Acta Biochim Biophys Sin 2022, 54(11): 1619–1629
https://doi.org/10.3724/abbs.2022167
Advance Access Publication Date: 10 November 2022
Original Article

Original Article

LncRNA-Airn alleviates acute liver injury by inhibiting hepatocyte apoptosis via the NF-κB signaling pathway

Shuai Shao[1,†], Yu Zhang[2,†], Feng Zhou[2,†], Xiaoxiang Meng[3,†], Zhenjun Yu[2], Guantong Li[2], Lina Zheng[3], Kun Zhang[3], Yuhan Li[2], Beichen Guo[2], Qi Liu[3], Mengxia Zhang[3], Xiaoxiao Du[3], Wei Hong[3,*], and Tao Han[1,2,4,5,*]

[1]The School of Medicine, Nankai University, Tianjin 300071, China, [2]Department of Hepatology and Gastroenterology, the Third Central Clinical College of Tianjin Medical University; Department of Histology and Embryology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin 300121, China, [3]Department of Histology and Embryology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin 300121, China, [4]Department of Gastroenterology and Hepatology, Tianjin Union Medical Center Affiliated to Nankai University, Tianjin 300122, China, and [5]Department of Hepatology and Gastroenterology, Tianjin Third Central Hospital Affiliated to Nankai University, Tianjin 300170, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-22-27557228; E-mail: hantaomd@126.com (T.H.) / Tel: +86-22-83336819; E-mail: hongwei@tmu.edu.cn (W.H.)

Received 20 February 2022 Accepted 19 April 2022

Abstract

Acute liver injury is a common and serious syndrome caused by multiple factors and unclear pathogenesis. If the injury persists, liver injury can lead to cirrhosis and liver failure and ultimately results in the development of liver cancer. Emerging evidence has indicated that long noncoding RNAs (lncRNAs) play an important role in the development of liver injury. However, the role of antisense Igf2r RNA (Airn) in acute liver injury and its underlying mechanism remain largely unclear. In this study, we show that Airn is upregulated in liver tissue and primary hepatocytes from an acute liver injury mouse model. Consistently, Airn is also overexpressed in serum samples of patients with acute-on-chronic liver failure and is negatively correlated with the Model for End-Stage Liver Disease (MELD) score. Moreover, gene knockout and rescue assays reveal that Airn alleviates CCl_4-induced liver injury by inhibiting hepatocyte apoptosis and oxidative stress *in vivo*. Further investigation reveals that Airn decreases H_2O_2-induced hepatocyte apoptosis *in vitro*. Mechanistically, we reveal that Airn represses CCl_4- and H_2O_2-induced enhancement of phosphorylation of p65 and IκBα, suggesting that Airn inhibits hepatocyte apoptosis by inactivating the NF-κB pathway. In conclusion, our results demonstrate that Airn can alleviate acute liver injury by inhibiting hepatocyte apoptosis via inactivating the NF-κB signaling pathway, and Airn could be a potential biomarker for acute liver injury.

Key words acute liver injury, lncRNA, apoptosis, oxidative stress, NF-κB signaling

Introduction

Acute liver injury is a common pathological basis for the development and progression of many liver diseases [1]. If the injury persists, when the extent of hepatocyte death exceeds the regenerative capacity of the liver, liver injury can progress to cirrhosis and liver failure, ultimately resulting in the development of liver cancer [2,3]. It has been found that aggravated hepatocyte apoptosis in acute liver injury is induced by sepsis, and that the inhibition of apoptosis could efficiently alleviate acute liver injury [4]. Another study revealed that shikonin attenuates acetaminophen (APAP)-induced acute liver injury via inhibiting oxidative stress [5]. Consequently, these studies indicated that inhibiting oxidative stress and hepatocyte apoptosis is important to alleviate acute liver injury.

Long noncoding RNAs (lncRNAs) are a class of non-protein-coding RNA transcripts that are longer than 200 nt and are involved in numerous physiological and pathological processes, including epigenetic regulation, transcription, and posttranscriptional regulation. lncRNAs have been found to play a key role in liver diseases [6,7]. For example, downregulation of lncRNA LINC00472 amelio-

rates sepsis-induced acute hepatic injury by regulating the miR-373-3p/TRIM8 axis [8]. LncRNA XIST silencing protects against sepsis-induced acute liver injury via inhibition of BRD4 expression [9]. LncRNA-Airn, an antisense RNA of IGF2R, is an imprinted and paternally expressed gene [10]. Recent studies have found that Airn plays an important role in many diseases. Knockdown of LncRNA-Airn restrained hepatocellular carcinoma (HCC) cell proliferation and boosted cell apoptosis by restraining the CUL4A-mediated ubiquitination of STAT1 protein [11]. It has been reported that Airn locates upstream of Igf2 bp2 to control the translation of many mRNAs, including genes involved in apoptosis and directly related to cell survival in cardiomyocytes [12]. Nevertheless, the underlying role of Airn in acute liver injury has not been investigated.

In the present study, we found that Airn was upregulated in CCl_4-induced acute liver injury and serum samples of patients with acute-on-chronic liver failure (ACLF). Further experiments revealed that Airn could alleviate CCl_4-induced ALI by mitigating liver apoptosis and oxidative stress. Mechanistically, Airn represses CCl_4- or H_2O_2-induced upregulation of phos-p65 and phos-IκBα, indicating that Airn alleviates hepatocyte apoptosis via inactivating the NF-κB signaling pathway. Our results indicated that Airn might play a protective role and could be a potential biomarker for acute liver injury.

Materials and Methods

Study population

In total, we collected serum samples from 27 healthy people and 31 patients diagnosed with acute-on-chronic liver failure at Tianjin Third Central Hospital (Tianjin, China). All subjects were of the same ethnicity. Clinical and pathological characteristics, including age, sex, alanine transaminase (ALT) and aspartate aminotransferase (AST), were recorded and summarized in Supplementary Table S1. The study was approved by the local Ethical Committee of Tianjin Third Central Hospital. Written informed consent was obtained from each patient according to the policies of the committee. The study methodologies conformed to the standards set by the Declaration of Helsinki.

In vivo animal study

All animal experiments were approved by the Animal Experiments Ethical Committee of Nankai University (Tianjin, China). *Airn*-knockout C57BL/6N mice generated by using the CRISPR/Cas9 system were obtained from Cyagen (Suzhou, China). All Balb/c male mice aged at eight weeks with a body weight of approximately 20 g were obtained from the Institute of Laboratory Animal Sciences, CAMS and PUMC (Beijing, China). All mice were housed in a specific-pathogen-free environment with 12/12-h dark/light cycles and had free access to chow and water. After one week of acclimatization, the acute liver injury mouse model was established. Forty-eight male mice were randomly divided into eight groups. For the control group, mice were injected intraperitoneally (i.p.) with olive oil. For the CCl_4 groups, the mice received one injection of a 20% solution of CCl_4 (Sigma-Aldrich, St Louis, USA) in olive oil (1 mL/kg body weight). After injection, mice were sacrificed at 6 h, 12 h, 24 h, 48 h, 72 h, 96 h and 8 days under anesthesia with 3% sodium pentobarbital (45 mg/kg, i.p.). Liver tissues and serum were collected for analysis. For Airn functional experiments, 20 wild-type (WT) mice and 20 Airn-knockout (Airn-KO) mice were randomly divided into four groups: (1) WT mice injected i.p. with olive oil (WT, $n = 10$), (2) WT mice injected i.p. with CCl_4 (WT + CCl_4, $n = 10$), (3) Airn-KO mice injected i.p. with olive oil (Airn-KO, $n = 10$), and (4) Airn-KO mice injected i.p. with CCl_4 (Airn-KO + CCl_4, $n = 10$). They were administered with 20% CCl_4 (v/v) dissolved in olive oil (1 mL/kg body weight). Twenty-four hours later, all mice were sacrificed under anesthesia with 3% sodium pentobarbital (45 mg/kg, i.p.). Liver tissues and serum were collected for analysis.

