Arsenic trioxide preconditioning attenuates hepatic ischemia-reperfusion injury in mice: Role of ERK/AKT and autophagy

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Abstract

Background: Arsenic trioxide (ATO) is indicated as a broad-spectrum medicine for a variety of diseases, including cancer and cardiac disease. While the role of ATO in hepatic ischemia/reperfusion injury (HIRI) has not been reported. Thus, the purpose of this study was to identify the effects of ATO on HIRI.

Methods: In the present study, we established a 70% hepatic warm I/R injury and partial hepatectomy (30% resection) animal models *in vivo* and hepatocytes anoxia/reoxygenation (A/R) models *in vitro* with ATO pretreatment and further assessed liver function by histopathologic changes, enzyme-linked immunosorbent assay, cell counting kit-8, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Small interfering RNA (siRNA) for extracellular signal-regulated kinase (ERK) 1/2 was transfected to evaluate the role of ERK1/2 pathway during HIRI, followed by ATO pretreatment. The dynamic process of autophagic flux and numbers of autophagosomes were detected by green fluorescent protein-monomeric red fluorescent protein-LC3 (GFP-mRFP-LC3) staining and transmission electron microscopy.

Results: A low dose of ATO (0.75 μmol/L *in vitro* and 1 mg/kg *in vivo*) significantly reduced tissue necrosis, inflammatory infiltration, and hepatocyte apoptosis during the process of hepatic I/R. Meanwhile, ATO obviously promoted the ability of cell proliferation and liver regeneration. Mechanistically, *in vitro* studies have shown that nontoxic concentrations of ATO can activate both ERK and phosphoinositide 3-kinase-serine/threonine kinase (PI3K-AKT) pathways and further induce autophagy. The hepatoprotective mechanism of ATO, at least in part, relies on the effects of ATO on the activation of autophagy, which is ERK-dependent.

Conclusion: Low, non-toxic doses of ATO can activate ERK/PI3K-AKT pathways and induce ERK-dependent autophagy in hepatocytes, protecting liver against I/R injury and accelerating hepatocyte regeneration after partial hepatectomy.

Keywords: Autophagy; Arsenic trioxide; Liver; Ischemia/reperfusion; Regeneration; Phosphoinositide 3-kinase-serine/threonine kinase; PI3K-AKT; ERK

Introduction

Hepatic ischemia/reperfusion injury (HIRI) is a pathophysiological process that commonly occurs during hemorrhagic shock, severe trauma, liver resection, or liver transplantation, and is a critical factor in liver dysfunction and even liver failure after hepatectomy.[1,2] The underlying pathogenesis of HIRI is complex and involves several

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mechanisms. Although current studies have revealed that the generation of reactive oxygen species (ROS), endoplasmic reticulum stress (ERS), overload of calcium, sterile inflammatory response, and autophagy are all related to its pathophysiological process,[3–5] there are still no effective prevention methodologies against HIRI.

Arsenic trioxide (ATO), as the effective ingredient of the traditional Chinese medicine arsenic, has been used in China for thousands of years and was reported to have a significant therapeutic effect on acute promyelocytic leukemia (APL) as early as the 1970s by Harbin Medical University.[6] Currently, ATO has become one of the first-line therapies for young, non-high-risk APL.[7,8] Furthermore, recent studies have demonstrated that ATO can inhibit the progression of a variety of solid tumors by blocking the cell cycle, inducing apoptosis, reducing angiogenesis, and suppressing invasion and metastasis.[9,10] However, the effects of ATO on HIRI have not yet been reported and the precise regulation of ATO on HIRI is not fully understood.

Autophagy is another type of programmed cell death that is different from apoptosis and is involved in the degradation of malformed proteins and organelles by lysosomes.[11,12] Thus, autophagy is considered the molecular basis for modulating the occurrence and development of different diseases, including cancer, cardiovascular dysfunction, and metabolic syndromes. Due to the enrichment of lysosomes in the liver, autophagy is strongly associated with many liver diseases (i.e., hepatocellular carcinoma, I/R injury, and non-alcoholic steatohepatitis).[4,13,14] Motiño *et al*[4] found that cyclooxygenase-2 (COX-2) attenuated hepatic injury during the I/R process through activation of autophagy. Xue *et al*[13] suggested that pituitary adenylate cyclase activating polypeptide (PACAP) promoted the autophagic process in hepatocellular homeostasis to combat HIRI. Interestingly, several studies have also reported that ATO induces autophagy.[15–17]

Therefore, in this study, we aimed to further clarify the underlying role of autophagy and the extracellular signal-regulated kinase/phosphoinositide 3-kinase-serine/threonine kinase (ERK/PI3K-AKT) pathways in HIRI with ATO treatment.

Methods

Animals and in vivo design

Male C57BL/6 mice (8 weeks old) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Mice were randomized into sham + normal saline solution (NSS), I/R + NSS, sham + ATO, and I/R + ATO groups ($n = 7$ per group) using simple randomization. Mice were housed in specific pathogen-free (SPF) conditions and raised following institutional guidelines for animal care. All animal experiments were in accordance with the standard protocols of the Ethic Committee on the Use of Live Animals in Teaching and Research of Harbin Medical University, Harbin, China (No. 2019035).

Mice were subjected to an intraperitoneal injection (ip) of NSS or ATO (1 mg/kg, SL PHARM, Beijing, China) 1 h before the liver I/R operation, respectively. The administration of U0126 (an inhibitor of mitogen-activated protein kinase kinase-MAPKK [MEK]1/2, MedChemExpress, Monmouth Junction, NJ, USA) (HY-12031A) (20 mg/kg) and LY294002 (an inhibitor of PI3K, MedChemExpress) (HY-10108) (1.5 mg/kg) was used in the corresponding groups via tail vein injection 0.5 h before ATO intraperitoneal injection and subsequent liver I/R. Liver and serum were collected for the subsequent experiment [Figure 1A].

Mouse HIRI and hepatectomy model

Mice fasted for 12 h and were given free access to water before surgery. The procedures of partial hepatic ischemia have been described previously.[18] Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg), and a midline laparotomy was performed. An atraumatic clip was placed across the left lateral and median lobes of the liver (about 70%). After 75 min of partial hepatic ischemia, the clip was removed for reperfusion for 6 h. Besides, animal models combined with HIRI and partial hepatectomy (30% resection) were also construted. For the combined hepatectomy model, the non-ischemic lobes (30%) were excised at the beginning of reperfusion. Sham control mice simply underwent the same operation without vascular clamp. Mice were then observed daily for survival analysis.

