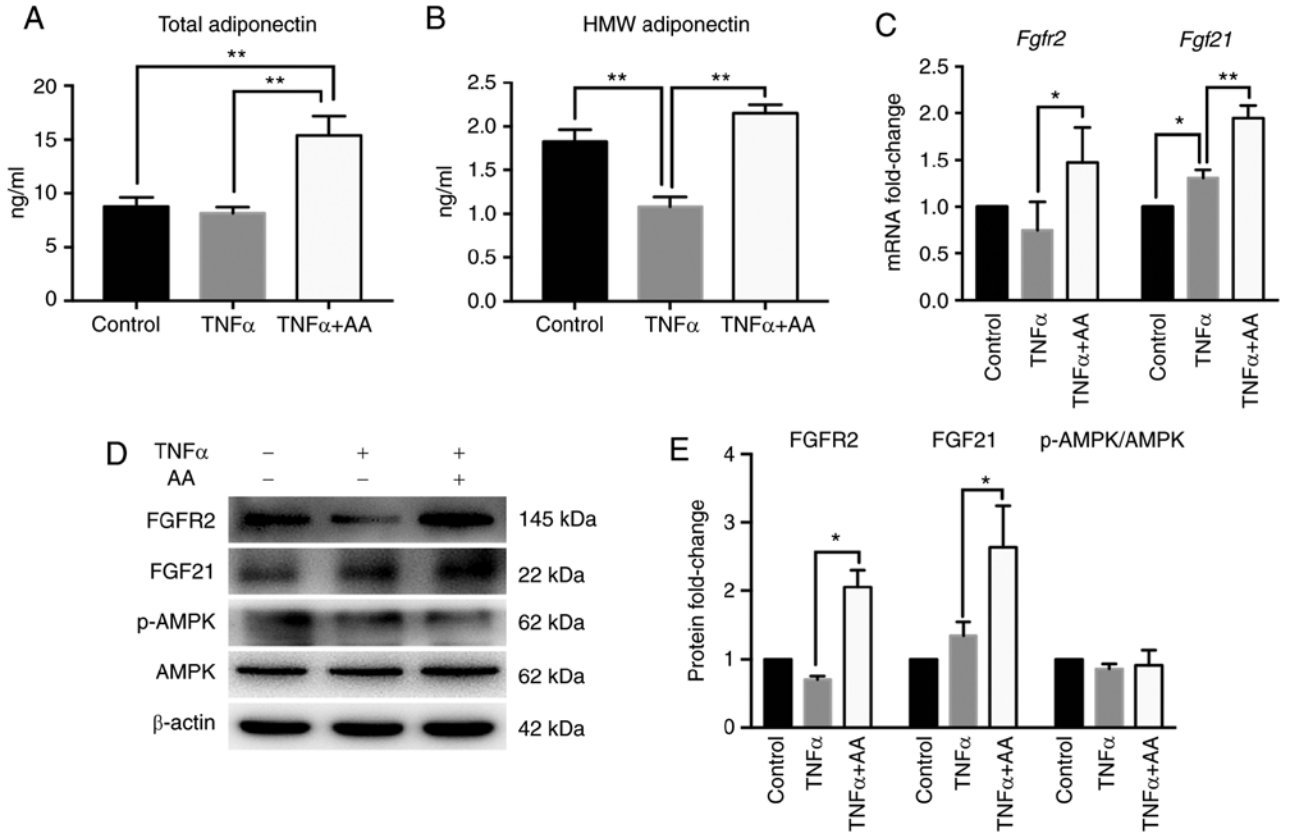

Figure 2. AA activates the FGF21/FGFR2/adiponectin pathway in HepG2 cells. ELISA of (A) total adiponectin and (B) HMW adiponectin secretion in all groups. (C) Reverse transcription-quantitative polymerase chain reaction analysis of *Fgf21* and *Fgfr2* gene expression in HepG2 cells. (D) Representative images of FGFR2, FGF21, p-AMPK and AMPK protein levels from 3 independent experiments and (E) fold changes of protein levels relative to the control group are shown. Control group, cultured in DMEM medium; TNFα group, treated with TNFα (10 ng/ml); and TNFα + AA group, co-cultured with TNFα (10 ng/ml) and AA (100 μM). All data are normalized to the band density of β-actin. *P<0.05; **P<0.01. AA, ascorbic acid; AMPK, 5'AMP-activated protein kinase; Fgf21, fibroblast growth factor 21; Fgfr2, fibroblast growth factor receptor 2; HMW, high molecular weight; p, phosphorylated; TNFα, tumor necrosis factor α.