For the rescue experiment, adeno-associated virus (AAV8) vectors were used to overexpress Airn in mice, and AAV8-GFP was used as a control. AAV8-GFP and AAV8-Airn were produced by AAV-293 cells and purified by using iodixanol density gradient ultra-centrifugations. Forty male Airn-KO mice were divided into four groups: (1) Airn-KO mice treated with olive oil in combination with injection of AAV8-GFP (Airn-KO + AAV8-GFP, $n = 10$), (2) Airn-KO mice treated with CCl_4 in combination with injection of AAV8-GFP (Airn-KO + AAV8-GFP + CCl_4, $n = 10$), (3) Airn-KO mice treated with olive oil in combination with injection of AAV8-Airn (Airn-KO + AAV8-Airn, $n = 10$), and (4) Airn-KO mice treated with CCl_4 in combination with injection of AAV8-Airn (Airn-KO + AAV8-Airn + CCl_4, $n = 10$). Mice were injected with AAV8-GFP or AAV8-Airn (1×10^{12} pfu/mouse) via the tail vein 2 weeks before oil or CCl_4 injection. CCl_4 was diluted to 20% (v/v) with olive oil before use, and used at 1 mL/kg body weight. After 24 h, all mice were sacrificed under anesthesia with 3% sodium pentobarbital (45 mg/kg, i.p.). Liver specimens and serum were obtained for subsequent analysis.

Isolation and culture of primary hepatocytes

Primary hepatocytes were isolated from the 8-week-old male mice by sequential *in sit*u perfusion with 30 mL of SC1 solution and then with 30 mL of 0.05% collagenase IV (Sigma-Aldrich) solution. Cells were collected after centrifugation at 50 *g* for 4 min three times. Cell viability was determined by the trypan blue exclusion method. Mouse primary hepatocytes were plated onto collagen-coated six-well plates at a density of 2×10^5 cells/well. Primary HCs were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, USA) and 1% penicillin/streptomycin (Gibco) and maintained in a humidified incubator with 5% CO_2 at 37°C.

Hematoxylin and eosin (H&E) staining

The liver specimens were fixed in 10% formalin for 2 days, dehydrated with a graded series of ethanol, and embedded in paraffin. For H&E staining, sections (5 μm) were stained with hematoxylin for 5 min at room temperature, and after extensive wash slices were stained with eosin for 1 min at room temperature. After that, the sections were dehydrated in gradient ethanol, transparently treated with xylene, and sealed with neutral gum. Images from randomly selected areas were captured at 100× magnification under a light microscope.

Measurement of serum biochemical markers

Blood samples were collected from CCl_4-treated mice, and serum levels of AST, ALT, and lactate dehydrogenase (LDH) were measured using commercially available diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The final data are presented as units/liter (U/L).

Determination of SOD, CAT and GSH activities

Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities in serum samples or mouse livers were analyzed using a SOD assay kit, a CAT assay kit and a GSH assay kit (Jiancheng Bioengineering Institute), respectively. All experiments were carried out according to the manufacturer's instructions.

TUNEL assay

For the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining assay, an *in situ* cell detection kit (Roche, Basel, Switzerland) was used according to the manufacturer's protocol. After dewaxing and rehydration, sections were treated with 3% H_2O_2 and subsequently permeabilized with proteinase K. DNase I was used as a positive control, and a TUNEL reaction mixture lacking terminal transferase (TdT) was used as a negative control. Samples were randomly selected and analyzed by light microscopy.

Small interfering RNA (siRNA) transfection

Mouse primary hepatocytes were plated onto collagen-coated six-well plates at a density of 2×10^5 cells/well. Then, the cells were transfected for 48 h with siRNA-Airn or siRNA-control. After that, total RNA and protein were extracted from the cells. Small interfering RNA (siRNA)-targeting Airn (siAirn) and negative control siRNA (NC) were designed and synthesized by GenePharma (Shanghai, China), and the sequences are as follows: siAirn (mouse),sense 5′-CCGUCACCAUGUGUCCUUUTT-3′ and antisense 5′-AAAGGACACAUGGUGACGGTT-3′; and NC (mouse), sense 5′-UUCUCCGAACGUGUCACGUTT-3′, and antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

Western blot analysis

Liver tissues or primary hepatocytes were lysed with cell lysis buffer (Cell Signaling Technology, Beverly, USA) supplemented with protease inhibitor cocktail, 1% phenylmethanesulfonyl fluoride (PMSF) and 1% phosphatase inhibitor. A BCA protein quantitative kit (Bio-Rad Laboratories, Hercules, USA) was used to detect the protein concentration. The resulting protein was boiled with SDS-PAGE sample buffer, separated by 12% SDS-PAGE, and transferred to a PVDF membrane (Millipore, Billerica, USA). The PVDF membrane was blocked with 5% skimmed milk in TBST for 1 h at room temperature and then incubated with primary antibodies at 4°C overnight. The primary antibodies used in this study are as follows: Caspase-3 (9664S, 1:1000; Cell Signaling Technology), BCL2 associated X (BAX) (ab32503, 1:1000; Abcam, Cambridge, UK), Bcl-2 (26593-1-AP, 1:1000; Proteintech, Chicago, USA), poly ADP-ribose polymerase 1 (PARP1) (13371-1-AP, 1:1000; Proteintech), p65 (mouse monoclonal, sc-8008, 1:200; Santa Cruz, Santa Cruz, USA), phospho-p65 (rabbit monoclonal, 3033S, 1:1000; Cell Signaling Technology), IκBα (mouse monoclonal, 4814S, 1:1000; Cell Signaling Technology), phospho-IκBα (mouse monoclonal, 2859S, 1:1000; Cell Signaling Technology) and GAPDH (mouse monoclonal, ab8245, 1:8000; Abcam). Then, all membranes were incubated with the HRP-conjugated secondary antibody for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence (ECL) reagent (WBKLS0500; Millipore) and quantified. using ECL solutions A and B were mixed (ratio 1:1), and incubated with the PVDF membrane in the dark room for 3–5 min prior to exposure.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR analysis was performed as described previously [13]. In brief, total RNA was extracted from liver tissues or cells with Trizol reagent (Takara, Dalian, China), and all RNA was digested with DNase I (Takara). Next, the first-strand cDNA was synthesized using AMV Reverse Transcriptase (Thermo Fisher Scientific, Basingstoke, UK) according to the manufacturer's instructions. The sequences of the primers are shown in Table 1.

Hoechst/PI double-staining

Cell apoptosis was analyzed using a Hoechst 33342-propidium iodide (PI) double-staining apoptosis-detection kit (SuoLaibao Technology, Beijing, China) according to the manufacturer's instructions. Briefly, primary hepatocytes were plated in 6-well plates. Treated cells were washed with ice-cold PBS, and then 1 mL reaction buffer was added to each well. Then, 5 μL Hoechst 33342 solution and 5 μL PI solution were added and incubated for 30 min at 4°C. Next, the wells were washed with 1 × PBS to remove excess stain. The cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Dual-luciferase reporter assay

Primary hepatocytes were seeded in 6-well plates at 5×10^5 cells/well and cotransfected with 500 ng pNF-κB-Luc (Hunan Fenghui Biotechnology, Changsha, China) and 50 ng pRL-TK (Hunan Fenghui Biotechnology) using Lipofectamine 3000 (Invitrogen, Carlsbad, USA) for 24 h. Lentivirus-Airn was transfected for another 48 h, followed by treatment with 3% H_2O_2 for 3 h. Luciferase assays were performed using the Dual-luciferase reporter assay system (Promega, Madison, USA) following the manufacturer's instructions. The relative firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, USA). Comparisons were performed using either Student's *t* test (between two groups) or one-way analysis of variance (among more than two groups), followed by post hoc comparison test.

Table 1. Sequences of primers used in this study

Gene	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
GAPDH (mouse)	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGCTTAGC
Airn (mouse)	GAAAGCACAGCACCGCCAGT	CCATGTCCTTTCTTTTCCACTACC
TNF-α (mouse)	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Mcp-1 (mouse)	GTTAACGCCCCACTCACCTG	GGGCCGGGGTATGTAACTCA
IL-6 (mouse)	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
GAPDH (human)	ACCCAGAAGACTGTGGATGG	TTCAGCTCAGGGATGACCTT
Airn (human)	GGAAAAGGGATGCGGTGTTT	CCTTTTCAGGACGTGACCCG

$P < 0.05$ was considered significantly different.