Primary hepatocyte isolation and anoxia/reoxygenation (A/R) model

Mouse hepatocytes were isolated by a modified *in situ* collagenase perfusion technique as we described previously.[19] The viability and purity of the cells were determined by trypan blue exclusion. To simulate mouse I/R, cellular anoxic conditions were established and maintained in a modular incubator chamber (Biospherix, Lacona, NY, USA) by continuous gas flow with a 1% O_2, 5% CO_2, and 94% N_2 gas mixture. After incubation under hypoxia for 6 h, cells were incubated under normoxic conditions with 95% air and 5% CO_2 for the indicated times. Besides, U0126 (U0, 10 μmol/L) and LY294002 (LY, 25 μmol/L) were used to pretreat hepatocytes before ATO treatment.

Exploration of optimal dose of ATO

To identify the optimal dose of ATO *in vitro* and *in vivo*, dose-response experiments were conducted in hepatocytes, animal, A/R model *in vitro*, and I/R model *in vivo*. And the detailed information is presented in Supplementary File, http://links.lww.com/CM9/C248.

Small interfering RNA (siRNA) transfection

The siRNA for *ERK1/2* (siG150819095637-1-5, siG150819100854-1-5) and respective negative controls were purchased from RiboBio (Guangzhou, China). For specific *ERK1/2* knockdown, primary hepatocytes were seeded in 6-well plates, and 50 nmol/L siRNA was transfected into cells using X-tremeGene siRNA Transfection Reagent (No. 04476093001, Roche, Basel, Switzerland).

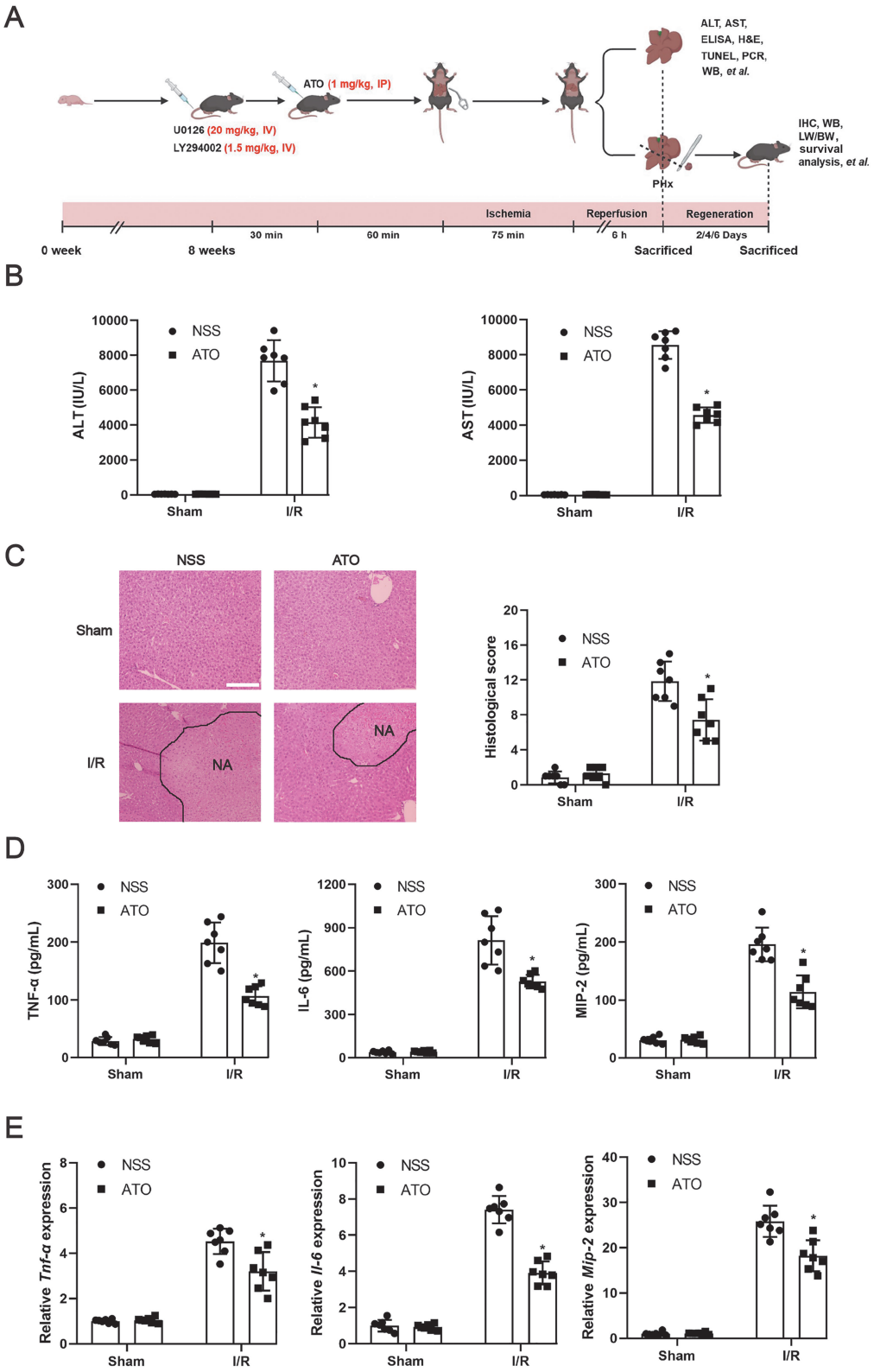

Figure 1: ATO pretreatment ameliorates hepatic I/R injury and inhibits the inflammatory response in mice. (A) The scheme of animal study. (B) Serum levels of aminotransferases (ALT and AST) were detected in the mice subjected to I/R (6 h) after ATO pretreatment ($n = 7$). (C) Images of H&E-stained (100× magnification) liver sections and representative histopathological scores after ATO pretreatment ($n = 7$). The scale bar was 200 μm. (D) TNF-α, IL-6, and MIP-2 levels after liver I/R were measured by ELISA ($n = 7$). (E) Relative mRNA expression of *Tnf-α*, *Il-6*, and *Mip-2* after liver I/R were examined by qRT-PCR ($n = 7$). All statistical analyses were performed by Student's *t*-test. $^*P < 0.05$ when compared with NSS-I/R group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATO: Arsenic trioxide; BW: Body weight; ELISA: Enzyme-linked immunosorbent assay; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry; IL-6: Interleukin-6; IP: Intraperitoneal injection; I/R: Ischemia/reperfusion; IV: Intravenous injection; LW: Liver weight; MIP-2: Macrophage inflammatory protein 2; mRNA: Messenger RNA; NA: Necrotic area; NSS: Normal saline solution; PHx: Partial hepatectomy; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; TNF-α: Tumor necrosis factor; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; WB: Western blotting.

Green fluorescent protein-monomeric red fluorescent protein-LC3 (GFP-mRFP-LC3) staining

GFP-mRFP-LC3 adenovirus was purchased from Hanbio (Shanghai, China). Transfection was performed with a multiplicity of infection (MOI) of 200. After adenovirus infection, mouse primary hepatocytes were cultured for 48 h or 72 h before measurement with a fluorescence microscope. The number of GFP and mRFP dots was detected in high-power fields (40×).

