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Gas6/AXL Alleviates Hepatic Ischemia/Reperfusion Injury by Inhibiting Ferroptosis via the PI3K/AKT Pathway

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Background. Hepatic ischemia/reperfusion (I/R) injury is a major cause of complications in clinical liver surgery. AXL receptor tyrosine kinase (AXL) is a member of the TAM receptor tyrosine kinase family (TYRO3, AXL, and MERTK). Our previous study has shown that AXL expression was markedly upregulated in liver transplantation patients. However, the underlying mechanism of AXL in hepatic I/R injury remains unclear. **Methods.** A mouse liver warm I/R model and a primary hepatocyte hypoxia/reoxygenation model were established to investigate the role of AXL activation and ferroptosis in hepatic I/R injury by pretreating with recombinant mouse growth arrest-specific protein 6 (AXL activator) or R428 (AXL inhibitor). Moreover, we used LY294002 (phosphatidylinositol 3-kinase [PI3K] inhibitor) to evaluate the relationship between the PI3K/AKT (the Ser and Thr kinase AKT) pathway and ferroptosis in hepatic I/R injury. **Results.** Hepatic I/R injury decreased phosphorylation AXL expression and enhanced ferroptosis in liver transplantation patients and hepatic I/R-subjected mice. AXL activation attenuated lipid peroxidation and ferroptosis in hepatic I/R injury in vivo and in vitro. Inhibition of AXL activation exacerbated liver pathological damage and liver dysfunction, as well as iron accumulation and lipid peroxidation in hepatic I/R injury. Mechanistically, activated growth arrest-specific protein 6/AXL and its downstream PI3K/AKT signaling pathway inhibited ferroptosis during hepatic I/R injury. **Conclusions.** AXL activation protects against hepatic I/R injury by preventing ferroptosis through the PI3K/AKT pathway. This study is the first investigation on the AXL receptor and ferroptosis, and activating AXL to mitigate ferroptosis may be an innovative therapeutic strategy to combat hepatic I/R injury.

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INTRODUCTION

Hepatic ischemia/reperfusion (I/R) injury is one of major cause of complications in a range of liver surgeries, including hepatectomy, liver transplantation, and trauma, which can induce severe liver dysfunction or functional failure and a range of multiple organ dysfunctions. Cells and factors involved in hepatic I/R injury include cell death, mitochondria, anaerobic metabolism, oxidative stress, intracellular calcium overload, and inflammation. Hepatocyte death plays a crucial role in the occurrence and progress of hepatic I/R injury.[1,2]

Ferroptosis is a nonapoptotic regulated cell death associated with oxidative damage,[3] which is characterized by the accumulation of redox-active iron and lipid peroxides.[4,5] Growing evidence shows that ferroptosis has been implicated in the occurrence and progression of various diseases, such as drug-resistant cancers[6] and neurodegenerative diseases, ischemic organ injury,[7-9] and other degenerative

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J.D. and L.C. designed the study. M.Z. and D.L. performed the experiments. L.Y. performed data analysis. M.Z. produced the initial draft of the article. W.W., R.Z., Y.X., and Z.W. drafted the article. Q.Y. and Q.F. revised the article. All authors have read and approved the final submitted article.

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diseases associated with extensive lipid peroxidation.[10] Impaired iron homeostasis triggers ferroptosis, which plays a key role in the pathogenesis of various diseases. The liver plays a critical role in iron homeostasis, and iron is stored in hepatocytes as ferritin in a soluble nontoxic form, the ferritin includes ferritin light chain and ferritin heavy chain 1 (FTH1).[11] Transferrin receptor 1 (TFR1) is a key regulator of ferroptosis,[12] and increased iron uptake and decreased iron stores may lead to iron overload and subsequent ferroptosis. In addition, lipid peroxidation severely disrupts cell function, and the imbalance between the generation and degradation of lipid reactive oxygen species (ROS) induced the occurrence of ferroptosis.[13] Ferroptosis can be antagonized by lipophilic radical trapping, iron depletion, and antioxidants by decreasing glutathione and malondialdehyde (MDA) levels, increasing glutathione peroxidase 4 (GPX4) activity, or using iron chelation such as vitamin E, liproxstatin-1, ferrostatin-1 (Fer-1), or ubiquinone.[14] Studies have suggested that ferroptosis plays an on-off switching regulatory role in I/R injury of different organs, such as the kidney,[8] heart,[15] intestines,[9] and lung.[16] Furthermore, a recent study suggested that ferroptosis plays a major role in postoperative liver injury compared with other programmed cell death pathways, such as apoptosis, necrosis, and pyroptosis.[17] Given that the liver is the main site for iron and lipid metabolism, metabolic disturbances induced by hepatic I/R can activate ferroptosis.[17,18] Therefore, ferroptosis is considered a potential therapeutic target for hepatic I/R injury.[19,20]

