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Inositol-requiring enzyme 1 alpha endoribonuclease specific inhibitor STF-083010 protects the liver from thioacetamide-induced oxidative stress, inflammation and injury by triggering hepatocyte autophagy

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ABSTRACT

Acute liver injury caused by toxins or drugs is a common condition that threatens patients' lives. Inositol-requiring enzyme 1 alpha (IRE1α), the most conserved endoplasmic reticulum (ER) stress sensor, has been implicated in the pathophysiology of liver injury. Activated IRE1α endoribonuclease (RNase) can splice X-box binding protein 1 (*XBP1*) mRNA to produce the sXBP1 transcription factor. STF-083010, a specific inhibitor of IRE1α RNase, has recently been suggested to exhibit anti-oxidant and anti-inflammatory properties in multiple injury models. However, it remains unknown whether STF-083010 has a protective effect against thioacetamide (TAA)-induced acute liver injury. Here, we demonstrated that IRE1α-sXBP1 signaling is involved in the development of TAA-induced acute liver injury and correlates with the severity of liver damage. STF-083010 protected against TAA-induced liver injury, as evidenced by higher survival rates in response to a lethal dose of TAA and less severe liver injury in response to a toxic dose of TAA. Mechanistic exploration showed that STF-083010 triggered hepatocyte autophagy in response to TAA stimulation both in vivo and in vitro, leading to reduced reactive oxygen species (ROS) production and attenuated hepatic inflammation. We also found that Beclin-1 played a critical role in STF-083010-mediated autophagy in response to TAA stimulation. Autophagy inhibition by chloroquine (CQ) in vivo and Beclin-1 knockdown in vitro markedly abrogated the protective role of STF-083010 against TAA-induced oxidative stress, inflammation and hepatotoxicity. Our results suggested STF-083010 as a potential therapeutic application to prevent TAA-induced acute liver injury.

1. Introduction

Acute liver injury is a common liver disease in daily clinical practice. The major causes of acute liver injury include drug overdose, toxin exposure, hepatitis virus infection and ischemia/reperfusion (IR). Although acute liver injury has been broadly investigated over the past decade, its pathophysiological process remains unclear. It is generally believed that a complex interplay of endoplasmic reticulum (ER) stress, oxidative stress and subsequent inflammatory response participates in the progression of acute liver injury caused by various stimuli [1,2].

Thioacetamide (TAA) is a frequently used toxin to induce liver injury and chronic fibrosis in a mouse model. In the liver, cytochrome P450 enzymes bioactivate TAA to produce toxic metabolites that cause direct damage to the liver [3]. ER stress and oxidative stress are also involved in the pathogenesis of TAA-induced liver injury. Superoxide dismutase 1 (SOD1)-deficient mice exhibited less severe liver damage caused by TAA due to decreased reactive oxygen species (ROS) production [4]. ER stress was attenuated in aldehyde reductase 1a (Akr1a)-deficient mice, leading to mitigated TAA-induced liver injury [5]. Indeed, ER stress and oxidative stress coexist in many pathologic processes and trigger each other in a positive feed-forward loop, resulting in ROS accumulation [6–8]. Increased ROS accumulation in hepatocytes is directly toxic to cells. Moreover, ROS and oxidized molecules released from injured or dead cells further activate inflammatory responses, leading to exacerbated liver damage [9].

ER stress has been well documented in various liver diseases [10].

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Inositol-requiring enzyme 1 alpha (IRE1α), which is the most conserved branch of (unfolded protein response) UPR, contains dual enzyme activities of protein kinase and endoribonuclease (RNase). Activation of IRE1α RNase by autophosphorylation cleaves the unconventional splicing of the mRNA of X-box binding protein 1 (*XBP1*) to produce active transcription factor spliced-XBP1 (sXBP1) [11]. IRE1α-sXBP1 signaling has been implicated in many types of liver diseases, including toxin-induced liver injury, liver IR injury, non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH), viral hepatitis, and alcoholic liver disease [12–14]. STF-083010, a novel specific inhibitor of IRE1α RNase, has been recently suggested to play a beneficial role in preventing liver injury and diseases, including carbon tetrachloride (CCl_4)-induced liver injury and NASH [15,16].

Autophagy, an evolutionarily conserved cellular process of the lysosomal degradation of proteins and organelles, is activated in response to multiple stresses [17]. Compelling evidence has shown that autophagy participates in both the response to oxidative stress and ER stress, resulting in ROS elimination [18–22]. Induction of autophagy by rapamycin represses acetaminophen (APAP)-induced hepatotoxicity, while the inhibition of autophagy by chloroquine (CQ) increases intracellular ROS production, leading to exacerbated APAP-induced liver injury [23]. In APAP-treated mice, hepatic ROS production is increased and liver damage is aggravated as a result of adiponectin deficiency-mediated autophagy inhibition [24]. IRE1α-sXBP1 signaling has also been proposed as an autophagy modulator in multiple studies [17,25–28]. However, no direct evidence has been reported on the role of STF-083010 in regulating autophagy in the context of TAA-induced liver injury.

The objective of this study was to investigate the protective effects of STF-083010 against TAA-induced oxidative stress, inflammation and liver injury and to further explore the potential mechanism with a focus on hepatocyte autophagy.

2. Materials and methods

2.1. Animals

Six- to eight-week-old male C57BL/6J mice were purchased from the Laboratory of Animal Resources of Nanjing Medical University. All animals received humane care, and all animal procedures were carried out in accordance with the relevant legal and ethical requirements according to a protocol (number NMU08-092) approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

2.2. Animal treatment

Acute liver injury was induced by intraperitoneal injection of either a lethal dose of 500 mg/kg of thioacetamide (TAA) (Sigma, St. Louis, MO, USA) or a toxic dose of 200 mg/kg of TAA dissolved in sterile phosphate-buffered saline (PBS). Normal control mice were injected intraperitoneally with the same volume of PBS. To justify the effective dose of STF-083010 (MedChemexpress, New Jersey, MO, USA), separate groups of mice received intraperitoneal injection of different concentrations of STF-083010 (30, 50, 70 mg/kg) dissolved in PBS 2 h prior to TAA administration. For survival experiments, 48 mice were divided into the TAA group, TAA + STF (30 mg/kg) group, TAA + STF (50 mg/kg) group and TAA + STF (70 mg/kg) group (n = 12/group) and were monitored for 4 days. For acute liver injury experiments, the mice were treated with a toxic dose of TAA (200 mg/kg, n = 6/group) and were divided into four groups: CON group, STF group, TAA group and TAA + STF group. To block autophagic flux, chloroquine (CQ, 60 mg/kg) dissolved with PBS was injected intraperitoneally 1 h prior to TAA administration in some mice.