Transmission electron microscopy

Fresh tissues were flushed with 1 mL saline and fixed in 2.5% glutaraldehyde. The tissues were embedded in spur resin and were cut as thin sections. The sectioned grids were examined by transmission electron microscopy (TEM, JEOL 1200EX, Tokyo, Japan) at 80 kV or 60 kV. Thirty fields of low magnification (×1000) were selected from tissue sections randomly for quantification.

Others assays

In addition, liver tissue underwent histological examination and immunohistochemistry (IHC) staining. Parameters in sera were measured, western blotting analysis was conducted, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was carried out. Detailed information can be found in the Supplementary File, http://links.lww.com/CM9/C248.

Cell death assay was conducted with cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) and cytoplasmic histone-associated DNA-fragmentations (mono- and oligonucleosomes) due to cell death were measured using enzyme-linked immuno sorbent assay (ELISA). Caspase-3 activity was assessed. Detailed information can be found in the Supplementary File, http://links.lww.com/CM9/C248.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Two-tailed Student's *t*-test was used for the comparison of a normally distributed continuous variable between two groups. The level of significance was set at a *P*-value less than 0.05 for all analyses. All calculations of significance were determined using GraphPad Prism 8 software (https://www.graphpad.com/scientific-software/prism/).

Results

A small dose of ATO had no hepatotoxic effect on liver in vitro or in vivo

Before validating the effect of ATO on HIRI, our first priority was to determine whether a small dose of ATO is toxic to liver tissue. *In vitro*, primary hepatocytes were pretreated with ATO at escalating concentrations for 24 h, and a CCK-8 assay was then conducted to measure the cytotoxicity of ATO to hepatocytes. As shown in Supplementary Figure 1A, http://links.lww.com/CM9/C248, concentrations less than 1 μmol/L did not cause noticeable changes in cell viability. *In vivo*, C57BL/6 mice were given an IP injection of 0.9% saline or various doses of ATO for 1 h before liver functions were evaluated by biochemical analysis. As shown by the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), there were no significantly statistical differences in the levels of serum liver enzymes among the ATO groups and NSS group [Supplementary Figure 1B, http://links.lww.com/CM9/C248]. In our preliminary experiment, we also evaluated the hepatoprotective effect of these ATO doses in A/R model *in vitro* and I/R model *in vivo*. Finally, 0.75 μmol/L *in vitro* and 1 mg/kg *in vivo* were used for further investigation [Supplementary Figure 1C, D, http://links.lww.com/CM9/C248].

ATO pretreatment ameliorates HIRI and inhibits the inflammatory response in mice

To elucidate the role of ATO in HIRI, a partial (70%) warm liver ischemia mouse model was established. The levels of ALT and AST were measured to evaluate liver function at 6 h after hepatic I/R. ATO pretreatment significantly decreased the levels of enzymes induced by I/R injury [Figure 1B]. Furthermore, as shown by histological analysis, I/R mice treated with ATO exhibited less tissue necrosis, inflammatory cell infiltration, and hemorrhagic alterations than saline-treated mice [Figure 1C].

A close connection was found between the sterile inflammatory response and I/R injury,[20] and the release of cytokines and chemokines was accompanied by the progression of hepatic I/R. Thus, ELISA was performed to detect the levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and macrophage inflammatory protein 2 (MIP-2) in serum. The results demonstrated that I/R significantly increased the release of TNF-α, IL-6, and MIP2 compared with sham mice. Pretreatment with a low ATO-concentration attenuated these elevations [Figure 1D]. Furthermore, similar results were shown in the mRNA expressions levels of *Tnf-α*, *Il-6*, and *Mip-2* [Figure 1E].

ATO protects hepatocytes from hepatic I/R injury in vivo and in vitro

A sustained inflammatory response will cause cell death and necrosis of liver.[26] Therefore, we determined the levels of apoptosis by TUNEL assay, caspase-3 activity assay, and DNA fragmentation by ELISA. As shown in Figure 2A, fewer TUNEL-positive cells were detected in the ATO-treated mice than in the saline group. The results of the caspase-3 activity assay and DNA fragmentation demonstrated dramatic decreases in apoptotic levels with ATO treatment in I/R mice [Figure 2B, C]. Furthermore, we performed quantitative real-time PCR (*Bcl2*, *Bax*) and western blotting (BCL2, BAX, and C-CASP3) to analyze the expression of apoptotic markers and found that ATO pretreatment reduced cell death during I/R injury