AXL receptor tyrosine kinase (AXL) is a member of the TAM receptor tyrosine kinases family, which includes TYRO3, AXL, and MERTK.[21] AXL and other TAM receptor tyrosine kinases can be activated by ligands including growth arrest-specific protein 6 (Gas6), protein S, Tubby, Tubby-like protein 1, and Galectin-3, among which Gas6 exhibits the highest affinity for the AXL receptor. AXL consists of a transmembrane, extracellular, and intracellular kinase domain.[15] The Gas6/AXL signaling pathway activates numerous critical downstream pathways, such as nuclear factor kappa B, PI3K (phosphatidylinositol 3-kinase)/AKT (the Ser and Thr kinase AKT)/mTOR (mechanistic target of rapamycin), RAS/RAF/MEK (mitogen-activated and extracellular signal-regulated kinase)/ERK (extracellular signal-regulated kinase), and JAK (Janus kinase)/STAT (signal transducer and activator of transcription), which contribute to protumoral processes, including cancer cell growth, migration, survival, apoptosis, and invasion.[22,23] In our group's previous studies, proteomic analysis through quantitative high-throughput mass spectrometry has shown that AXL expression is upregulated in human hepatectomy with I/R injury.[2] Our recent research has suggested that AXL activation attenuates inflammation and apoptosis during hepatic I/R injury.[24] This is noteworthy, because recent study has revealed that R428, which is a specific inhibitor of AXL, is critical in inhibiting cell growth through ferroptosis induction in metastatic melanoma cells.[25] However, it remains unclear how ferroptosis occurs in hepatocytes during hepatic I/R injury. Therefore, we hypothesized that AXL activation attenuates ferroptosis during hepatic I/R injury.

In this study, we found that AXL activation significantly attenuated ferroptosis during hepatic I/R injury via the PI3K/AKT signaling pathway. The study suggests that AXL activation may be a novel therapeutic strategy for suppressing ferroptosis during hepatic I/R injury.

MATERIALS AND METHODS

Human Samples

A total of 30 liver transplant grafts were enrolled between September 2018 and August 2022 in The First Affiliated Hospital of Anhui Medical University. The human liver transplant grafts were biopsied after liver transplantation 3 h after reperfusion (before abdominal closure). Control liver samples were obtained from patients with hepatic hemangiomas who underwent hepatectomy. All experiments were approved by The Review Committee of the First Affiliated Hospital of Anhui Medical University, Anhui Province, China (No. 20180145). All patients and their family members provided written informed consent.

Animals

Male C57 mice (6–8 wk, 23–26 g) were produced by the Anhui Experimental Animal Center (Hefei, China). The mice were housed in an independent ventilation system with appropriate temperature, regulated light and provided adequate food and water. The Ethics Committee of Anhui Medical University approved the procedures for the care and use of experimental animals, and we followed all applicable institutional and governmental regulations concerning the ethical use of animals.