2.3. Serum biochemical examination and liver histopathology

In acute liver injury experiments, the mice were sacrificed at 24 h after TAA treatment, and then the liver tissues and serum were collected. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using an automated chemical analyzer (Olympus Company, Tokyo, Japan). Liver specimens were fixed in 10% buffered formalin and embedded in paraffin. The specimens were sectioned in 4-μm slices and were stained with hematoxylin and eosin (H&E). The liver damage extent was assessed by the necrotic area using ImageJ software.

2.4. TUNEL staining

Paraffin sections of liver specimens were stained by terminal deoxynucleotidyltransferase-mediated dUTP nick-end (TUNEL) staining using a fluorescent detection kit (Roche Diagnostics) according to the manufacturer's instructions.

2.5. Western blot analysis

Tissue and cellular proteins were extracted with ice-cold lysis buffer. Proteins (20 μg) were subjected to 10% SDS-PAGE and were transferred to polyvinylidene difluoride nitrocellulose membrane. The monoclonal rabbit antibodies against LC3B, p62, Beclin-1, sXBP1, and β-actin (Cell Signaling Technology, MA, USA) and rabbit antibodies against IRE1α and p-IRE1α (Abcam, Cambridge, MA, USA) were used. The protein bands were analyzed using ImageJ software.

2.6. Quantitative real-time PCR

Total RNA was extracted from the liver tissues according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the Transcription First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Real-time PCR was performed using SYBR green (Roche, Indianapolis, IN, USA). The relative mRNA levels were normalized to *GAPDH*, and the results were calculated using the $2^{-\triangle\triangle Ct}$ method. The following primers were used: *GAPDH*, forward, 5′-*ATTCCACCCATGGCAAATTC*-3′, reverse, 5′-*CGCTCCTGGAAGATGGTGAT*-3′; *TNF-α*, forward, 5′-*ACTGAACTTCGGGGTGATCG*-3′, reverse, 5′-*ACAGGCTTGTCACTCGAATTTTG*-3′; *IL-6*, forward, 5′-*AGTGGCTAAGGACCAAGAC*-3′, reverse, 5′-*ATAACGCACTAGGTTTGCCGA*-3′; *IL-1β*, forward, 5′-*GCACTAC-AGGCTCCGAGATGAA*-3′, reverse, 5′-*GTCGTTGCTT-GGTTCTCCTTGT*-3′.

2.7. Primary mouse hepatocytes and treatment

Primary mouse hepatocytes were isolated using the two-step collagenase perfusion protocol. Briefly, the mouse liver was perfused via the portal vein with Hank's balanced salt solution, followed by DMEM containing collagenase type IV (Sigma, St. Louis, MO, USA). Primary hepatocytes were then plated and cultured with Williams E medium and hepatocyte maintenance supplements.

To determine the impact of STF-083010 on the inhibition concentration (IC50) of TAA, primary hepatocytes were treated with different concentrations of TAA (0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 μM) dissolved in PBS with STF-083010 pretreatment (10 mM) and were cultured for 6 h. To evaluate the toxic effect of TAA, primary hepatocytes were treated with TAA at 70 μM for 6 h in the presence or absence of STF-083010 (10 mM). In some experiments, hepatocytes were incubated with CQ (50 μM) to block autophagic flux. STF-083010 and CQ were both dissolved in PBS. For Beclin-1 knockdown, non-specific siRNA (NS-siRNA) or siRNA targeting Beclin-1 (Beclin-1-siRNA) (RiboBio, Guangzhou, China) were transiently transfected using Lipofectamine 2000 siRNA Transfection Reagent (Roche, Diagnostics, Mannheim, Germany). Hepatocellular viability was examined by the

Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocols (Dojindo Molecular Technologies, Inc., USA). Hepatocellular cytotoxicity was measured by the lactate-dehydrogenase (LDH) release assay following the manufacturer's instructions (Promega, Madison, WI, USA).

2.8. ROS detection

ROS production in primary hepatocytes was measured using the DCFDA ROS Detection Assay Kit according to the manufacturer's instructions (Abcam, Cambridge, MA, USA). Cell fluorescence was detected using a fluorescence plate reader with an excitation wavelength at 485 nm and an emission wavelength at 535 nm. Hepatic ROS levels were detected using DHE staining. Frozen liver sections were stained with DHE (10 μM) (Sigma, St Louis, MO, USA) at 37 °C for 30 m. The sections were observed under a fluorescence microscope. The fluorescence intensity was quantified using ImageJ software.

2.9. Statistical analysis

The data are shown as means ± standard error of the mean (SEM). Comparison of different groups was performed using one-way analysis of variance followed by Tukey's post-hoc test. Survival rates were compared using a log-rank test. Statistical analysis was performed using GraphPad Prism 6.0 software. Two-tailed $p < 0.05$ was considered statistically significant.

3. Results

3.1. IRE1α-sXBP1 signaling is involved in the development of TAA-induced acute liver injury

First, we evaluated the time course of TAA-induced acute liver injury. As shown in Fig. 1A–C, compared with the CON group, TAA-induced liver injury was gradually exacerbated within 24 h, as evidenced by elevated serum alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels and worse-damaged liver histological features with increased necrotic areas. To assess the involvement of IRE1α-sXBP1 signaling in TAA-treated liver samples, we detected the protein levels of IRE1α, p-IRE1α and sXBP1 by Western blotting. The results showed that IRE1α-sXBP1 signaling was activated in response to TAA stimulation in a time-dependent manner, as demonstrated by increased protein levels of p-IRE1α and sXBP1 accompanied by unchanged protein levels of IRE1α (Fig. 1D). These results suggested the involvement of IRE1α-sXBP1 signaling in the development of TAA-induced acute liver injury.