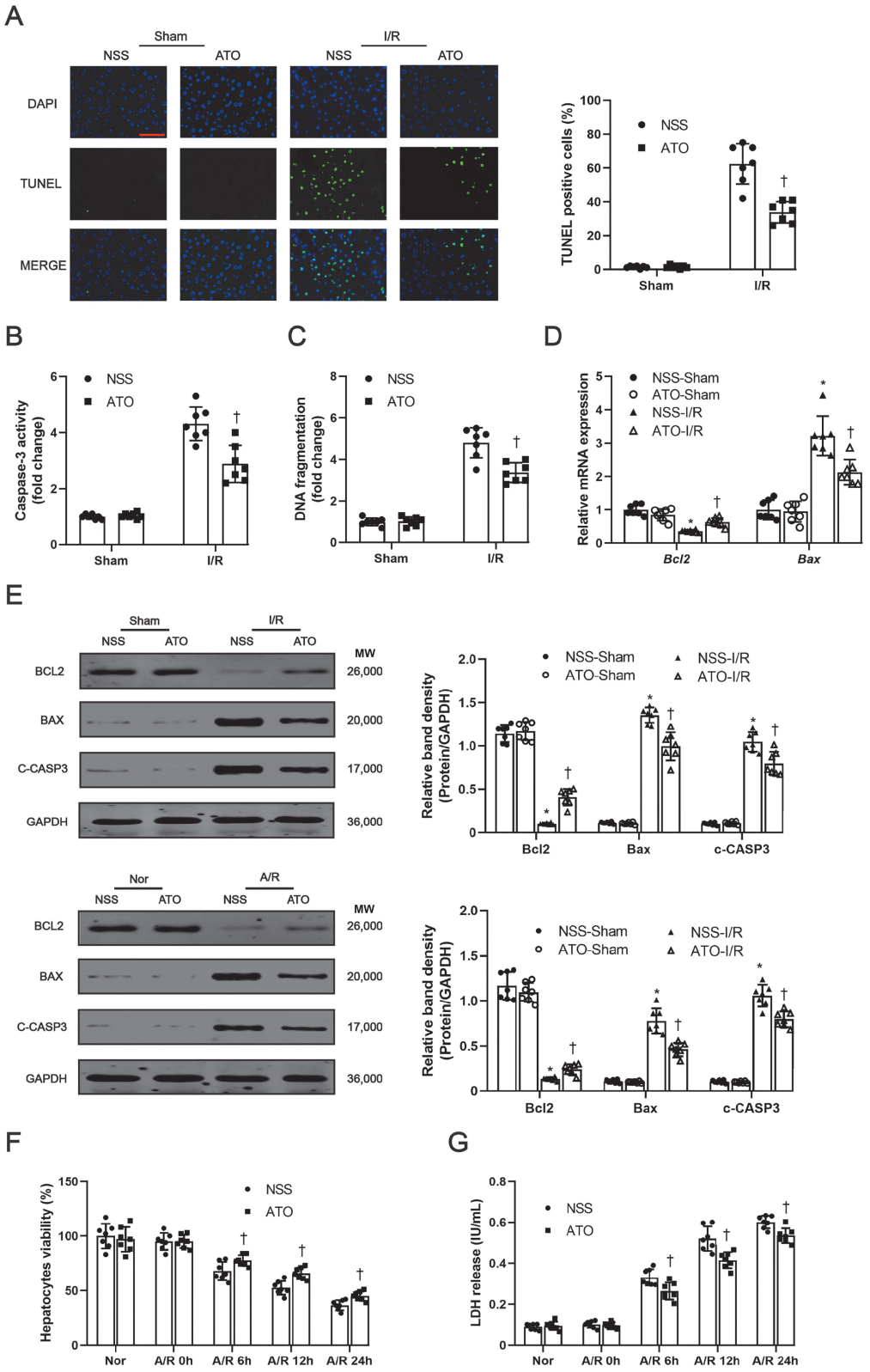

Figure 2: ATO protects hepatocytes from hepatic I/R injury *in vivo* and *in vitro*. (A) Representative images of liver sections stained by TUNEL and the quantification of TUNEL-positive cells percentage ($n = 7$). The scale bar was 50 μm. (B, C) Caspase-3 activity (B) and DNA fragmentation (C) in mice liver extract were determined by ELISA ($n = 7$). (D) Relative mRNA expression levels of *Bcl2* and *Bax* were examined by qRT-PCR ($n = 7$). (E) Western blot analysis of BCL2, BAX, and C-CASP3 and relative band density (animal study: $n = 7$). (F) Hepatocyte viability was determined at different timepoints after A/R treatment by CCK-8 assay. (G) LDH release of hepatocyte was measured after A/R treatment. All statistical analyses were performed by Student's *t*-test. $^{*}P < 0.05$ when compared with NSS-Sham (Nor) group; $^{\dagger}P < 0.05$ when compared with NSS-I/R (A/R) group. A/R: Anoxia/reoxygenation; ATO: Arsenic trioxide; BAX: Bcl-2 Associated X-protein; BCL2: B-cell lymphoma-2; C-CASP3: Cleaved-caspase-3; CCK-8: Cell counting kit-8; ELISA: Enzyme-linked immunosorbent assay; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL-6: Interleukin-6; I/R: Ischemia/reperfusion; LDH: Lactate dehydrogenase; mRNA: Messenger RNA; MW: Molecular weight; Nor: Normal control; NSS: Normal saline solution; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; WB: Western blotting.

[Figure 2D, E]. *In vitro*, the viability of hepatocytes subjected to A/R operation was measured by CCK-8 assay and found that ATO promoted cell proliferation, compared to the control groups [Figure 2F]. Meanwhile, less LDH was released from ATO-treated hepatocyte culture, compared to the corresponding saline-treated groups [Figure 2G].

ATO accelerates hepatocyte regeneration after I/R injury and partial hepatectomy (PHx)

Given that ATO attenuates hepatocyte apoptosis during I/R injury, we established a mouse model of PHx after I/R injury to further determine whether ATO plays critical roles in liver regeneration [Figure 1A]. As shown by the immunohistochemical staining of BrdU, pretreatment with ATO markedly promoted hepatocyte proliferation in comparison with the NSS group at day 2 after PHx (30% resection) and I/R injury, while in the sham group, there was almost no difference between NSS and ATO groups [Figure 3A]. In addition, western blot analysis confirmed that ATO-preconditioning exerted a pro-regeneration effect on hepatocyte growth by analyzing the expression levels of proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin E1 [Figure 3B]. Furthermore, a liver-to-body weight ratio (LW/BW) analysis demonstrated that ATO treatment could improve liver regeneration after hepatectomy [Figure 3C]. All of the control mice died within 6 days of liver resection, whereas nearly half of the mice treated with ATO survived for more than one week [Figure 3D]. Overall, these data supported the idea that ATO pretreatment can accelerate liver regeneration after major hepatectomy and I/R injury.

ATO decreases susceptibility to I/R injury by activating the ERK and PI3K/AKT signaling pathways

Mitogen-activated protein kinase (MAPK—ERK, P38, Jun N-terminal kinase [JNK]) and PI3K-AKT signaling are two well-known pathways involved in the cell survival,[2,21,22] and ERK/AKT signaling has been reported to promote hepatocyte proliferation and ameliorate HIRI.[23] Furthermore, low dose of ATO was reported to activate both ERK and PI3K-AKT pathways.[24,25] Therefore, to confirm the underlying role of ATO in hepatic I/R injury, we tested whether the low-dose of ATO had an effect on the activation of the ERK and PI3K-AKT pathways *in vivo* and *in vitro*. As expected, there was moderate activation of ERK/AKT signaling accompanied by I/R operation; intriguingly, the phospho-ERK (p-ERK) and phospho-AKT (p-AKT) levels were further increased with ATO treatment [Figure 4A]. In line with the *in vivo* findings, the *in vitro* results also demonstrated that primary hepatocytes subjected to A/R and ATO treatment had upregulated p-ERK and p-AKT expression [Figure 4B].

To test the roles of the ERK and PI3K-AKT signaling pathways in ATO-mediated I/R injury, the administration of specific inhibitors of either ERK1/2 (U0126) or PI3K-AKT (LY294002) was applied for further investigation. Both of the antagonists dramatically reversed the activation of ERK/PI3K-AKT pathways induced by ATO *in vivo* and *in vitro* [Figure 4A, B]. Notably, the liver damage after I/R was deteriorated after U0126/LY294002 treatment, as determined by measuring histopathological changes and the levels of cleaved caspase 3, serum aminotransferases, cytokine (TNF-α and IL-6) release, and hepatocyte viability [Figure 4A–F]. Taken together, ATO relieves I/R-induced liver injury in an ERK/AKT-dependent manner.