Results

Airn is upregulated in acutely injured livers

We have previously identified systemic variations of lncRNAs from fibrotic and normal mouse livers with microarray [13]. In that study, we found that Airn was significantly upregulated in fibrotic liver according to the microarray data. Since liver fibrosis is a consequence of wound healing caused by acute or chronic liver injury, we initially detected the level of Airn in a mouse model of acute liver injury. The constructed acute liver injury mouse models were confirmed and showed that the most severe injury was at 24 h after injection of CCl_4 (Figure 1A–E). qPCR analysis showed that *Airn* was dramatically increased in CCl_4-induced acute injured liver tissues (Figure 1F). The primary hepatocytes were subsequently isolated from the injured liver to detect the expression of *Airn*,

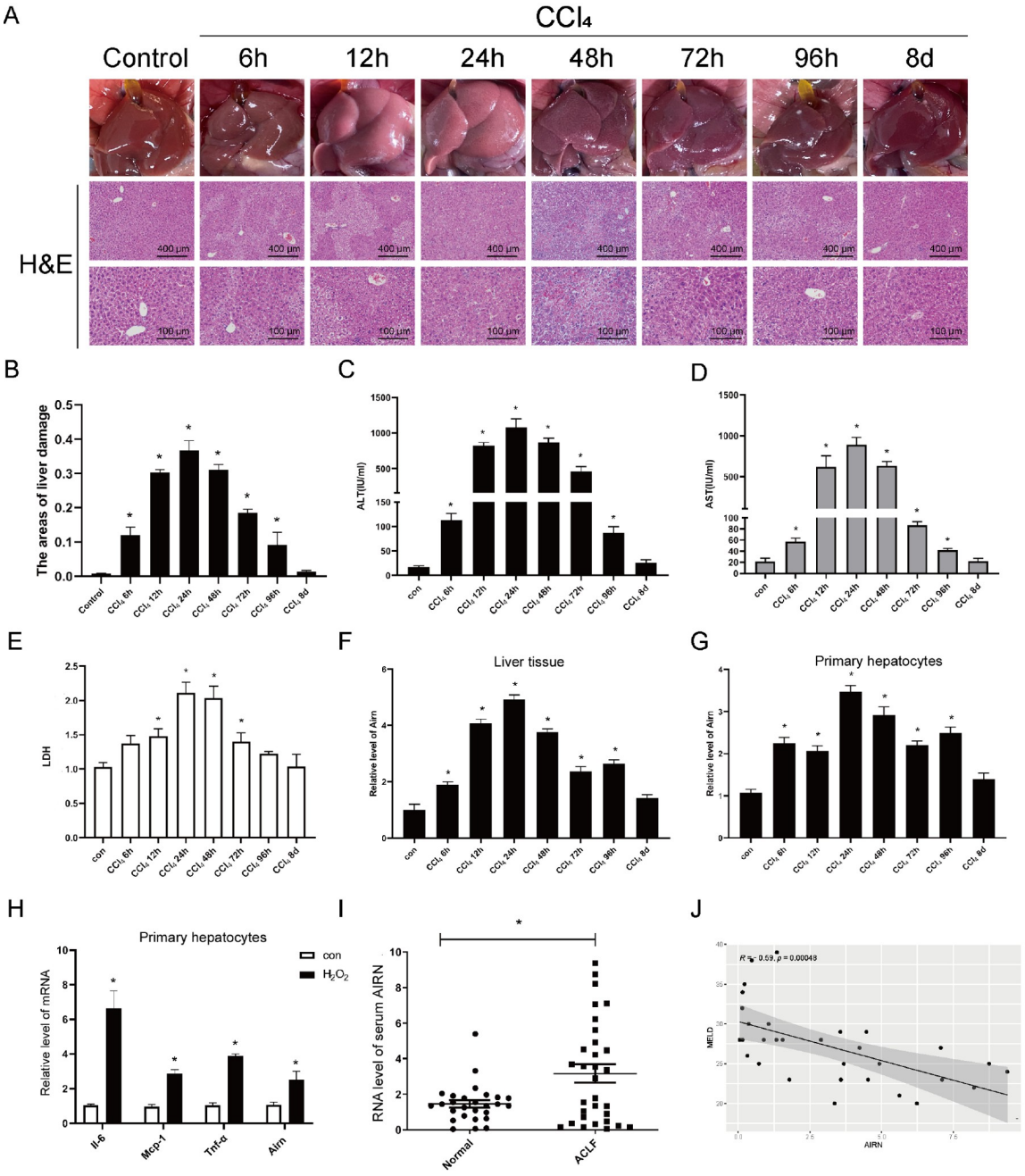

Figure 1. CCl_4-induced acute liver injury mouse model and the expression of Airn Mice were injected with CCl_4 (olive oil as a control) to induce acute liver injury and were sacrificed at different time points. Macroscopic appearance and H&E staining of liver tissues from CCl_4-treated mice (A). The areas of liver damage for H&E staining (B), serum ALT (C), AST (D), and LDH (E) were detected. qPCR analysis of the *Airn* transcript in injured liver tissues (F) and primary HCs (G). Primary HCs were isolated from the livers of Balb/c mice and treated with 3% H_2O_2 for 3 h. Then, the expressions of *Airn*, *IL-6*, *Mcp-1*, and *TNF-α* were detected by qPCR (H). qPCR analysis of the *Airn* transcript in serum samples of healthy people and patients with ACLF (I). The correlation between the Airn level and the MELD score of ACLF patients was assessed using Pearson's correlation analysis, $n = 58$ (J). Data are expressed as the mean ± SD from at least three experiments, $n = 6$. *$P < 0.05$ vs control.

which showed an enhanced level of this molecule (Figure 1G). To confirm this finding *in vitro*, primary hepatocytes were treated with H_2O_2 to generate an acute hepatocytic injury model, which was used for the detection of Airn, and the qPCR results indicated that *Airn* was overexpressed (Figure 1H). As the human homolog of the mouse Airn has been identified [14], we then detected the serum level of Airn in patients with acute-on-chronic liver failure, and the result demonstrated that Airn was increased in these patients compared with that in healthy people (Figure 1I). Moreover, the Airn level was negatively correlated with the MELD score of ACLF patients (Figure 1J). Taken together, these data revealed that Airn was upregulated in acutely injured liver and hepatocytes and could be a potential marker for acute liver injury.

Deletion of Airn aggravates CCl_4-induced acute liver injury

To investigate the function of Airn during acute liver injury *in vivo*, Airn-KO mice were generated using the CRISPR/Cas9 system. As shown in Figure 1A–E, liver injury reached the greatest severity after administration of CCl_4 for 24 h, accompanied by a peak level of Airn. We therefore selected this time point to investigate the biological function of Airn. The expression of Airn was enhanced in CCl_4-treated WT mice but not in Airn-KO mice (Figure 2A). The macroscopic examination and H&E staining showed that the CCl_4-treated WT group developed severe liver damage, while knockout of *Airn* notably aggravated CCl_4-induced liver injury (Figure 2B). In addition, serum levels of ALT, AST and LDH were significantly increased after CCl_4 injection, however, knockout of *Airn* led to further increase of these enzymes in the CCl_4-treated group (Figure 2C–E). Taken together, these results indicated that Airn deficiency aggravates CCl_4-induced acute liver injury.

Airn alleviates CCl_4-induced acute liver injury and oxidative stress

To investigate whether Airn could alleviate CCl_4-induced acute liver injury *in vivo*, a rescue experiment was performed. The qPCR results confirmed that *Airn* was overexpressed (Figure 3A), and macroscopic examination and H&E staining showed that overexpression of *Airn* attenuated CCl_4-induced acute liver injury (Figure 3B). In addition, overexpression of Airn reduced CCl_4-induced ALT, AST and LDH levels (Figure 3C–E), suggesting that Airn alleviates CCl_4-induced acute liver injury.

It has been reported that CCl_4-induced liver injury is always accompanied by serious oxidative stress [15]. To explore whether Airn alleviates hepatic injury induced by CCl_4 through inhibition of hepatic oxidative stress, we next measured the levels of oxidative stress-associated indexes. As shown in Figure 4A–C, the levels of SOD, GSH and CAT were decreased after CCl_4 treatment, and knockout of *Airn* aggravated CCl_4-induced oxidative stress. However, forced expression of Airn increased CCl_4-reduced levels of SOD, GSH and CAT (Figure 4D–F). Taken together, these results suggested that Airn alleviates CCl_4-induced oxidative stress.