3.2. STF-083010 attenuates TAA-induced acute liver injury

To further study the role of IRE1α-sXBP1 signaling in TAA-induced acute liver injury, STF-083010, a specific IRE1α RNase inhibitor, was used to suppress IRE1α-sXBP1 signaling in vivo. We first evaluated the effect of STF-083010 on the mortality of mice treated with a lethal dose of TAA (500 mg/kg). As shown in Fig. 2A, all the mice treated with a lethal dose of TAA alone died within 48 h. The mice in the low-dose STF-083010 (30 mg/kg) group showed similar mortality to those in the TAA group. However, a higher dose of STF-083010 (50 mg/kg) significantly decreased TAA-induced mortality (67%), a finding that was comparable to that in the group treated with 70 mg/kg of STF-083010. The effective dose of STF-083010 was justified as 50 mg/kg. The results indicated that STF-083010 significantly decreased TAA-induced mortality.

Since the most severe liver damage was present at 24 h after a toxic dose of TAA administration, we collected the liver tissues and serum samples at 24 h after TAA treatment in subsequent experiments. As shown in Fig. 2B–D, compared with the TAA group, STF-083010 markedly alleviated TAA-induced liver injury, as evidenced by reduced serum ALT (~70% reduction) /AST (~50% reduction) levels and decreased liver necrotic areas (~60% decrease). We also assessed hepatocyte cell death by TUNEL staining. The results showed that STF-083010 significantly inhibited TAA-induced hepatocyte cell death in the TAA + STF group as manifested by fewer TUNEL-positive cells (~55% decrease) (Fig. 2E). Thus, STF-083010 protected the liver from TAA-induced injury.

3.3. STF-083010 inhibits TAA-induced oxidative stress and hepatic inflammation

Oxidative stress and subsequent hepatic inflammation were also considered to play important roles in TAA-induced hepatotoxicity. Hepatic ROS production in different groups was measured by DHE staining. The results showed that TAA treatment significantly increased hepatic ROS production compared with that in the CON group (~3.3-fold). Importantly, TAA + STF-treated liver samples exhibited markedly reduced hepatic ROS levels compared with TAA treatment alone (~33% reduction) (Fig. 3A). We further detected the transcriptional levels of hepatic pro-inflammatory cytokines, including *TNF-α*, *IL-6* and *IL-1β*, by qRT-PCR. The results indicated that the markedly increased transcriptional levels of *TNF-α*, *IL-6* and *IL-1β* in TAA-treated liver samples were significantly decreased in the TAA + STF-treated liver samples (~61%, 57% and 69% reduction, respectively) (Fig. 3B–D). These results suggested that STF-083010 protected the liver from TAA-induced oxidative stress and inflammation.

3.4. STF-083010 triggers autophagy in response to TAA treatment

To validate the inhibitory effect of STF-083010 on IRE1α-sXBP1 signaling, we measured the protein levels of p-IRE1α and sXBP1 by Western blotting. Consistent with a previous study [29], our results indicated that STF-083010 specifically inhibited IRE1α RNase activity with no impact on IRE1α kinase activity in TAA-treated liver samples, as demonstrated by decreased sXBP1 levels (~72% decrease) accompanied by unchanged p-IRE1α levels (Fig. 4; TAA + STF vs TAA).

Increasing evidence supports that autophagy is an adaptive response to ER stress activated by various stimuli and plays critical roles in maintaining homeostasis [25]. Therefore, we analyzed whether activation of IRE1α-sXBP1 signaling affects hepatic autophagy in TAA-treated liver samples. Autophagy markers were measured by Western blotting. As shown in Fig. 4, neither TAA nor STF-083010 alone influenced hepatic autophagy, as demonstrated by unaltered protein levels of LC3B, p62 and Beclin-1. However, markedly increased LC3B levels (~2.7-fold) and decreased p62 levels (~52% decrease) were observed in the TAA + STF group, suggesting STF-083010 as an autophagy promoter upon TAA stimulation (Fig. 4; TAA + STF vs TAA). To further verify the role of STF-083010 in triggering autophagy, CQ was used to block autophagic flux in vivo. Western blot analysis indicated that LC3B levels were further increased (~1.4-fold) in the TAA + STF + CQ group compared with those in the TAA + STF group, but the decreased p62 levels in the TAA + STF group were significantly restored by CQ, indicating that STF-083010 facilitated autophagic flux in TAA-treated liver samples. The expression of Beclin-1, a critical autophagy marker, was also markedly elevated in the TAA + STF group, implicating that STF-083010 triggered autophagy probably due to Beclin-1 activation (Fig. 4).

3.5. Autophagy activation contributes to the protective effects of STF-083010 against TAA-induced oxidative stress, inflammation and liver injury

To better understand the role of autophagy in STF-083010-mediated protection against TAA-induced liver injury, we investigated the function of CQ in vivo. In the present study, CQ pretreatment alone did not cause liver injury (data not shown). Functionally, compared with the