Autophagy activation mediates the hepatoprotective role of ATO

Autophagy is a metabolic process that plays a prosurvival role under histopathological conditions.[26] Given that ATO was reported to have a stimulatory effect on autophagy, we next assessed the hepatocellular autophagic response upon

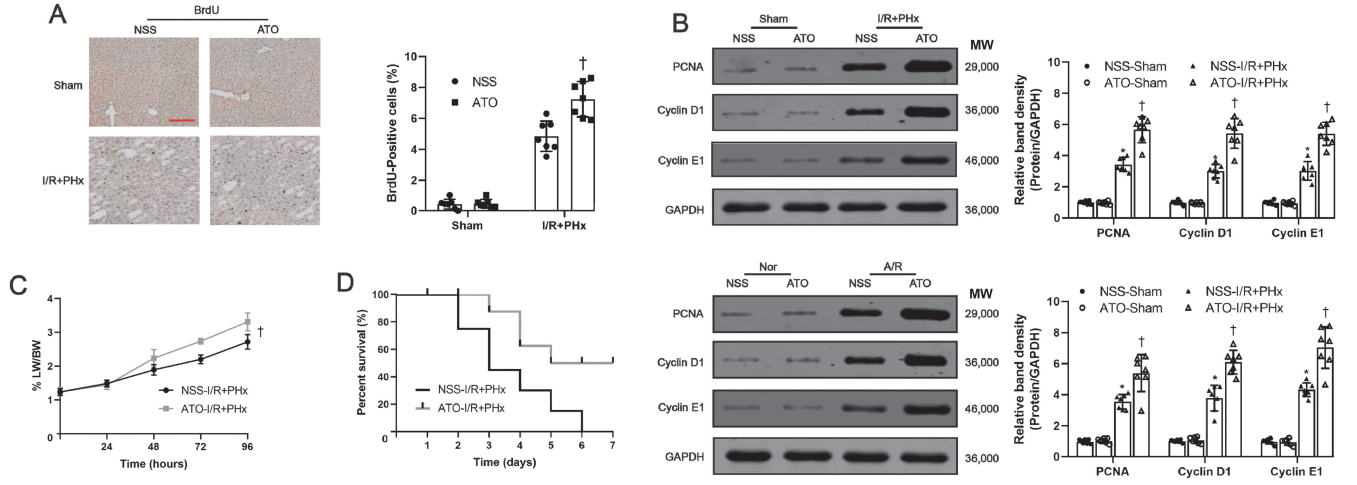

Figure 3: ATO accelerates hepatocyte regeneration after I/R injury and PHx. (A) Representative images of Brdu staining (100× original magnification) and the quantification of Brdu-positive cells ratio ($n = 7$). The scale bar was 200 μm. (B) Western blot analysis of PCNA, Cyclin D1, Cycling E1, and relative band density (animal study: $n = 7$). (C) Percent liver weight after PHx in NSS and ATO preconditioning mice ($n = 3$) (D) Survival curves after PHx with I/R injury of NSS and ATO preconditioning mice ($n = 20$). All statistical analyses were performed by Student's *t*-test. * means $P < 0.05$ when compared with NSS-Sham (Nor) group; † means $P < 0.05$ when compared with NSS-I/R +PHx or (A/R) group. A/R: Anoxia/reoxygenation; ATO: Arsenic trioxide; BW: Body weight; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; LW: Liver weight; MW: Molecular weight; Nor: Normal control; NSS: Normal saline solution; PCNA: Proliferating cell nuclear antigen; PHx: Partial hepatectomy.

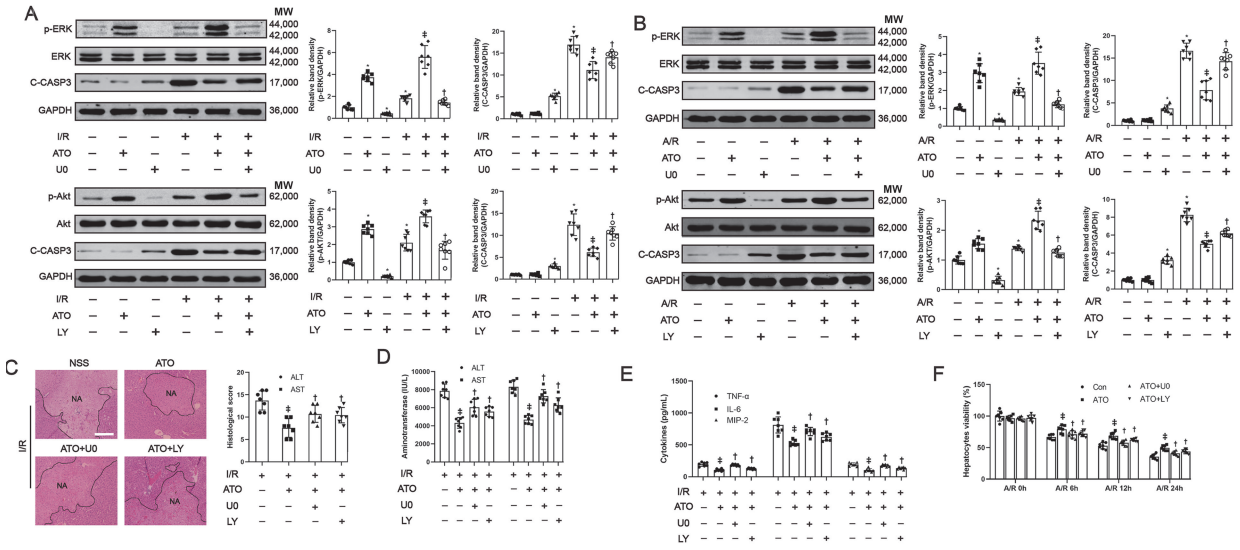

Figure 4: ATO decreases susceptibility to I/R injury by activating the ERK and PI3K/AKT signaling pathways. (A, B) Western blot analysis of p-ERK, ERK, p-AKT, AKT, and C-CASP3 and relative band density (animal study: $n = 7$). (C) Images of H&E-stained liver sections and representative histopathological scores ($n = 7$). The scale bar was 200 μm. (D) Serum levels of aminotransferases (ALT and AST) were detected in the mice subjected to I/R after ATO, U0126 and LY294002 pretreatment. (E) TNF-α and IL-6 levels in the mice subjected to I/R after ATO, U0126 and LY294002 pretreatment ($n = 7$). (F) Hepatocyte viability was determined after A/R(6h) treatment and ATO, U0126 and LY294002 preconditioning by CCK-8 assay. U0126 (U0) and LY294002 (LY) pretreatment for 24h, and pretreatment concentration: 10 μmol/L and 25 μmol/L, perspectively. All statistical analyses were performed by Student's *t*-test.* means $P < 0.05$ when compared with blank; ‡ means $P < 0.05$ when compared with NSS-I/R (A/R) group; † means $P < 0.05$ when compared with compared with NSS-I/R (A/R) + ATO group. A/R: Anoxia/reoxygenation; AKT: Protein kinase B; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATO: Arsenic trioxide; C-CASP3: Cleaved-caspase-3; CCK-8: Cell Counting Kit-8; ERK: Extracellular signal-regulated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; H&E: Hematoxylin and eosin; I/R: Ischemia/reperfusion; IL-6: Interleukin-6; MIP-2: Macrophage inflammatory protein 2; MW: Molecular weight; NA: Necrotic area; p-AKT: Phosphor-AKT; p-ERK: Phosphor-ERK; TNF-α: Tumor necrosis factor.