the effects of AA on hepatocyte stress in obesity-related NAFLD *in vitro*. Our results revealed that AA: i) Significantly ameliorated TNFα-induced cell stresses, including hypoxia, inflammation and ER stress; ii) significantly promoted the secretion of HMW adiponectin from TNFα-treated cells; and iii) activated the FGF21/FGFR2/adiponectin signaling pathway to exert these beneficial effects as mentioned above.

Our data demonstrated that AA co-treatment effectively attenuated TNFα-induced cell stress. The expression of TNFα, a known pro-inflammatory factor, is significantly elevated in patients with NAFLD/NASH, and is involved in liver lipid metabolism and inflammation (33,34). Thus, TNFα-induced HepG2 cells were employed in the present study to establish an *in vitro* model in order to mimic the unhealthy state of hepatocytes in the obesity-related NAFLD/NASH population. Our results revealed that TNFα successfully induced hepatocyte stress in the form of increased hypoxia, inflammation and ER stress.

The human body cannot synthesize vitamin C; a lower consumption of vitamin C has been associated with a decreased plasma level of AA, which may increase the risk of developing metabolic diseases, including NAFLD/NASH (27). In addition, hepatocytes from patients with NAFLD/NASH suffer from various types of stress, such as hypoxia, inflammation and ER stress (35-37). Therefore, we aimed to investigate the effects of AA on NAFLD/NASH beyond its traditional characteristic as an excellent antioxidant. Our results reported that AA reversed the aforementioned stresses triggered by TNFα. Similar findings have also been reported in human intervention studies. For example, overweight adults supplemented with AA (1,000 mg/day) for 2 months exhibited significantly reduced plasma c-reactive protein levels (38). Of note, we reported AA to reduce the levels of HIF1α protein. Generally, the total level of HIF1α does not usually change under hypoxia (39); although the mechanism associated with the expression of HIF1α under these conditions was not explored in our study, further investigation is required. AA can inhibit the stability and transcriptional activity of HIF1α protein. As mentioned in a previous study (32), HIF-1 is downregulated by iron-containing 2-oxoglutarate-dependent enzymes that require AA as a cofactor; HIF-1-dependent gene expression is effectively suppressed by AA and is inhibited even under conditions that allow HIF-1α protein stabilization. Additionally, Vissers *et al* (40) have found that AA is the main regulator of the hypoxic response in normal cells, and the optimal level of AA has an important impact on HIF-1 regulation (41). Taken together, these results demonstrate that AA alone may be beneficial for the amelioration of obesity-induced NAFLD/NASH by attenuating a wide range of hepatocyte stresses.

This study also measured the secretion of total adiponectin and HMW adiponectin levels, as obesity-associated