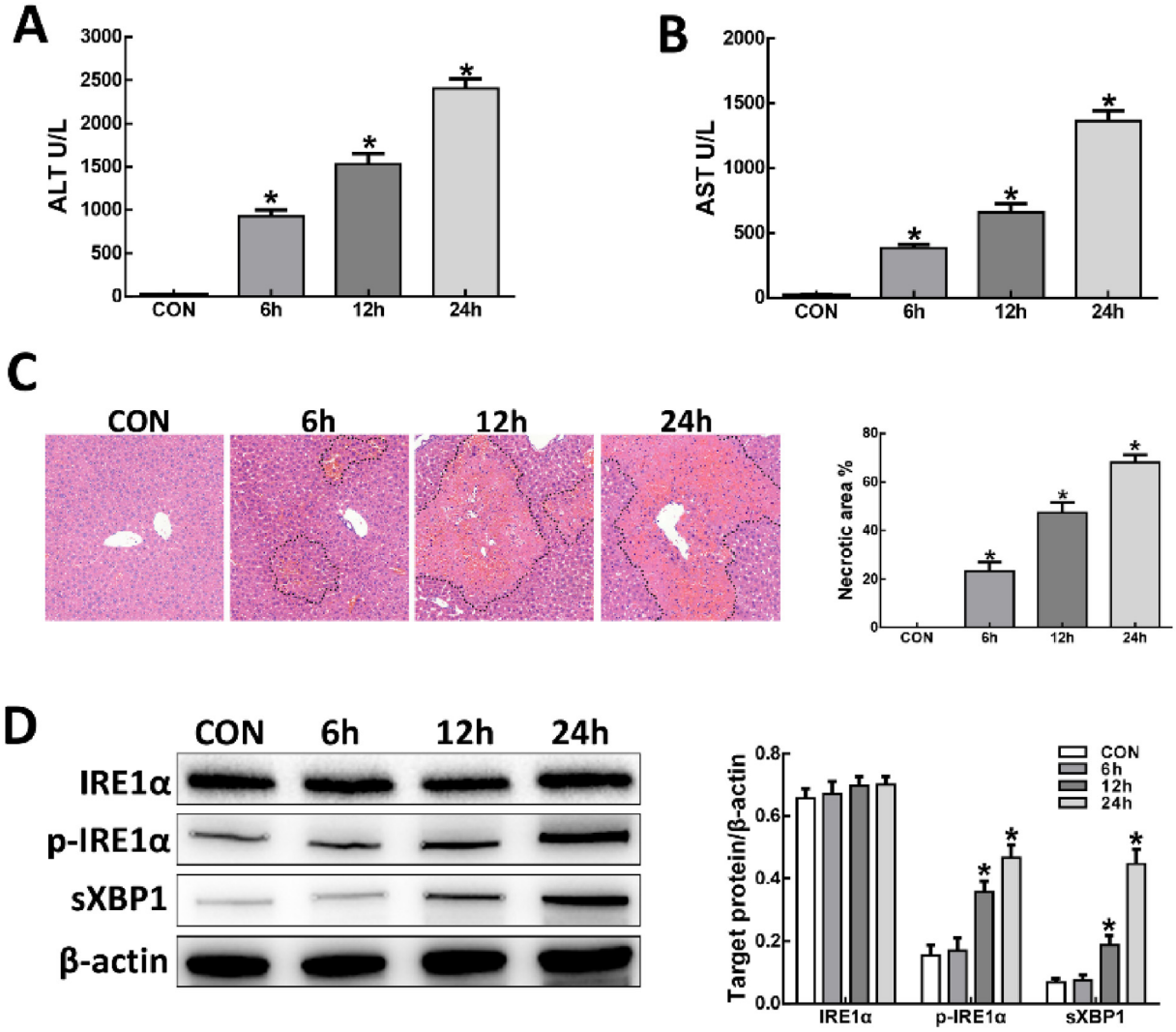

Fig. 1. IRE1α-sXBP1 signaling was activated in the livers of TAA-treated mice. The mice were subjected to a toxic dose of TAA administration (200 mg/kg, n = 6/group). The liver tissues and serum samples were collected at 6, 12 and 24 h after TAA treatment. (A, B) Serum ALT and AST levels. (C) Representative images of liver histopathology by H&E staining (magnification ×100); necrotic areas were exhibited. (D) Western blot analysis for the expression levels of IRE1α, p-IRE1α, sXBP1 and β-actin. The relative intensity was normalized to that of β-actin. The data are represented as the means ± SEM. $^{*}p < 0.05$ compared with the CON group.

TAA + STF group, CQ pretreatment significantly abrogated the protective effect of STF-083010 against TAA-induced liver injury, as evidenced by elevated serum ALT/AST levels (~2.2- and 2.4-fold, respectively), increased liver necrotic areas (~2-fold) and increased TUNEL-positive hepatocytes (~2.3-fold) in the TAA + STF + CQ group (Fig. 5A–D). Furthermore, DHE staining showed that the reduced ROS levels in the TAA + STF group were markedly restored by autophagy inhibition (Fig. 5E, TAA + STF vs TAA + STF + CQ). A similar effect of CQ pretreatment on hepatic inflammation was also observed. As shown in Fig. 5F–H, the decreased transcriptional levels of pro-inflammatory cytokines *TNF-α*, *IL-6* and *IL-1β* in the TAA + STF group were significantly reversed in the TAA + STF + CQ group. Thus, our results indicated that STF-083010 inhibited TAA-induced oxidative stress, inflammation and liver injury by facilitating autophagy.

3.6. STF-083010 ameliorates TAA-induced oxidative stress and hepatotoxicity by promoting autophagy in vitro

To verify our in vivo findings, we isolated primary hepatocytes from mice and treated them with TAA in the presence or absence of STF-083010 in vitro. We first assessed the impact of STF-083010 on the IC50 of TAA. As shown in Fig. 6A, STF-083010 obviously increased the IC50 value of TAA from 47.32 μM to 62.19 μM. Consistent with the in vivo results, TAA treatment significantly activated IRE1α-sXBP1 signaling (Fig. 6B, TAA vs CON). STF-083010 obviously attenuated the elevation of sXBP1 levels with no impact on the p-IRE1α levels in TAA-treated cells (Fig. 6B, TAA + STF vs TAA). Western blot analysis also showed that, combined with TAA treatment, STF-083010 obviously increased the expression of LC3B (~2.8-fold) and Beclin-1 (~2.0-fold) but decreased the expression of p62 (~70% decrease), indicating that STF-083010 promoted hepatocyte autophagy in response to TAA stimulation (Fig. 6B, TAA + STF vs TAA).

Importantly, TAA-induced oxidative stress and hepatotoxicity were markedly extenuated by STF-083010 as demonstrated by lower ROS production (~30% reduction), lower LDH release (~55% reduction) and higher cell viability measured (~1.2-fold) by ROS detection, the LDH release assay and the CCK-8 assay, respectively (Fig. 6C–E, TAA + STF vs TAA). To determine whether the protective role of STF-083010 was directly related to autophagy induction, Beclin-1-siRNA was used to inhibit autophagy in vitro using nonspecific-siRNA (NS-siRNA) as the control. As shown in Fig. 6B, Beclin-1 knockdown significantly inhibited autophagy in the TAA + STF + Beclin-1-siRNA group compared with that in the TAA + STF and TAA + STF + NS-siRNA groups, as evidenced by decreased protein levels of LC3B (~75%