Airn inhibits hepatocyte apoptosis

CCl_4-induced liver injury is accompanied by increased hepatocyte apoptosis [16]. Thus, we detected whether Airn is involved in this process. Western blot analysis showed that the expressions of the proapoptotic proteins BAX, cleaved Caspase-3 and PARP1 were upregulated after CCl_4 treatment, while the antiapoptotic protein Bcl-2 was downregulated. Further study revealed that CCl_4-induced hepatocyte apoptosis was exacerbated after knockout of *Airn* but decreased upon Airn overexpression (Figure 5A,B). To further elucidate the role of Airn in hepatocyte apoptosis, a TUNEL apoptosis detection assay was performed. The results showed that CCl_4 exposure led to greatly increased numbers of TUNEL-positive hepatocytes, while the numbers were further increased when *Airn* was knocked out and decreased after overexpression of Airn (Figure 5C,D). In an *in vitro* study, we used siRNA to silence the expression of Airn and lentivirus to overexpress Airn in primary hepatocytes

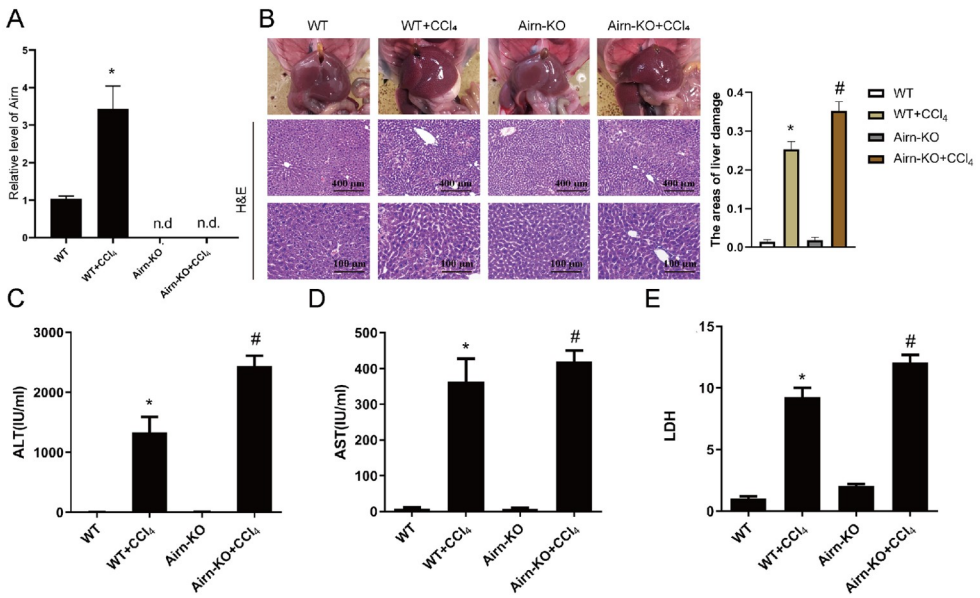

Figure 2. Deletion of Airn aggravates CCl_4-induced acute liver injury C57BL/6N mice were divided into four groups. (A) qPCR analysis of the *Airn* transcript in liver tissues of the acute liver injury mouse model. (B) Macroscopic appearance and H&E staining of liver tissues from control and CCl_4-treated mice. (C–E) Serum ALT, AST and LDH levels were detected. Data are expressed as the mean ± SD from at least three experiments, $n = 6$. P values were analyzed by Student's t test. $^*P < 0.05$, WT + CCl_4 vs WT; $^\#P < 0.05$, Airn-KO + CCl_4 vs WT + CCl_4. n.d., not detected.

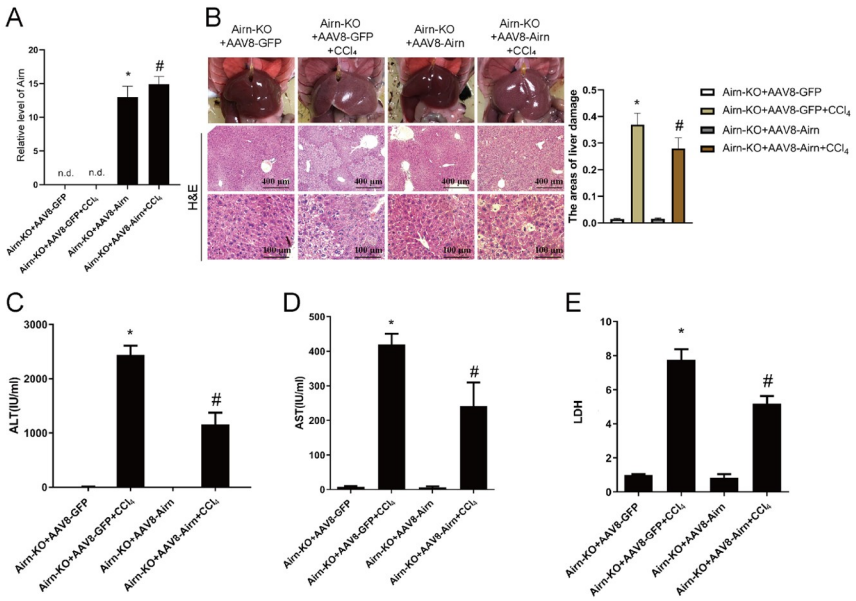

Figure 3. Rescue experiments confirming the role of Airn in CCl_4-induced acute liver injury Airn-KO mice were divided into four groups. (A) The expression of *Airn* in liver tissues was detected by qPCR. (B) Macroscopic examination and H&E staining of liver tissues from control and CCl_4-treated mice. (C–E) The serum levels of ALT, AST and LDH were determined. Data are expressed as the mean ± SD from at least three experiments, $n = 6$. *P* values were analyzed by Student's *t* test. $^*P < 0.05$, Airn-KO + AAV8-GFP + CCl_4 vs Airn-KO + AAV8-GFP; $^{\#}P < 0.05$, Airn-KO + AAV8-Airn + CCl_4 vs Airn-KO + AAV8-GFP + CCl_4.

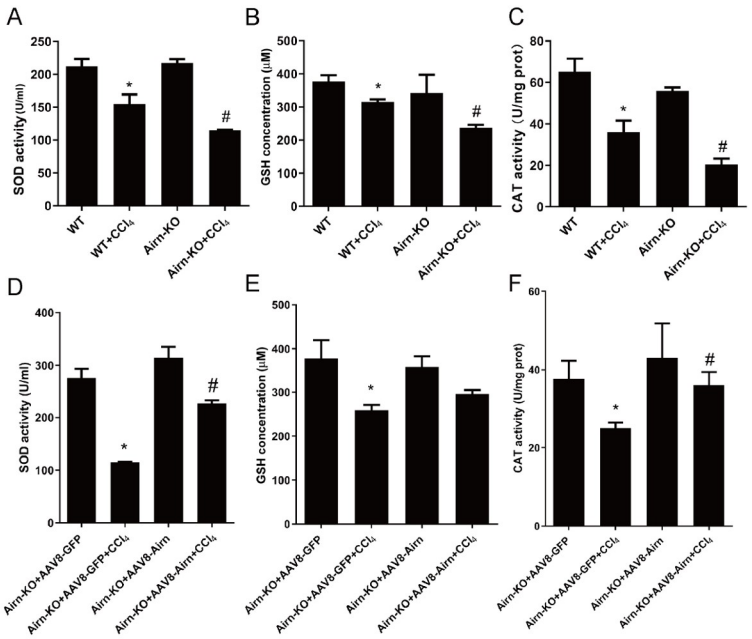

Figure 4. Airn alleviates CCl_4-induced oxidative stress (A–C) Mice were divided into four groups: WT, WT + CCl_4, Airn-KO and Airn + KO + CCl_4. The activities of SOD, CAT and GSH were detected. (D–F) Airn-KO mice were divided into four groups: Airn-KO + AAV8-GFP, Airn-KO + AAV8-GFP + CCl_4, Airn-KO + AAV8-Airn, and Airn-KO + AAV8-Airn + CCl_4. The activities of SOD, CAT and GSH were detected. Data are expressed as the mean ± SD from at least three experiments, $n = 6$. *P* values were analyzed by Student's *t* test. $^*P < 0.05$, WT + CCl_4 vs WT or Airn-KO + AAV8-GFP + CCl_4 vs Airn-KO + AAV8-GFP; $^{\#}P < 0.05$, Airn-KO + CCl_4 vs WT + CCl_4 or Airn-KO + AAV8-Airn + CCl_4 vs Airn-KO + AAV8-GFP + CCl_4.