Murine Hepatic I/R Model

The mouse liver ischemia model was established as previously described.[26] After 60 min of liver ischemia, followed by reperfusion at various time points. The mice were divided into the following groups: Sham, I/R (0 h), I/R (3 h), I/R (6 h), I/R (12 h), and I/R (24 h). The mice were euthanized after being anesthetized, and liver tissues and serums were obtained for analysis. The Sham-operated mice underwent the same protocol except that the vessels were not clipped. The endogenous AXL activator recombinant mouse growth arrest-specific protein 6 (rmGas6) (5 μg), the AXL specific inhibitor R428 (125 mg/kg) and PBS (vehicle) were intraperitoneally injected 2 h before liver ischemia. Ferroptosis inhibitor Fer-1 (10 mg/kg) dissolved in a solution (5% Tween-80 + 10% dimethyl sulfoxide + 40% polyethylene glycol 300 + 45% saline) and was intraperitoneally injected 60 min before liver ischemia, and dimethyl sulfoxide (vehicle) was administered 1 h before liver ischemia, and then reperfusion for 6 h. The specific experimental groups were as follows (n = 4–6): (1) Sham, I/R, I/R + vehicle, and I/R + Fer-1 (10 mg/kg) and (2) Sham, I/R, I/R + vehicle, I/R + rmGas6 (5 μg), I/R + R428 (125 mg/kg), and I/R + rmGas6 + R428. The PI3K/AKT pathway inhibitor LY294002 (10 mg/kg, MedChemExpress [MCE]) was intraperitoneally administered 36 h before liver ischemia and then reperfusion for 6 h. The experimental mice were randomly divided into 6 groups (n = 4–6): Sham, I/R, I/R + vehicle, I/R + rmGas6 (5 μg), I/R + LY294002 (10 mg/kg), and I/R + rmGas6 + LY294002. RmGas6 (R&D Systems, 8310-GS, United States), Fer-1 (MCE, HY-100579, United States), R428 (MCE, HY-15150, United States), and LY294002 (MCE, HY-10108, United States).

Primary Hepatocyte Hypoxia/Reoxygenation Model

Primary hepatocytes were extracted from 6- to 8-wk-old male mice as described in previous article[27] and

were then cultured in 1640 medium for 24 h. The primary hepatocytes were cultured in serum/glucose-free DMEM incubator 5% CO_2 and 95% N_2 at 37 °C for 1 h and then transferred to a normal culture environment and medium for various times to determine the appropriate reoxygenation time point. rmGas6 or R428 pretreatment was performed for 2 h, and LY294002 was added 1.5 h before rmGas6 pretreatment and then reoxygenated for 6 h. The primary hepatocytes were grouped as follows (n = 4–6): (1) Control, hypoxia/reoxygenation (H/R) (0 h), H/R (3 h), H/R (6 h), H/R (12 h), and H/R (24 h); (2) Control, H/R, H/R + vehicle, H/R + rmGas6 (200 ng/mL), H/R + R428 (200 nmol/L), and H/R+ rmGas6 (200 ng/mL) + R428 (200 nmol/L); and (3) Control, H/R, H/R+ vehicle, H/R+ rmGas6 (200 ng/mL), H/R + LY294002 (10 μM), and H/R + rmGas6 (200 ng/mL) + LY294002 (10 μM).

Analysis of Serum Samples

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected by commercial kit (Nanjing Jiancheng, China).

Histopathology and Immunohistochemistry

Liver sections were stained with hematoxylin and eosin. Immunohistochemical staining for 4-Hydroxynonenal (4-HNE) (R&D Systems) and phosphorylation AXL (p-AXL) (Cell Signaling Technology) was performed on the paraffin sections.

Western Blotting

Primary hepatocytes and liver tissues were lysed to prepare protein samples. The proteins were separated by SDS-PAGE and transferred to the nitrocellulose filter membrane. The membrane was incubated with primary antibody at 4 °C overnight. The antibodies are listed in Table 1. Then, the membrane was incubated with secondary antibody (1: 10000, Proteintech).

Immunofluorescence Analysis

Primary hepatocytes were exposed to hypoxia for 1 h followed by reoxygenation for 6 h. Before hypoxia, the hepatocytes were pretreated with either the AXL activator rmGas6 or the AXL inhibitor R428 for 2 h. The hepatocytes were incubated with GPX4 antibody at 4 °C overnight, and incubated with secondary antibodies for 1 h at 37 °C. The slides were imaged by a fluorescence microscope (Olympus DX51, Tokyo, Japan).

Transmission Electron Microscope

Liver samples were processed as previously described[28] and were observed using a transmission electron microscope (TEM).

Measurement of MDA Levels

MDA levels in liver tissues and hepatocytes were assessed by an MDA Assay Kit (S0131, Beyotime, China) according to the manufacturer's instructions.

TABLE 1.