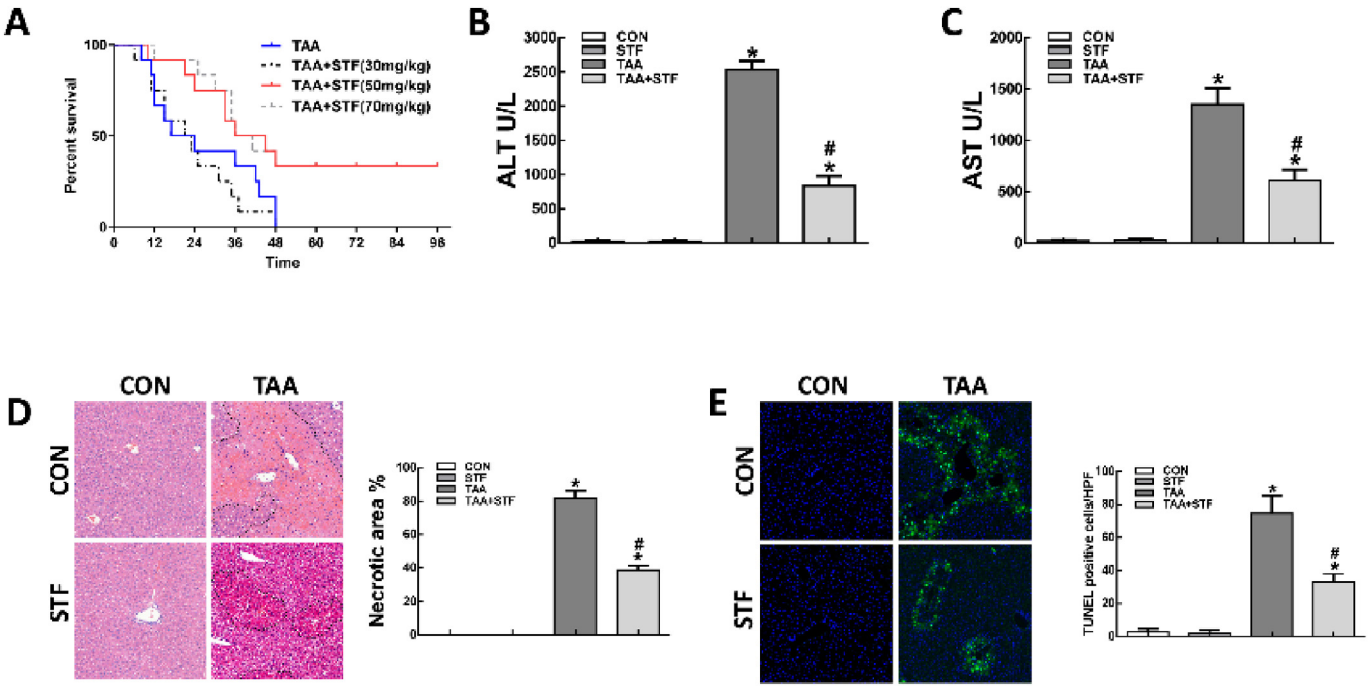

Fig. 2. STF-083010 protects mice from TAA-induced liver injury. For survival experiments, the mice were subjected to a lethal dose of TAA administration (500 mg/kg, n = 12/group). (A) Survival rate of TAA-treated mice with different concentrations of STF-083010 pretreatment at various time points. For injury experiments, the mice were subjected to a toxic dose of TAA administration (200 mg/kg, n = 6/group), and the liver tissues and serum samples were collected at 24 h after TAA treatment. (B, C) Serum ALT and AST levels. (D) Representative images of liver histopathology by H&E staining (magnification, ×100); the necrotic areas were exhibited. (E) Representative images of liver sections stained with TUNEL in green and DAPI in blue (magnification, ×100) and the quantification of TUNEL-positive cells. The data are represented as the means ± SEM. $^{*}p < 0.05$, compared with the CON group. $^{\#}p < 0.05$, compared with the TAA group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decrease) and increased protein levels of p62 (~2.6-fold). Functionally, as shown in Fig. 6C–E (TAA + STF + Beclin-1-siRNA vs TAA + STF or TAA + STF + NS-siRNA), more ROS production, higher LDH release and lower cell viability were observed in the TAA + STF + Beclin-1-siRNA group, suggesting that the protective role of STF-083010 was significantly abrogated by autophagy inhibition. Thus, these results demonstrated that the protective effects of STF-083010 against TAA-induced oxidative stress and hepatotoxicity resulted from facilitating hepatocyte autophagy via Beclin-1 activation.

4. Discussion

Acute liver injury caused by drugs or toxins is a common clinical syndrome that threatens patient lives with few effective therapies. Therefore, a clearer understanding of the molecular mechanisms underlying the pathogenesis of acute liver injury and the discovery of new pharmacological treatment are urgently needed. A complex crosstalk among ER stress, oxidative stress and autophagy has been broadly suggested to engage in the development of acute liver injury. STF-083010, a specific inhibitor of IRE1α RNase, has been reported to exert anti-oxidant and anti-inflammation capability in several injury models, including liver injury [15,16,30,31]. However, few studies have reported on the autophagy modulatory effect of STF-083010. The present study revealed that the activation of IRE1α-sXBP1 signaling is related to the severity of liver damage in TAA-treated mice. Our results also uncovered a protective role for STF-083010 in TAA-induced oxidative stress, inflammation and liver injury and demonstrated a potential mechanism by which STF-083010 facilitates hepatocyte autophagy in response to TAA stimulation.

IRE1α has been proven to determine cell fate under ER stress in response to various stimuli [32]. Activation of IRE1α-sXBP1 signaling has been well documented in the hepatic metabolic response such as glucose and lipid metabolism [10]. However, in terms of liver injury, the function of IRE1α-sXBP1 signaling has remained controversial. It was previously reported that IRE1α-sXBP1 signaling was activated in the liver of APAP-treated mice [33], and 4-PBA (an ER stress inhibitor) significantly prevented APAP-induced hepatotoxicity [34]. However, Hur KY et al. reported that XBP1-deficient mice that exhibit constitutive activation of IRE1α was resistant to APAP-induced liver injury, while they did not observe UPR activation in APAP-treated normal mice [12]. It was notable that APAP was administered by intraperitoneal injection in the above studies. Another study indicated that ER stress and UPR were activated by the oral administration of APAP as a late event [35]. Additionally, IRE1α was markedly activated in the CCl_4-treated liver samples during the early stage, and hepatic IRE1α deficiency exacerbated CCl_4-induced liver injury [13]. By contrast, it was recently reported that the inhibition of IRE1α-sXBP1 signaling by STF-083010 obviously attenuated CCl_4-induced liver injury by reversing hepatic miR-122 downregulation [16]. These results indicated that IRE1α-sXBP1 signaling may play distinct roles under different conditions such as the stress environment and different administration routes.