(Figure 6A,B). Next, we examined the changes in apoptosis-related genes at the protein level. Western blot analysis demonstrated that the proapoptotic proteins BAX and cleaved Caspase-3 were upregulated after H_2O_2 treatment, and the expression of the antiapoptotic protein Bcl-2 was downregulated, while the H_2O_2-induced increase in apoptosis was aggravated when *Airn* was knocked down. In contrast, overexpression of Airn mitigated H_2O_2-induced apoptosis (Figure 6C,D). To further assess the influence of

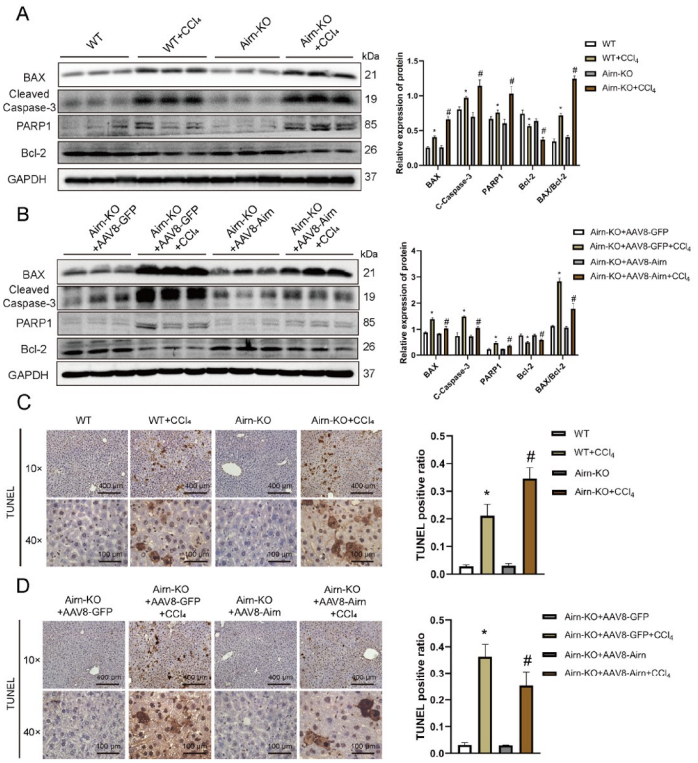

Figure 5. Airn inhibits CCl_4-induced hepatocyte apoptosis *in vivo* (A,C) Mice were divided into four groups: WT, WT + CCl_4, Airn-KO and Airn-KO + CCl_4. (B,D) Airn-KO mice were divided into four groups: Airn-KO + AAV8-GFP, Airn-KO + AAV8-GFP + CCl_4, Airn-KO + AAV8-Airn and Airn-KO + AAV8-Airn + CCl_4. Western blot and TUNEL assays were used to detect the level of apoptosis in liver tissues and liver tissue sections, respectively. GAPDH was used as an internal control. The data are expressed in at least three experiments, $n = 3$. *P* values were analysed by Student's *t* test. $^*P < 0.05$, WT + CCl_4 vs WT or Airn-KO + AAV8-GFP + CCl_4 vs Airn-KO + AAV8-GFP; $^\#P < 0.05$, Airn-KO + CCl_4 vs WT + CCl_4 or Airn-KO + AAV8-Airn + CCl_4 vs Airn-KO + AAV8-GFP + CCl_4.

Airn on hepatocyte apoptosis, Hoechst/PI double-staining assay was performed, and the results showed that H_2O_2 provoked an increase in apoptosis, while H_2O_2-induced hepatocyte apoptosis was aggravated under the condition of *Airn* knockdown. However, overexpression of Airn mitigated H_2O_2-induced hepatocyte apoptosis (Figure 6E). The above results suggest that Airn inhibits hepatocyte apoptosis.

Airn inhibits hepatocyte apoptosis through NF-κB signaling

Next, we investigated the mechanisms of Airn in hepatocyte apoptosis. It has been extensively reported that NF-κB has proinflammatory functions and promotes apoptosis [16]. Therefore, further experiments were performed to investigate whether Airn is involved in NF-κB signaling. The results revealed that phos-p65 and phos-IκBα, which are degraded and subsequently enable NF-κB dimers to translocate into the nucleus to regulate gene expression, were markedly increased after CCl_4 treatment. Knockout of *Airn* aggravated CCl_4-induced increase of phos-p65 and phos-IκBα (Figure 7A). On the other hand, CCl_4-induced increase of phos-p65 and phos-IκBα was decreased after overexpression of Airn (Figure 7B). *In vitro*, silencing *Airn* promoted H_2O_2-induced upregulation of phos-p65 and phos-IκBα. Overexpression of Airn decreased the H_2O_2-induced increase of phos-p65 and phos-IκBα (Figure 7C). Moreover, BAY11-7082, an antagonist of NF-κB, was used to further confirm that Airn is involved in NF-κB signaling. Western blot analysis and Hoechst/PI double-staining assays showed that H_2O_2-induced upregulation of apoptosis-related genes and apoptosis-positive cells were abrogated by the antagonist of NF-κB (Figure 7D,E). Next, we performed a Dual-luciferase reporter assay to confirm whether Airn could repress H_2O_2-induced activation of NF-κB. The results showed that H_2O_2 promoted the activation of NF-κB, while overexpression of Airn repressed H_2O_2-induced NF-κB activation, as shown by the decreased luciferase activity (Figure 7F). Taken together, these results suggest that Airn inhibits hepatocyte apoptosis through NF-κB signaling.

Discussion

Acute liver injury results in the massive death or loss-of-function of hepatocytes, and severe or persistent liver injury eventually leads to liver failure, which represents a stage of sudden deterioration of liver function that is characterized by cell necrosis, inflammation and oxidative damage [17,18]. It is a severe clinical syndrome caused by many factors, such as drugs, viruses, alcohol, and autoimmunity [19]. A recent study also revealed that intestinal flora disorder inhibited the recovery of liver function to aggravate acute liver injury [20]. In this study, CCl_4 was used to induce acute liver injury in a mouse model, and we found that Airn was upregulated in injured mouse livers and primary hepatocytes. Consistently, Airn was also overexpressed in serum samples of patients with acute-on-chronic liver failure and negatively correlated with the MELD score. Furthermore, Airn could alleviate CCl_4-induced acute liver injury by