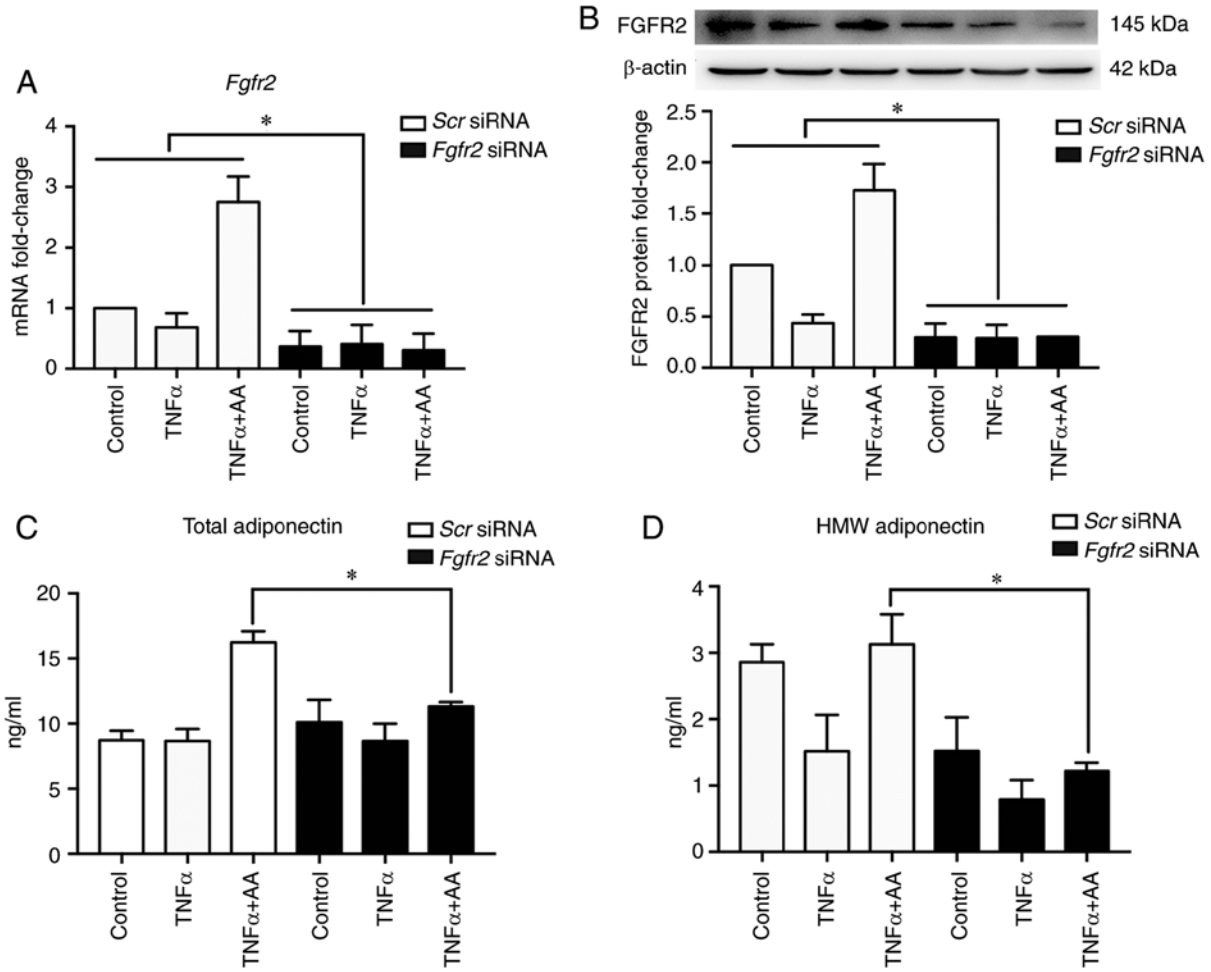

Figure 3. *Fgfr2* knockdown decreases the AA-induced secretion of adiponectin. (A) *Fgfr2* mRNA and (B) protein levels from *Scr* siRNA or *Fgfr2* siRNA-transfected cells. (C) Total adiponectin and (D) HWM adiponectin levels were determined by ELISA. All data are expressed as the mean ± standard error of the mean. *P<0.05. AA, ascorbic acid; Fgfr2, fibroblast growth factor receptor 2; HMW, high molecular weight; Scr, scramble; siRNA, small interfering RNA; TNFα, tumor necrosis factor α.

NAFLD/NASH has been associated with decreased levels of circulating adiponectin and HMW adiponectin (42). We found that TNFα reduced the secretion of HMW adiponectin, but exerted no notable effects on total adiponectin levels. Treatment with AA not only increased total adiponectin secretion, but also attenuated reductions in HMW adiponectin secretion induced by TNFα. Our results were consistent with those of a recent study, which indicated that the beneficial effects of AA are closely associated with improvements in adiponectin profiles rather than changes in total adiponectin levels (43). Therefore, the findings of the present study may suggest that AA may exert its beneficial effects on hepatocyte stress by increasing the levels of adiponectin levels, particularly HMW adiponectin.