I/R injury following ATO treatment. The expression of the autophagy markers LC3, Beclin 1, and P62 (a protein which is degraded when autophagy is induced) was subsequently examined by immunoblotting analysis. Not surprisingly, overtly elevated levels of LC3 Ⅱ (lipidated form of LC3) and Beclin 1 were detected during I/R injury [Figure 5A], the P62 protein levels were decreased, and intriguingly, the activation of autophagic flux was more significant with ATO-preconditioning both *in vivo* and *in vitro*.

As a dynamic feature of hepatic autophagic flux, GFP-mRFP-LC3 staining was further used to detect the autophagic response. Consistent with the western blot results, LC3-fluorescence images demonstrated that the ratios of autolysosomes (red dots) to autophagosomes (yellow dots) were dramatically elevated in ATO-treated hepatocytes subjected to A/R *vs*. control one [Figure 5B]. To confirm the activation of autophagy by ATO, we examined the accumulation of autophagosomes by TEM. Compared with the respective control group, more autophagosomes were found following ATO treatment by evaluating the number of autophagic vacuoles, which was characterized by multiple-membrane structures containing fragments of cell components, per 100 μm cytoplasmic area [Figure 5C]. Collectively, these findings indicate that autophagy can be activated by ATO during hepatic I/R injury *in vivo* and *in vitro*.

The ERK pathway dominates the ATO-induced activation of autophagy

Several studies have suggested that ERK is an important signaling pathway in regulating autophagic activity. Xiong *et al*[27] reported that ERK-dependent autophagy triggers cell death in bladder cancer, and the PI3K-AKT pathway is known for its suppressive effect on the autophagic process.[28,29] Due to the data mentioned above, we concluded that ATO-preconditioning induces coactivation of autophagy and ERK/PI3K-AKT pathways, which contradicted previous findings. Hence, to further elucidate the correlation between ERK/PI3K-AKT activation and autophagy, I/R-mice and primary hepatocytes subjected to A/R operation were treated with selective inhibitors of ERK (U0126) and PI3K-AKT (LY294002). As indicated by western blot, U0126 pretreatment remarkably abrogated the elevated levels of LC3 and Beclin 1 and reversed the reduction in P62 protein induced by ATO, whereas LY294002 had no effect on the autophagic process [Figure 5A]. In accordance with the protein immunoblotting results, GFP-mRFP-LC3 assays also illustrated a dramatically reduced autophagic flux in U0126-treated hepatocytes, and the additional administration of LY294002 did not affect the level of autophagy [Figure 6A]. Moreover, the results obtained from TEM images were consistent with the above findings [Figure 6B]. To eliminate the off-target effects of ERK1/2 inhibitors, siRNA-mediated ERK pathway silencing was performed. *ERK1/2* knockdown markedly suppressed autophagic flux, as shown by the western blot, LC3-fluorescence, and TEM results [Figure 6C–E]. These data strongly suggest that ERK-dependent autophagy in the hepatoprotective effect of ATO-preconditioning upon I/R injury.

Discussion

ATO has long been known to have toxicological effects on cell growth and further used for the anticancer activity during the treatment of hepatocellular carcinoma, breast cancer,