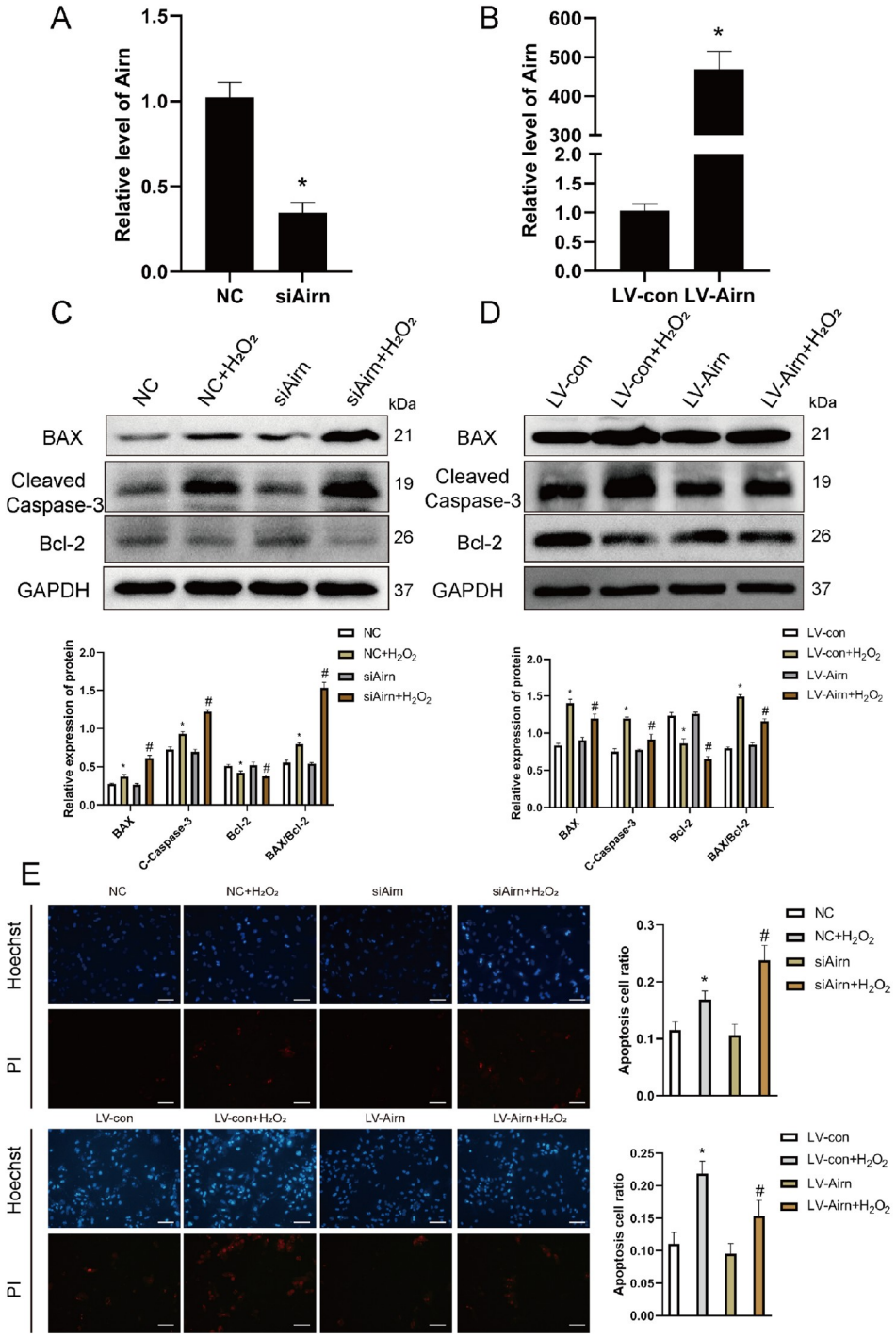

Figure 6. Airn inhibits H_2O_2-induced hepatocyte apoptosis *in vitro* Primary HCs were transfected with siRNA or lentivirus for 48 h, followed by treatment with 3% H_2O_2 for another 3 h. (A,B) The mRNA level of Airn was detected by qPCR. (C,D) The protein levels of apoptosis-related genes were detected by western blot analysis. GAPDH was used as an internal control. (E) Hoechst/PI double-staining was used to detect cell apoptosis. Scale bar: 100 μm. Data are expressed as the mean ± SD from at least three experiments. *P* values were analyzed by Student's *t* test. $^*P < 0.05$, siAirn vs siRNA-control or LV-Airn vs LV-control or NC + H_2O_2 vs NC or LV-con + H_2O_2 vs LV-con; $^\#P < 0.05$, siAirn + H_2O_2 vs NC + H_2O_2 or LV-Airn + H_2O_2 vs LV-con + H_2O_2.

reducing hepatocyte apoptosis and oxidative stress. Mechanistically, Airn represses CCl_4- and H_2O_2-induced increase of phos-p65 and phos-IκBα, suggesting that Airn inhibits hepatocyte apoptosis via NF-κB signaling.

CCl_4 is a well-known hepatotoxin that causes serious liver injury and cell death through death pathways such as apoptosis and necrosis, and prolonged exposure to CCl_4 leads to liver failure, cirrhosis and hepatocellular carcinoma [21]. Its principal mechan-

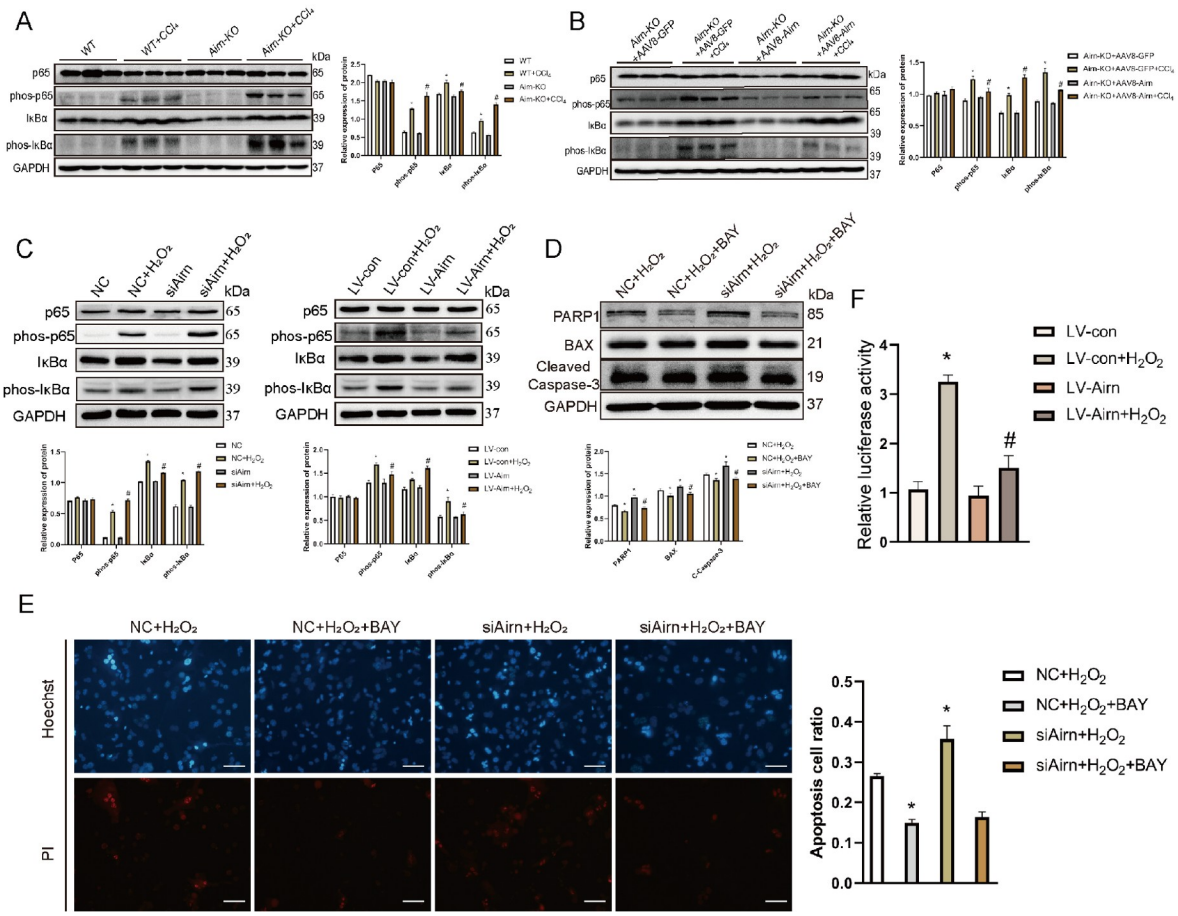

Figure 7. Airn inhibits hepatocyte apoptosis through NF-κB signaling (A) Mice were divided into four groups. The protein levels of p65, phos-p65, IκBα and phos-IκBα were detected by western blot analysis. GAPDH was used as an internal control. (B) Airn-KO mice were divided into four groups. The protein levels of p65, phos-p65, IκBα and phos-IκBα were detected by western blot analysis. GAPDH was used as an internal control. (C) Primary HCs were transfected with siRNA or lentivirus for 48 h, followed by treatment with 3% H_2O_2 for another 3 h. The protein levels of p65, phos-p65, IκBα and phos-IκBα were detected by western blot analysis. GAPDH was used as an internal control. (D,E) Primary hepatocytes were transfected with siRNA for 48 h, and then treated with 3% H_2O_2 and 10 μM of the NF-κB antagonist BAY11-7082 for another 3 h. The protein levels of PARP1, Bcl-2, BAX and cleaved caspase-3 were determined by western blot analysis. GAPDH was used as an internal control. Hoechst/PI double-staining was used to detect cell apoptosis. Scale bar: 100 μm. (F) pNF-κB-Luc and pRL-TK were cotransfected into primary hepatocytes using Lipofectamine 3000. Then, the cells were transfected with lentivirus-Airn and treated with 3% H_2O_2. NF-κB luciferase activity was detected using the Dual-luciferase reporter assay system, and the relative firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are expressed as the mean ± SD from at least three experiments, $n = 3$. *P* values were analyzed by Student's *t* test, $^*P < 0.05$, WT + CCl_4 vs WT or Airn-KO + AAV8-GFP + CCl_4 vs Airn-KO + AAV8-GFP or NC + H_2O_2 vs NC or LV-con + H_2O_2 vs LV-con or NC + H_2O_2 + BAY vs NC + H_2O_2; $^\#P < 0.05$, Airn-KO + CCl_4 vs WT + CCl_4 or Airn-KO + AAV8-Airn + CCl_4 vs Airn-KO + AAV8-GFP + CCl_4 or siAirn + H_2O_2 vs NC + H_2O_2 or LV-Airn + H_2O_2 vs LV-con + H_2O_2 or siAirn + H_2O_2 + BAY vs NC + H_2O_2 + BAY.