Over the past few years, the specific roles of FGF21/FGFR2 in modulating obesity and obesity-associated metabolic diseases, such as NAFLD/NASH and type 2 diabetes have undergone extensive study (44-46). Although FGF21 expression has been shown to be notably elevated in obese individuals, long-term treatment with FGF21 was determined to favorably improve the outcomes linked to obesity and NAFLD (47,48). Adiponectin is a downstream effector of FGF21/FGFR2 (49,50). A recent study also suggested that the FGF21/adiponectin/IL-17A pathway is crucial in alleviating hepatic steatosis and inflammation in a mouse model of NASH (24). Nevertheless, to the best of our knowledge, no studies have investigated the effects of AA on the FGF21/FGFR2 signaling pathway, although white pitaya juice, which contains AA, was proposed to attenuate hepatic steatosis in obese mice (51). The results of our study demonstrated that AA significantly increased the mRNA and protein expression levels of FGF21 and FGFR2, and adiponectin secretion. Furthermore, FGFR2 knockdown almost abolished the protective effects of AA in terms of blocking the reduction of HIF1α and the downregulation of cellular stress-related genes. Unexpectedly, TNFα also induced FGF21 expression; further investigations are warranted to determine the mechanisms underlying this effect. An overlap in energy metabolism has been reported between the AMPK and FGF21 signaling pathways (52-54). However, in our specific model, AA did not activate the AMPK pathway; this was inconsistent with the findings of another study which reported that strawberry extract attenuated lipid accumulation in HepG2 cells via AMPK activation (55). This inconsistency may be explained by the different agents used; strawberry extract contains other bioactive substances apart