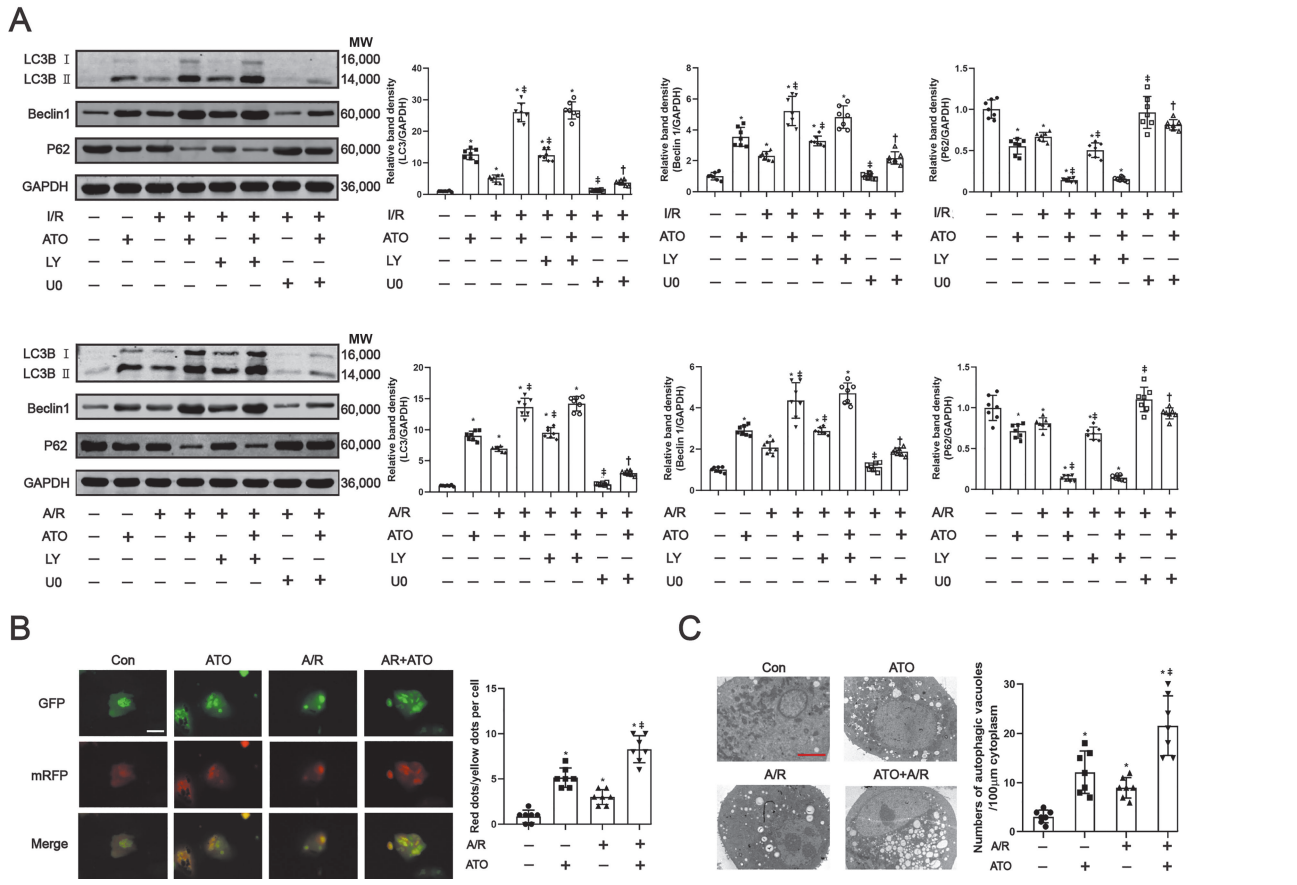

Figure 5: Autophagy activation mediates the hepatoprotective role of ATO. (A) Western blot analysis of LC3, Beclin 1, and P62 and relative band density in I/R model (upper) and A/R model (under) (animal study: $n = 7$). (B) Primary hepatocytes transfected with mRFP-GFP-LC3 adenovirus were determined by fluorescent puncta. The scale bar was 10 μm. (C) Representative electron micrographs showing autophagosomes in primary hepatocytes and the quantification of the number of autophagosomes in 100 μm cytoplasm. The scale bar was 5 μm. All statistical analyses were performed by Student's *t*-test. * means $P < 0.05$ when compared with blank; ‡ means $P < 0.05$ when compared with NSS-I/R (A/R) group; † means $P < 0.05$ when compared with compared with NSS-I/R (A/R) + ATO group. A/R: Anoxia/reoxygenation; ATO: Arsenic trioxide; Con: Control; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; LY: LY294002, an inhibitor of PI3K; mRFP-GFP-LC3: Tandem fluorescent-tagged LC3; MW: Molecular weight; U0: U0126, an inhibitor of MEK1/2.

non-small cell lung cancer, and glioma.[17,30–31] Meanwhile, ATO is limited for further therapeutic application due to cytotoxicity as well.[32] In addition, recent studies have paid more attention to the potential therapeutic effects of ATO on a range of benign diseases. It was reported that ATO-induced autophagic activation provides a perspective for the treatment of atherosclerosis (AS).[15] An *et al*[33] suggested that ATO exerts a neuroprotective function in experimental autoimmune encephalomyelitis (EAE). However, few investigations have focused on ischemic/reperfusion injury, especially hepatic I/R injury. Considering the toxicity of arsenic, we tried to detect the safe and lowest doses of ATO *in vivo* and *in vitro* firstly, and further explore the effects of ATO on HIRI.

Both ERK and PI3K-AKT signaling play potent roles in various cellular functions involved in cell proliferation and differentiation, and have been shown to actively participate in cell survival under ischemic conditions.[34,35] ERK activation is suggested to protect cardiomyocytes against apoptosis and promote angiogenesis under I/R operation, resulting in cardioprotective effects of infarcted hearts.[34] AKT and ERK signaling are also thought to alleviate acute kidney injury (AKI) induced by A/R treatment.[35] Moreover, ERK and PI3K-AKT signaling can contribute to hepatoprotection during liver IRI. AKT and ERK activation has been shown to attenuate cell death and ROS accumulation caused by HIRI.[36] Interestingly, ATO is reported to activate both ERK and PI3K-AKT pathways in different contexts.[24] Huang *et al*[37] suggested that ATO pretreatment caused significant increase of the level of phosphorylated ERK in mouse cardiomyocytes. Therefore, it is making ATO a potential candidate agent to protect against HIRI via ERK and AKT signaling. Several studies have shown that ATO is a potent inducer of autophagy.[38,39] Meanwhile, it was also reported that the activation of autophagic flux exerted an hepatoprotective effects on I/R induced liver injury in mice and even in human.[40] Thus, we hypothesized that ATO may be a promising agent for the treatment of liver IRI by activating both ERK and AKT signaling pathway.

In this work, ATO preconditioning dramatically induced ERK and AKT signaling, which alleviated liver damage and reduced inflammatory response during HIRI. Hepatocyte apoptosis and regeneration are regarded as two hallmarks of HIRI. In the early phase of reperfusion, the release of cytokines/chemokines and ROS accumulation directly trigger hepatocyte death, while