ism is associated with both the chemical itself and its reactive metabolites [22]. A previous study revealed that damage to hepatocyte membranes caused by reactive oxygen radicals (ROS) is the key characteristic of CCl_4-induced acute liver injury [23,24].

SOD and CAT activities, as well as GSH levels, are important antioxidant biomarkers, and synergistic activity between SOD and CAT has been found to enhance the clearance of free radicals. GSH can catalyze the degradation of H_2O_2 to strengthen the cell membrane and prevent cell damage [25–27]. A previous study reported that CCl_4 treatment reduced the levels of SOD and GSH and that reducing the level of oxidative stress could alleviate liver injury [28]. BRP pretreatment significantly decreased the enzyme activities of ALT, ALP and AST in the serum, markedly increased the activities of SOD and GSH-Px in the liver, reduced the MDA concentration in the liver and then alleviated CCl_4-induced liver injury [29]. Another study revealed that glutamine treatment effectively attenuated oxidative stress, ameliorated CCl_4-induced liver fibrosis and suppressed TGF-β1-induced EMT progression and apoptosis [15]. The most common resident cell types in the liver are hepatocytes and account for 75%–80% of the liver [30]. Hepatocyte apoptosis has emerged as an important pathological mechanism of acute liver injury or liver failure [31]. Effective blockade of hepatocyte apoptosis is one of the important ways to reduce liver damage and maintain hepatic function. For example, a previous study revealed that Parkin deficiency could aggravate chronic alcohol intake-induced liver injury by promoting apoptosis [32]. Some other studies have demonstrated that cellular apoptosis is often coupled with mitochondrial dysfunction, while mitochondrial dysfunction causes ROS overproduction. Excessive ROS can increase mitochondrial membrane permeability, allowing the

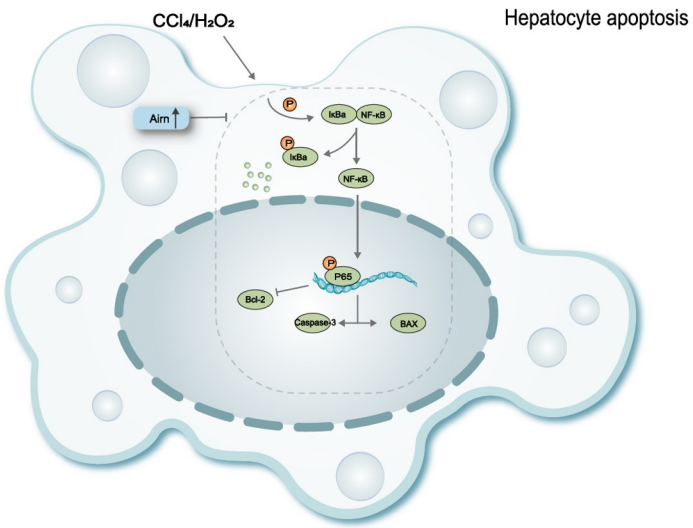

Figure 8. The mechanism of Airn in hepatocyte apoptosis Airn represses CCl_4- and H_2O_2-induced enhancement of phosphorylation of p65 and IκBα, suggesting that Airn inhibits hepatocyte apoptosis by inactivating the NF-κB pathway.

release of cytochrome C into the cytoplasm, followed by the activation of the mitochondria-dependent caspase signaling pathway, which ultimately leads to apoptosis [33]. In this study, CCl_4-induced hepatocyte apoptosis was aggravated under the condition of *Airn* knockout but decreased when Airn was overexpressed. Similarly, the levels of SOD, CAT and GSH showed the same results. These findings demonstrated that Airn could increase the levels of SOD, CAT and GSH to strengthen the antioxidant capacity and alleviate CCl_4-induced hepatocyte apoptosis.

Accumulated evidence indicates that the NF-κB signaling pathway plays an essential role in acute liver injury. Under normal physiological conditions, NF-κB and IκB exist in the cytoplasm as inactive complexes; when the liver is damaged, with the action of the kinases IKKα and IKKβ, IκB is phosphorylated and dissociated from NF-κB, and NF-κB transfers into the nucleus, activates the expressions of a series of cell inflammatory factors, and promotes necrosis and apoptosis of liver cells [23,24]. It has been reported that knockdown of either *STING* or *IRF3* leads to a significant reduction in FFA-induced hepatic inflammation and apoptosis, as evidenced by modulation of NF-κB signaling pathway [34]. In addition, liver kinase B1 (LKB1) suppresses the activation of the NLRP3 inflammasome by blocking the NF-κB signaling pathway, leading to alleviation of the inflammatory response to hypoxia/reoxygenation injury [35]. Tβ4 inhibits hepatic apoptosis and fibrosis induced by lincRNA-p21 via suppressing the PI3K-AKT-NF-κB pathway [36]. Some studies have reported that inhibiting NF-κB p65 and IκBα phosphorylation can block the activation of the NF-κB pathway, thereby reducing the expressions of inflammatory factors and the occurrence of apoptosis, which is important for the prevention of CCl_4-induced acute liver injury in mice [37–39]. Our results verified that Airn suppressed the activation of NF-κB signaling *in vivo* and *in vitro*. Moreover, by using an NF-κB antagonist, we demonstrated that Airn inhibits hepatocyte apoptosis through NF-κB signaling, as revealed by luciferase reporter assay.

In summary, our research demonstrated that Airn could alleviate CCl_4-induced acute liver injury by reducing hepatocyte apoptosis and oxidative stress. Mechanistically, Airn decreases hepatocyte apoptosis by inactivating the NF-κB signaling pathway (Figure 8). This study suggests that Airn may play a protective role and be used as a potential biomarker in acute liver injury.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81870429, 82170630, 32171125, and 81971331) and the Natural Science Foundation of Tianjin (No. 19JCZDJC36700).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Thawley V. Acute liver injury and failure. *Vet Clin N Am-Small Anim Pract* 2017, 47: 617–630
2. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. *Gastroenterology* 2014, 147: 765–783
3. Wang YH, Suk FM, Liu CL, Chen TL, Twu YC, Hsu MH, Liao YJ. Antifibrotic effects of a barbituric acid derivative on liver fibrosis by blocking the NF-κB signaling pathway in hepatic stellate cells. *Front Pharmacol* 2020, 11: 388
4. Chida Y. The hepatic sympathetic nerve plays a critical role in preventing Fas induced liver injury in mice. *Gut* 2005, 54: 994–1002
5. Guo H, Sun J, Li D, Hu Y, Yu X, Hua H, Jing X, ***et al.*** Shikonin attenuates acetaminophen-induced acute liver injury via inhibition of oxidative stress and inflammation. *Biomed Pharmacother* 2019, 112: 108704
6. Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. *Cell* 2013, 154: 26–46
7. Lu X, Ding Y, Bai Y, Li J, Zhang G, Wang S, Gao W, ***et al.*** Detection of allosteric effects of lncRNA secondary structures altered by SNPs in