It should be noted that CYP2E1 converts APAP to *N*-acetyl-p-benzoquinone imine (NAPOI), resulting in glutathione depletion [36,37], while glutathione levels are elevated in TAA-treated mice, suggesting different mechanisms by which the two toxins exert hepatotoxicity [4]. Regarding TAA-induced hepatotoxicity, the elevated TAA metabolites and ROS produced by CYP enzymes has been reported to promote TAA-induced liver damage as well as lipogenesis by activating ATF6 and CHOP [4]. Consistent with the previous study that reported the enhanced activation of JNK mediated by IRE1α in TAA-treated mice [5], our results also demonstrated the involvement of IRE1α-sXBP1 signaling in TAA-induced liver injury correlating with the severity of liver damage.

Some controversy exists regarding the autophagy level in TAA-treated liver samples. Mardi MA et al. revealed that autophagy is activated during TAA-induced liver fibrosis in rats [38]. However,

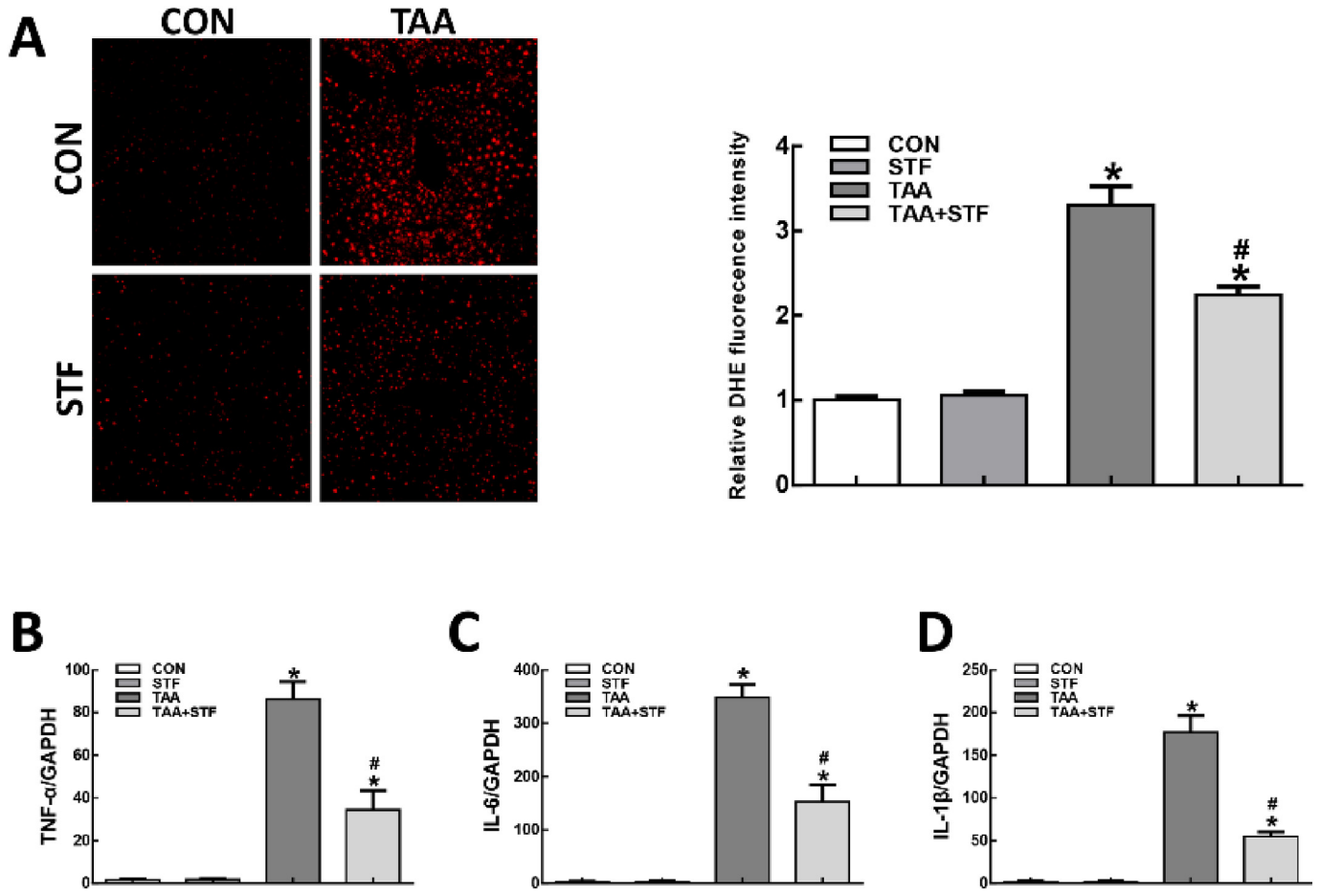

Fig. 3. STF-083010 alleviates TAA-induced oxidative stress and hepatic inflammation. (A) Representative images of liver sections stained with DHE (magnification ×100) and the relative fluorescence density. (B, C, D) The transcriptional levels of pro-inflammatory cytokines *TNF-α*, *IL-6* and *IL-1β* in the liver tissues. The data are represented as the means ± SEM. $^{*}p < 0.05$, compared with the CON group. $^{\#}p < 0.05$, compared with the TAA group.

consistent with our previous study [39], we observed no significant alteration of autophagy in C57BL/6J mice with TAA treatment alone. This discrepancy was probably attributed to the distinct time course of TAA treatment, as well as the different animals used in experiments. Intriguingly, although TAA or STF-083010 treatment alone had no obvious effect on hepatocyte autophagy, we found that STF-083010 triggered autophagy in the presence of TAA treatment. CQ is a lysosomal inhibitor, and our in vivo data (Fig. 4) showed that CQ obviously reduced the degradation of p62 but had no effect on the LC3B level, indicating that CQ blocked STF-083010-triggered autophagy flux.