The antibody information

Antibody name	Brand	Country	Catalog number	Dilution
AXL	Proteintech	China	13196-1-AP	1:1000
AXL	CST	United States	No. 8661	1:1000
p-AXL	CST	United States	No. 5724	1:500
TFR1	Affinity	China	AF5343	1:1000
FTH1	Affinity	China	DF6278	1:1000
ACSL4	Affinity	China	DF12141	1:1000
GPX4	Affinity	China	DF6701	1:1000
SLC7A11	Duoning-BIO	China	AB0138101	1:500
p-PI3K	Proteintech	China	AF3242	1:1000
PI3K	Wanleibio	China	WL03380	1:1000
p-AKT	Zenbio	China	381555	1:1000
AKT	Wanleibio	China	WL0003b	1:1000
4-HNE	R&D systems	United States	MAB3249	1:100
β-Actin	Proteintech	China	66009-1-Ig	1:1000

4-HNE, 4-hydroxynonenal; ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; AKT, the Ser and Thr kinase AKT; AXL, AXL receptor tyrosine kinase; CST, Cell Signaling Technology; FTH1, ferritin heavy chain 1; GPX4, glutathione peroxidase 4; p-AKT, phospho-AKT; p-AXL, phosphorylation AXL; PI3K, phosphatidylinositol 3-kinase; p-PI3K, phosphorylation phosphatidylinositol 3-kinase; SLC7A11, solute carrier family 7 member 11; TFR1, transferrin receptor 1.

Measurement of Iron Content

After hepatic I/R, fresh liver tissues were immediately collected, and liver iron content was measured by an iron assay kit (Nanjing Jiancheng, China). An iron colorimetric assay kit (Beijing Applygen, China) was used to measure levels of Fe^{2+} in hepatocytes.

Iron Staining

Liver tissues were stained with Prussian Blue by using an iron staining kit (Prussian Blue Stain, Solarbio).

Measurement of Reactive Oxygen Species

Primary hepatocytes were exposed to hypoxia for 1 h followed by reoxygenation for 6 h, and pretreated with the AXL activator rmGas6 or AXL inhibitor R428 for 2 h before H/R. The levels of ROS in hepatocytes were measured by the ROS assay kit (S0033, Beyotime, China). Hepatocytes were incubated with 10 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate for 30 min and observed by an inverted fluorescence microscope (Axio Observer 3, Germany).

Lipid Peroxidation Assay

Hepatocytes were cultured at 37 °C and pretreated with rmGas6 or R428 for 2 h before H/R. The hepatocytes were incubated with boron dipyrromethene C11 (ABclonal, RM02821) at 37 °C for 1 h, and the images were analyzed by measuring the excitation and emission at wavelengths of 460–495 nm and 510–550 nm, respectively, using flow cytometry (Beckman Coulter CytoFlex) and a fluorescence microscope (Olympus DX51, Tokyo, Japan).

Statistical Analysis

All data were presented as the mean ± SD and analyzed using GraphPad Prism software. Paired Student's *t* test or unpaired 2-tailed Student's *t* test were used to analyze the statistical significance between different groups. $P \leq 0.05$ was considered statistically significant.

RESULTS

Hepatic I/R Injury Decreases p-AXL Expression and Enhances Ferroptosis Activation

To investigate the potential role of p-AXL in hepatic I/R injury, we examined the expression of p-AXL in liver transplant grafts from liver transplant recipients and healthy livers, immunohistochemistry results showed that the level of p-AXL expression was downregulated in liver transplant grafts compared with healthy livers (Figure 1A). Therefore, liver transplant grafts were categorized into low and high p-AXL expression groups based on Western blotting analysis (**Figure S1A, SDC**, http://links.lww.com/TP/D48). Furthermore, we evaluated the correlation between p-AXL levels and liver damage in human liver transplant recipients after liver transplantation (n = 30). We found that serum transaminases (ALT, AST) within 48 h after liver transplantation were significantly lower in the high p-AXL group, suggesting less liver injury and better liver function after liver transplantation (**Figure S1B, SDC**, http://links.lww.com/TP/D48). These data implied that p-AXL may shed significant light on hepatic I/R injury. Interestingly, the cohort with lower levels of p-AXL expression had significantly lower levels of ferroptosis-related proteins FTH1, GPX4, and solute carrier family 7 member 11. The cohort with higher levels of p-AXL expression had lower levels of ferroptosis-related proteins TFR1 and ACSL4 (Figure 1B). The proteins TFR1 and FTH1 are key indicators of iron metabolism, and ACSL4 and GPX4 are regulators of lipid metabolism. There is some association between p-AXL expression and ferroptosis in liver transplant grafts as shown by Western blotting. To further explore the role of ferroptosis in hepatic I/R injury, mice were subjected to hepatic ischemia for 60 min, and then followed by reperfusion for various times. AXL expression increased, whereas p-AXL levels decreased with increasing reperfusion times and peaked at 6 h of reperfusion. The levels of ferroptosis-related proteins also changed significantly at 6 h of reperfusion (Figure 1C). In addition, the expression of p-AXL markedly reduced in hepatocytes after exposed to H/R. Correspondingly, increase in TFR1 and ACSL4 and decrease in FTH1 and GPX4, particularly after 6 h of reoxygenation, indicating that the induction of ferroptosis (Figure 1D). These results indicate that p-AXL may be involved in the pathophysiological process of hepatic I/R, which may be associated with ferroptosis.