human diseases. *Front Cell Dev Biol* 2020, 8: 242
8. Li L, He Y, He X, Bi M, Qi Y, Zhu W. Down-regulation of long noncoding RNA LINC00472 alleviates sepsis-induced acute hepatic injury by regulating miR-373-3p/TRIM8 axis. *Exp Mol Pathol* 2020, 117: 104562
9. Shen C, Li J. LncRNA XIST silencing protects against sepsis-induced acute liver injury via inhibition of BRD4 expression. *Inflammation* 2021, 44: 194–205
10. Nagano T, Mitchell JA, Sanz LA, Pauler FM, Ferguson-Smith AC, Feil R, Fraser P. The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. *Science* 2008, 322: 1717–1720
11. Cai H, Zheng Y, Wen Z, Yang Y, Yang S, Zhang Q. LncRNA AIRN influences the proliferation and apoptosis of hepatocellular carcinoma cells by regulating STAT1 ubiquitination. *Arch Pharm Res* 2021, 44: 414–426
12. Hosen MR, Militello G, Weirick T, Ponomareva Y, Dassanayaka S, Moore Iv JB, Döring C, ***et al.*** *Airn* regulates Igf2bp2 translation in cardiomyocytes. *Circ Res* 2018, 122: 1347–1353
13. Zhang K, Han X, Zhang Z, Zheng L, Hu Z, Yao Q, Cui H, ***et al.*** The liver-enriched lnc-LFAR1 promotes liver fibrosis by activating TGFβ and Notch pathways. *Nat Commun* 2017, 8: 144
14. Yotova IY, Vlatkovic IM, Pauler FM, Warczok KE, Ambros PF, Oshimura M, Theussl HC, ***et al.*** Identification of the human homolog of the imprinted mouse Air non-coding RNA. *Genomics* 2008, 92: 464–473
15. Shrestha N, Chand L, Han MK, Lee SO, Kim CY, Jeong YJ. Glutamine inhibits CCl4 induced liver fibrosis in mice and TGF-β1 mediated epithelial–mesenchymal transition in mouse hepatocytes. *Food Chem Toxicol* 2016, 93: 129–137
16. Liu B, Deng X, Jiang Q, Li G, Zhang J, Zhang N, Xin S, ***et al.*** Scoparone alleviates inflammation, apoptosis and fibrosis of non-alcoholic steatohepatitis by suppressing the TLR4/NF-κB signaling pathway in mice. *Int Immuno Pharmacol* 2019, 75: 105797
17. Wu S, Lu H, Wang W, Song L, Liu M, Cao Y, Qi X, ***et al.*** Prevention of D-GalN/LPS-induced ALI by 18β-glycyrrhetinic acid through PXR-mediated inhibition of autophagy degradation. *Cell Death Dis* 2021, 12: 480
18. Tao XM, Li D, Zhang C, Wen GH, Wu C, Xu YY, Kan Y, ***et al.*** Salvianolic acid B protects against acute and chronic liver injury by inhibiting Smad2C/L phosphorylation. *Exp Ther Med* 2021, 21: 341
19. Stravitz RT, Lee WM. Acute liver failure. *Lancet* 2019, 394: 869–881
20. Leng L, Ma J, Lv L, Gao D, Li M, Wang Y, Zhu Y. Serum proteome profiling provides a deep understanding of the 'gut-liver axis' in relation to liver injury and regeneration. *Acta Biochim Biophys Sin* 2021, 53: 372–380
21. Chang SN, Kim SH, Dey DK, Park SM, Nasif O, Bajpai VK, Kang SC, ***et al.*** 5-O-demethylnobiletin alleviates CCl4-induced acute liver injury by equilibrating ROS-mediated apoptosis and autophagy induction. *Int J Mol Sci* 2021, 22: 1083
22. Yi R, Wei Y, Tan F, Mu J, Long X, Pan Y, Liu W, ***et al.*** Antioxidant capacity-related preventive effects of shoumei (slightly fermented *camellia sinensis*) polyphenols against hepatic Injury. *Oxid Med Cell Longev* 2020, 2020: 1–17
23. Bruccoleri A, Gallucci R, Germolec DR, Blackshear P, Simeonova P, Thurman RG, Luster MI. Induction of early-immediate genes by tumor necrosis factor alpha contribute to liver repair following chemical-induced hepatotoxicity. *Hepatology* 1997, 25: 133–141
24. Weber LWD, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003, 33: 105–136
25. Huang QH, Xu LQ, Liu YH, Wu JZ, Wu X, Lai XP, Li YC, ***et al.*** Polydatin protects rat liver against ethanol-induced injury: involvement of CYP2E1/ROS/Nrf2 and TLR4/NF-κB p65 pathway. *Evid-Based Complement Alternat Med* 2017, 2017: 1–14
26. Li YG, Ji DF, Chen S, Hu GY. Protective effects of sericin protein on alcohol-mediated liver damage in mice. *Alcohol Alcoholism* 2008, 43: 246–253
27. Wang X, Liu M, Zhang C, Li S, Yang Q, Zhang J, Gong Z, ***et al.*** Antioxidant activity and protective effects of enzyme-extracted oudemansiella radiata polysaccharides on alcohol-induced liver injury. *Molecules* 2018, 23: 481
28. Tsai JC, Chen YA, Wu JT, Cheng KC, Lai PS, Liu KF, Lin YK, ***et al.*** Extracts from fermented black garlic exhibit a hepatoprotective effect on acute hepatic injury. *Molecules* 2019, 24: 1112
29. Li Q, Yang H, Wang W, Li N, Zou X, Li Y, Fan G, ***et al.*** *Brassica rapa* polysaccharides ameliorate CCl_4 induced acute liver injury in mice through inhibiting inflammatory apoptotic response and oxidative stress. *Chem Biodivers* 2020, 17: e1900534
30. Zhou Z, Xu MJ, Gao B. Hepatocytes: a key cell type for innate immunity. *Cell Mol Immunol* 2016, 13: 301–315
31. Kaplowitz N. Mechanisms of liver cell injury. *J Hepatol* 2000, 32: 39–47
32. Peng H, Qin X, Chen S, Ceylan AF, Dong M, Lin Z, Ren J. Parkin deficiency accentuates chronic alcohol intake-induced tissue injury and autophagy defects in brain, liver and skeletal muscle. *Acta Biochim Biophys Sin* 2020, 52: 665–674
33. Chen Q, Zhan Q, Li Y, Sun S, Zhao L, Zhang H, Zhang G. *Schisandra* lignan extract protects against carbon tetrachloride-induced liver injury in mice by inhibiting oxidative stress and regulating the NF- *κ*B and JNK signaling pathways. *Evid-Based Complement Alternat Med* 2017, 2017: 1–11
34. Qiao JT, Cui C, Qing L, Wang LS, He TY, Yan F, Liu FQ, ***et al.*** Activation of the STING-IRF3 pathway promotes hepatocyte inflammation, apoptosis and induces metabolic disorders in nonalcoholic fatty liver disease. *Metabolism* 2018, 81: 13–24
35. Dai J, Chen Q, Huang W, Shi K, Zhang Y, Li T, Mou T, ***et al.*** Liver kinase B1 attenuates liver ischemia/reperfusion injury via inhibiting the NLRP3 inflammasome. *Acta Biochim Biophys Sin* 2021, 53: 601–611
36. Yang L, Fu W, Zhu Y, Wang X. Tβ4 suppresses lincRNA-p21-mediated hepatic apoptosis and fibrosis by inhibiting PI3K-AKT-NF-κB pathway. *Gene* 2020, 758: 144946
37. Dai C, Xiao X, Li D, Tun S, Wang Y, Velkov T, Tang S. Chloroquine ameliorates carbon tetrachloride-induced acute liver injury in mice via the concomitant inhibition of inflammation and induction of apoptosis. *Cell Death Dis* 2018, 9: 1164
38. Zhang M, Hu X, Li S, Lu C, Li J, Zong Y, Qi W, ***et al.*** Hepatoprotective effects of ethyl pyruvate against CCl4-induced hepatic fibrosis via inhibition of TLR4/NF-κB signaling and up-regulation of MMPs/TIMPs ratio. *Clin Res Hepatol Gastroenterol* 2018, 42: 72–81
39. Son G, Iimuro Y, Seki E, Hirano T, Kaneda Y, Fujimoto J. Selective inactivation of NF-κB in the liver using NF-κB decoy suppresses CCl_4-induced liver injury and fibrosis. *Am J Physiol-Gastrointestinal Liver Physiol* 2007, 293: G631–G639