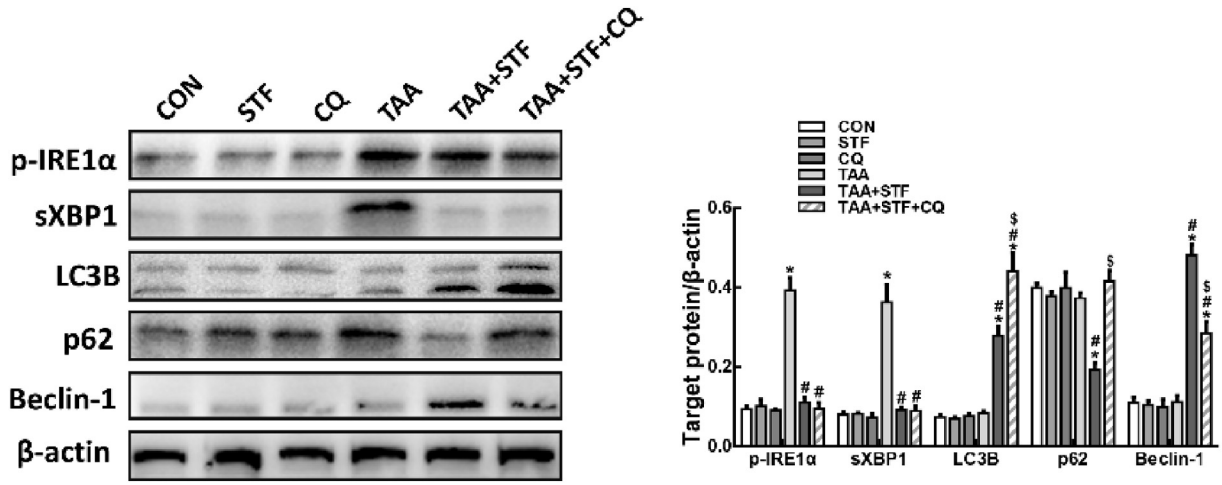

Fig. 4. STF-083010 triggers autophagy in TAA-treated liver samples. The mice were subjected to a toxic dose of TAA administration (200 mg/kg, n = 6/group) in the presence or absence of STF-083010 and/or CQ pretreatment. The liver tissues and serum samples were collected at 24 h after TAA treatment. Western blot analysis for the expression levels of p-IRE1α, sXBP1, LC3B, p62, Beclin-1 and β-actin. The relative intensity is normalized to β-actin. The data are represented as the means ± SEM. $^{*}p < 0.05$, compared with the CON group; $^{\#}p < 0.05$, compared with the TAA group; $^{\$}p < 0.05$, compared with the TAA + STF group.

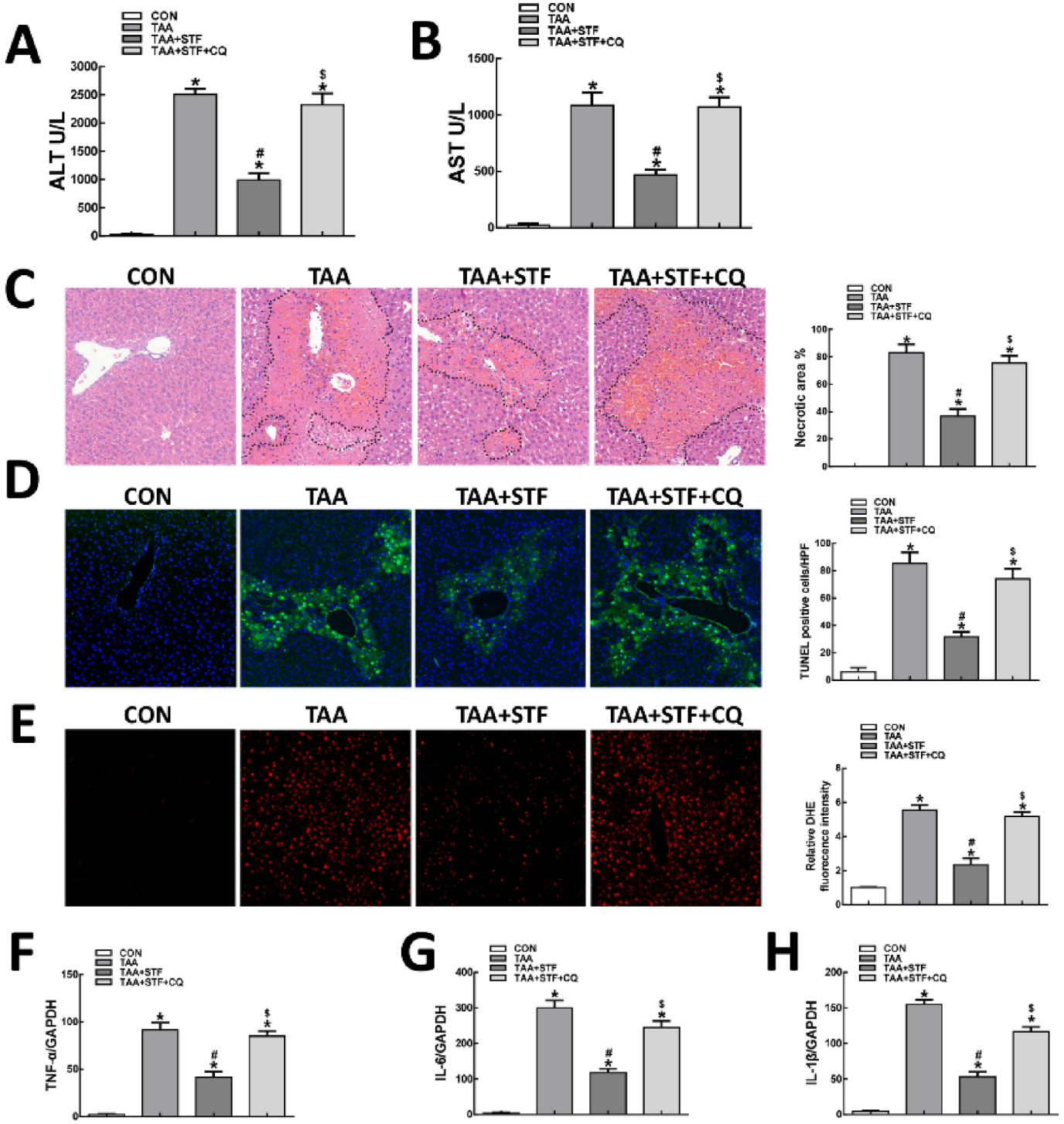

Fig. 5. STF-083010 alleviates TAA-induced oxidative stress, inflammation and liver injury by triggering autophagy in vivo. The mice were subjected to a toxic dose of TAA administration (200 mg/kg, n = 6/group) in the presence or absence of STF-083010 and/or CQ pretreatment. The liver tissues and serum samples were collected at 24 h after TAA treatment. (A, B) Serum ALT and AST levels. (C) Representative images of liver histopathology by H&E staining (magnification, ×100); necrotic areas were exhibited. (D) Representative images of liver sections stained with TUNEL in green and DAPI in blue (magnification, ×100) and the quantification of TUNEL-positive cells. (E) Representative images of liver sections stained with DHE (magnification ×100) and the relative fluorescence density. (F, G, H) The transcriptional levels of pro-inflammatory cytokines *TNF-α*, *IL-6* and *IL-1β* in the liver tissues. The data are represented as the means ± SEM. $^{*}p < 0.05$, compared with the CON group; $^{\#}p < 0.05$, compared with the TAA group; $^{\$}p < 0.05$, compared with the TAA + STF group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Beclin-1 plays a critical role in the initiation of autophagy, and our in vitro data (Fig. 6B) showed that Beclin-1 knockdown significantly inhibited LC3B activation, leading to p62 accumulation. Our results also suggested that the crosstalk among ER stress, oxidative stress and autophagy constitutes a complex regulatory pathway in the pathophysiological process of TAA-induced liver injury.