Ferroptosis Is Involved in Hepatic I/R Injury

The Fer-1 treatment group exhibited a reduction in the area of liver necrosis and lower serum levels of ALT and AST after hepatic I/R injury (Figure 2A and B). The Western blotting results showed that TFR1 and ACSL4 protein levels were upregulated, whereas FTH1, GPX4, and solute carrier family 7 member 11 were downregulated in hepatic I/R mice. However, the Fer-1 treatment reversed these effects (Figure 2C). The levels of liver iron were characteristically increased in I/R mice, but this effect was reversed by Fer-1 (Figure 2D). To characterize the hallmarks of ferroptosis during hepatic I/R injury, the ultrastructure of liver tissue was detected by TEM. Cytoplasmic expansion was observed, and the cytoplasm became electron lucent, the cristae of mitochondria were destroyed and the mitochondria exhibited high electron density in liver tissues after liver I/R injury. However, those mitochondrial changes were suppressed by Fer-1 (Figure 2E). Mitochondrial damage was consistent with previous reports.[3] These findings suggest that ferroptosis is involved in the development of hepatic I/R injury.

AXL Activation Attenuates Lipid Peroxidation in Hepatic I/R Injury In Vivo

AXL is a member of the TAM receptor tyrosine kinases family, which can be activated by its high-affinity ligand Gas6. The expression of p-AXL was decreased in hepatic I/R injury (Figure 1A); therefore, we used rmGas6, the endogenous AXL activators, to activate AXL. Hepatic I/R injury caused liver damage and elevated serum levels of ALT and AST compared with the Sham-operated group, rmGas6 treatment reversed these effects. However, pretreatment with R428 significantly exacerbated the area of liver necrosis and elevated serum levels of ALT and AST (Figure 3A and B). Moreover, 4-HNE and MDA are both secondary products of lipid peroxidation. The expression of 4-HNE and the MDA content of liver tissues were significantly elevated in liver I/R mice, pretreatment with rmGas6 reversed these effects (Figure 3C and D). These results indicated that lipid peroxidation is involved in hepatic I/R injury, and the activation of AXL by rmGas6 protects against hepatic I/R injury by attenuating lipid peroxidation.

AXL Activation Alleviates Iron Accumulation to Protect Against Hepatic I/R Injury In Vivo

Iron is an essential micronutrient for most life. However, iron overload can damage the organism by producing ROS through Fenton reactions, which can lead to cell death.[29] Ferroptosis, an iron-dependent regulatory form of cell death, is characterized primarily by iron overload and lipid peroxidation.[30] The maintenance of intracellular iron homeostasis depends on the balanced expression of ferritin and TFR.[31] Our results showed that the p-AXL expression level in the liver tissues was markedly decreased during hepatic I/R, accompanied by increased ACSL4, TFR1 and diminished FTH1, and GPX4 protein levels. When AXL was activated with rmGas6, the increase of p-AXL expression indicated that AXL was activated. AXL activation restored the levels of FTH1 and GPX4 proteins while decreasing the expression of ACSL4 and TFR1 after hepatic I/R (Figure 4A). Furthermore, iron overload exacerbated ferroptosis during hepatic I/R injury. Thus, liver iron content of mice was detected to evaluate the levels of iron overload. Liver iron contents were significantly elevated after liver I/R injury, which could be reversed by rmGas6 treatment (Figure 4B and C). In addition, we examined ultrastructural architecture in liver tissues by TEM, which is a recently identified criterion of ferroptosis in vivo.[31] TEM results revealed that the cristae of mitochondria were destroyed and the mitochondria exhibited high electron density after liver I/R injury, but rmGas6 treatment reversed this effect (Figure 4D). These results indicate that iron metabolism contributes a lot to the pathophysiological process of hepatic I/R injury, and AXL activation by rmGas6 partially reduces iron accumulation during hepatic I/R injury.