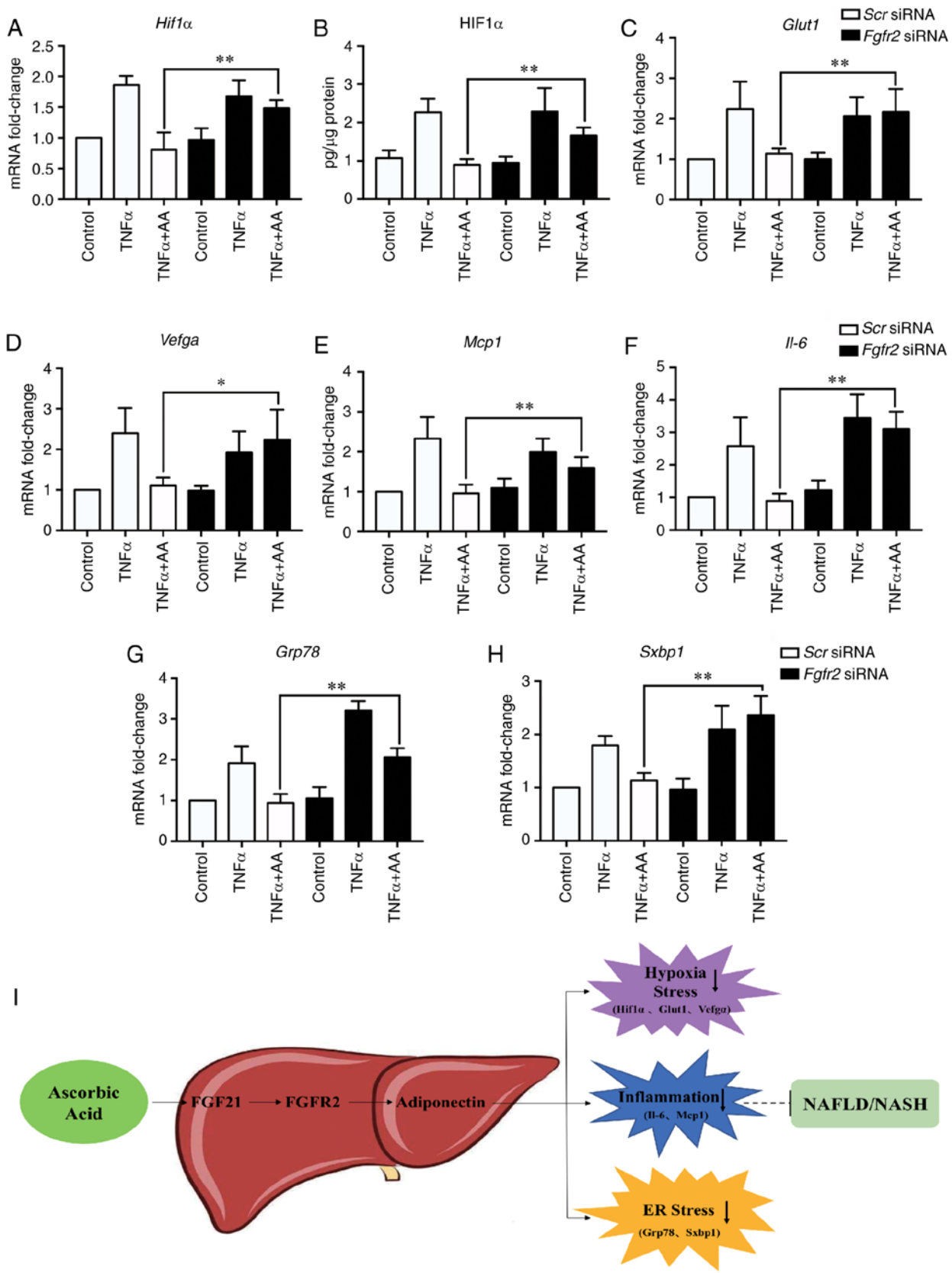

Figure 4. *Fgfr2* knockdown suppresses the inhibitory effects of AA on cell stress. *Hif1α* (A) mRNA and (B) protein expression levels were assessed in HepG2 cells by RT-qPCR and ELISA, respectively. The gene expression of (C) *Glut1* and (D) *Vegfa*, (E) *Mcp1* and (F) *Il-6*, (G) *Grp78* and (H) *Sxbp1* were determined by RT-qPCR. Data are expressed as fold changes compared with *Scr* siRNA. *P<0.05; **P<0.01. (I) Schematic model of the effects of AA on hepatocyte stress. AA treatment was proposed to be beneficial for relieving cell stress (hypoxia, inflammation and ER stress) in hepatocytes via the regulation of the FGF21/FGFR2/adiponectin pathway, which may improve NAFLD/NASH. AA, ascorbic acid; ER, endoplasmic reticulum; FGF21, fibroblast growth factor 21; Fgfr2, fibroblast growth factor receptor 2; Glut1, glucose transporter 1; Grp78, glucose-regulated protein, 78 kDa; HIF1α, hypoxia inducible factor 1α; IL-6, interleukin-6; Mcp1, monocyte chemoattractant 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Scr, scramble; siRNA, small interfering RNA; Sxbp1, spliced X-box-binding protein 1; TNFα, tumor necrosis factor α; Vegfa, vascular endothelial growth factor A.

from AA that may activate the AMPK pathway. Collectively, these results illustrated that the FGF21/FGFR2/adiponectin signaling pathway could serve a crucial role in attenuating obesity-related insult-associated hepatocyte stress by AA.

In conclusion, the present study demonstrated that AA effectively attenuated hepatocyte stress induced by obesity-related insults through activation of the FGF21/FGFR2/adiponectin pathway, which may suggest a novel mechanism of AA in alleviating NAFLD/NASH. Further investigation is required to verify the role of AA in NAFLD/NASH in future studies.

Our study has certain limitations in that HepG2 cells were employed to establish an *in vitro* model of NAFLD. Under certain culture conditions, HepG2 cells display robust morphological and functional differentiation. Therefore, this cell line can be important for the study of human liver diseases, yet this cell line represent as a model of cancer rather than normal human liver. Thus, the results of the present study require further verification *in vivo*.

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Availability of data and materials

All data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XQG, XL and XQL made substantial contributions to the design of this study. XQG, ZYH, KJW and YFZ performed the experiments. ZYH, YXW and XML conducted the RT-qPCR and western blot analysis experiments. YFY, JJ and XQG interpreted the results and wrote the draft of the manuscript. XQL and XL critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript and agreed to the publication of the final manuscript.

Ethics approval and consent to participate

Not applicable

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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