In this study, while we mainly focused on the role of STF-083010 in liver parenchymal cells, we also found that STF-083010 exerted a

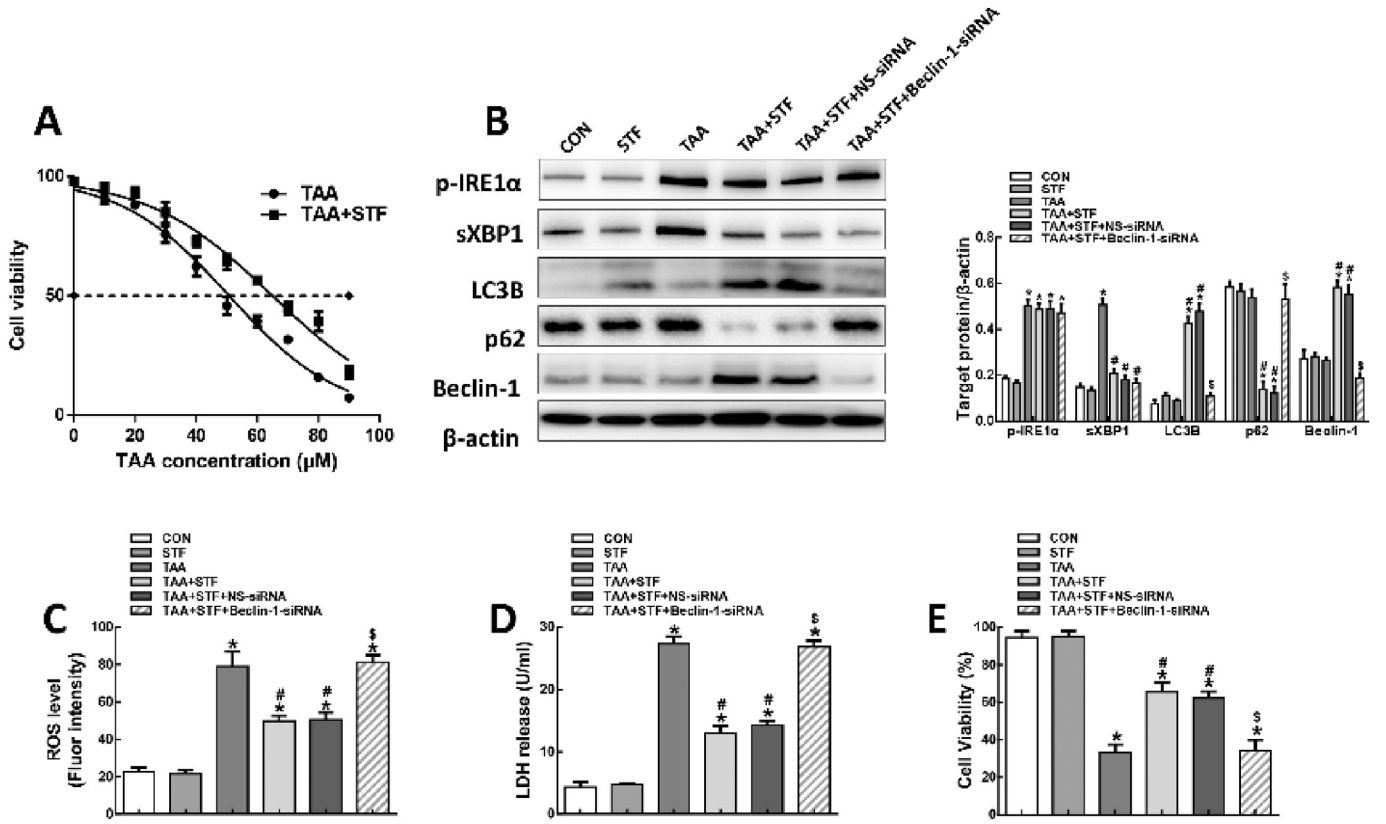

Fig. 6. STF-0830101 protects hepatocytes from TAA-induced oxidative stress and cell injury by facilitating autophagy in vitro. (A) Cell viability was measured by the CCK-8 assay, and IC50 was evaluated. (B) Western blot analysis for the expression levels of p-IRE1α, sXBP1, LC3B, p62, Beclin-1 and β-actin. The relative intensity was normalized to that of β-actin. (C) Hepatocellular ROS levels were measured by the ROS detection assay. (D) LDH produced by hepatocytes were measured by the LDH release assay. (E) Hepatocyte viability was measured by the CCK-8 assay. The data were represented as the means ± SEM. $^{*}p < 0.05$, compared with the CON group; $^{\#}p < 0.05$, compared with the TAA group; $^{\$}p < 0.05$, compared with the TAA + STF group.

beneficial effect on alleviating TAA-induced hepatic inflammation. Given that the inflammatory response also plays critical roles in various liver injuries, further studies need to assess the effect of IRE1α-sXBP1 signaling on liver nonparenchymal cells, such as Kupffer cells and neutrophils. Additionally, our results suggested the involvement of Beclin-1 in STF-083010-mediated autophagy both in vivo and in vitro. However, whether Beclin-1 activation is directly related to the inactivation of IRE1α-sXBP1 signaling mediated by STF-083010 remains unclear and may be our future study interests.

In conclusion, the present study revealed that IRE1α-sXBP1 signaling is involved in the development of TAA-induced acute liver injury. STF-083010 triggers hepatocyte autophagy via Beclin-1 activation, leading to the alleviation of TAA-induced oxidative stress, inflammation and liver injury. Thus, our study provides a promising therapeutic strategy to treat TAA-induced acute liver injury.

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Conflicts of interest

All authors declare no conflict of interest.

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