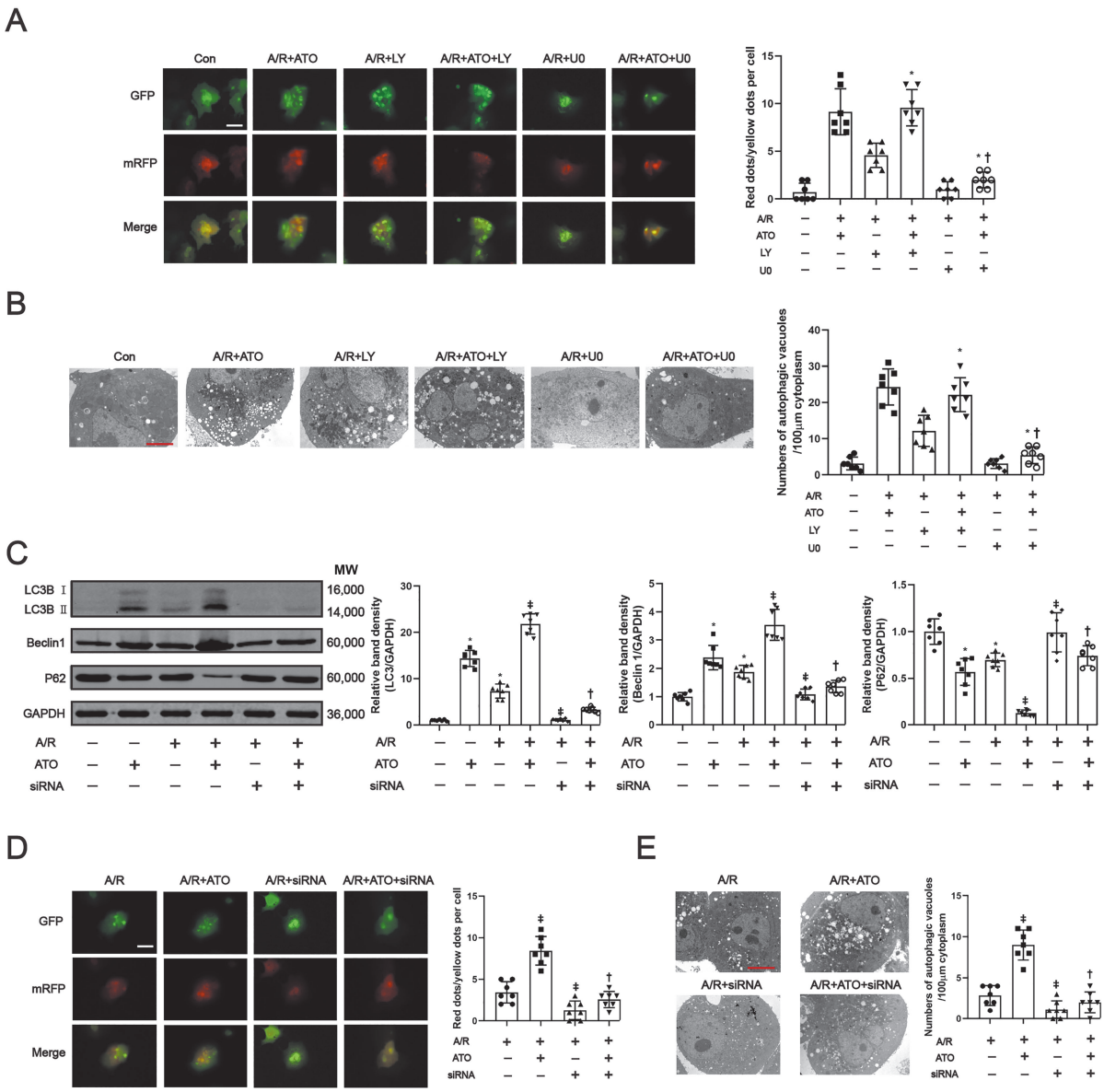

Figure 6: The ERK pathway dominates the ATO-induced activation of autophagy. (A) Primary hepatocytes transfected with mRFP-GFP-LC3 adenovirus were determined by fluorescent puncta after A/R (6h) treatment and ATO, U0126 and LY294002 preconditioning. The scale bar was 10 μm. (B) Representative electron micrographs showing autophagosomes in primary hepatocytes after A/R (6h) treatment and ATO, U0126, and LY294002 preconditioning and the quantification of the number of autophagosomes in 100 μm cytoplasm. The scale bar was 5 μm. (C) Western blot analysis of LC3, Beclin 1, and P62 after A/R (6h) treatment and ATO, *siERK1/2* (siRNA) preconditioning and relative band density. (D) Primary hepatocytes transfected with mRFP-GFP-LC3 adenovirus were determined by fluorescent puncta after A/R treatment and ATO and siRNA preconditioning. The scale bar was 10 μm. (E) Representative electron micrographs showing autophagosomes in primary hepatocytes after A/R (6h) treatment and ATO and siRNA preconditioning. The scale bar was 5 μm. All statistical analyses were performed by Student's *t*-test. * means $P<0.05$ when compared with blank; ‡ means $P<0.05$ when compared with NSS-I/R (A/R) group; † means $P<0.05$ when compared with compared with NSS-I/R (A/R) + ATO group. A/R: Anoxia/reoxygenation; ATO: Arsenic trioxide; ERK: Extracellular signal-regulated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; mRFP-GFP-LC3: Tandem fluorescent-tagged LC3; MW: Molecular weight; siRNA: Small interfering RNA.

in the late periods of reperfusion, the regenerative capacities of hepatocytes contribute to liver repair and further improve liver function.[41] In view of the cell survival and proliferation maintained by ERK/PI3K-AKT signaling and the activation of those two pathways by ATO, our results demonstrated that ATO might attenuate liver IRI and exert significant pro-proliferative effects in the late stages of HIRI to promote liver recovery.

Descriptions of the roles of autophagy in cell growth have been controversial. Recent reports have focused on the crosstalk between ATO and autophagy, elucidating the antiapoptotic details of autophagy following ATO pretreatment. Hsin *et al*[42] found that the combined application of ATO and ABT-737 (a target agent activating cell apoptosis) promoted autophagic flux, which ameliorated the antiapoptotic effects of ABT-737 on uterine cervical cancer. In contrast, ATO treatment also exhibited a strong inhibitory activity in glioma progression by activating autophagy and cell apoptosis.[30] To the best of our knowledge, autophagy is considered to be a metabolic process involved in cell survival, particularly in HIRI,[43] and ATO is known as an autophagic activator.[15,44] Hence, we hypothesized that ATO treatment

would enhance cell viability by promoting autophagy. This study reveal that hepatocytes undergo both cell survival and autophagy when exposed to ATO under I/R injury. We observed that the administration of ATO could significantly promote autophagy, which exerted great hepatoprotective properties in HIRI.

The relationship between autophagy activation and ERK/PI3K-AKT pathways is complicated, for example, ERK signaling activation can markedly promote autophagy,[30,45] whereas, in contrast, PI3K-AKT signaling is commonly characterized by autophagy suppression.[30,46] While it was also reported that class IA PI3K p110β subunit could promote cellular autophagy through small GTPase when ROS was accumulated.[47] Our data have demonstrated a coactivation of ERK/PI3K-AKT pathways and autophagy, which is likely incompatible with previous findings. Nevertheless, rescue experiments showed that autophagy activity could be abrogated only with the administration of U0126 and ERK1/2 siRNA, indicating that ERK-dependent autophagy dominates the hepatoprotective effect of ATO on I/R injury [Figure 7].

In summary, we found that ATO pretreatment can alleviate I/R-induced liver injury and promote liver proliferation both *in vivo* and *in vitro*. Our results uncover that the ERK and PI3K-AKT signaling pathways, which improve liver function and reduce cell apoptosis can be activated by ATO exposure during HIRI. Additionally, our data suggest that the protective effects of ATO are autophagy dependent and are activated by the ERK pathway.

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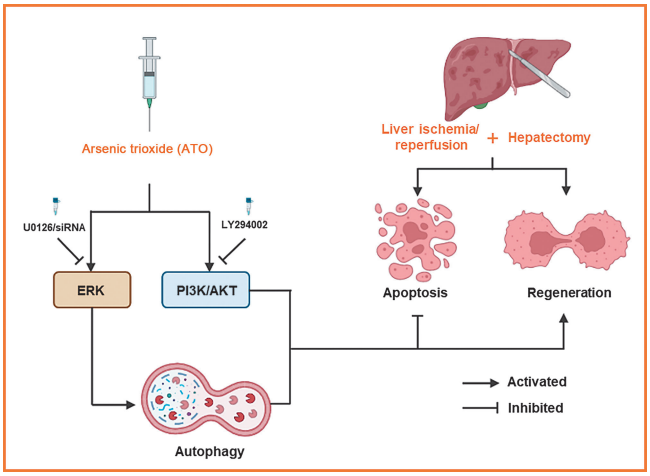

Figure 7: Graphical abstract of the arsenic trioxide preconditioning attenuates hepatic ischemia-reperfusion injury by activating the ERK and PI3K/AKT signaling pathways. ERK: Extracellular signal-regulated protein kinase; PI3K/AKT: Phosphoinositide 3-kinase-serine/threonine kinase; siRNA: Small interfering RNA.

Conflicts of interest

None.

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