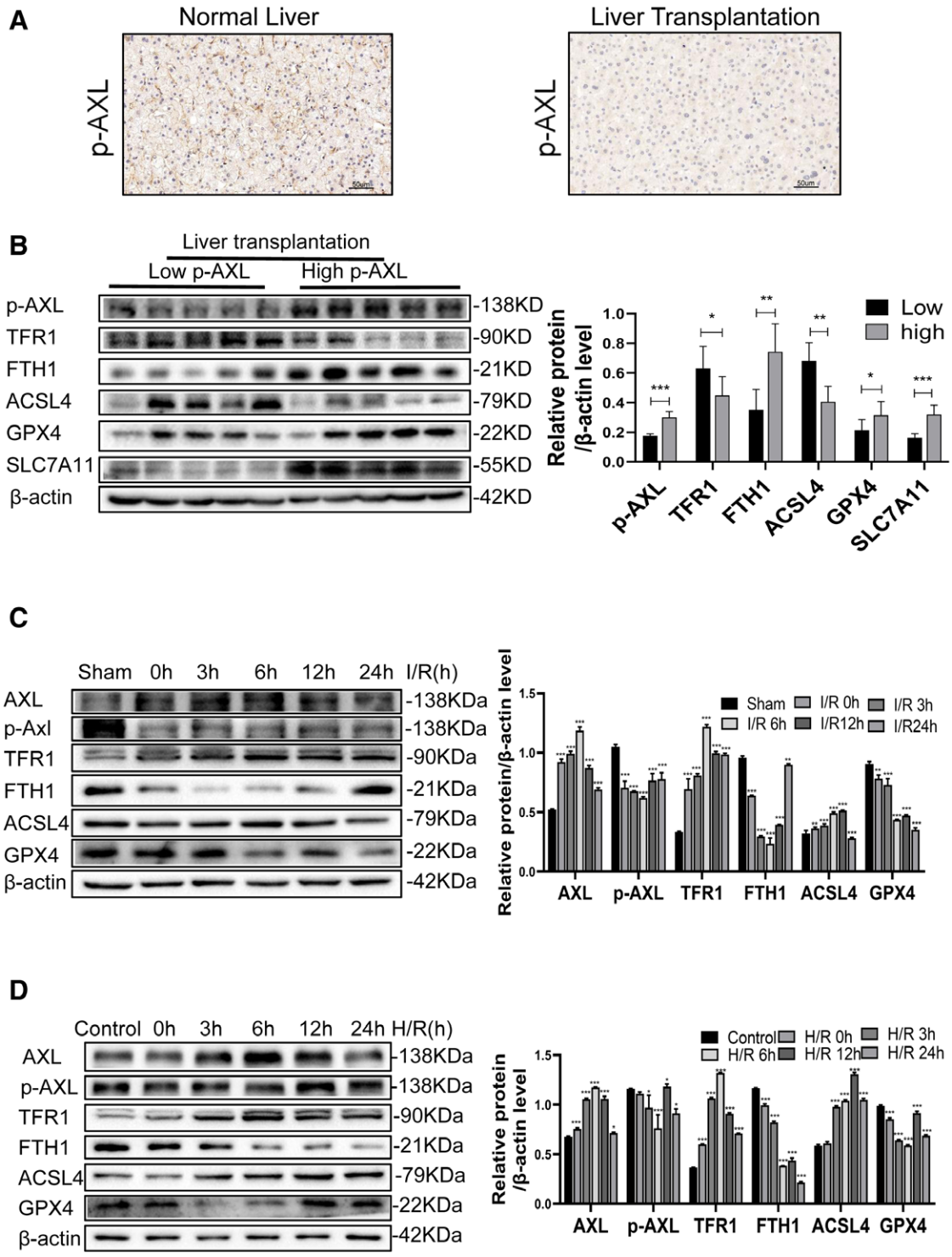

FIGURE 1. Hepatic I/R injury decreases p-AXL expression and enhances ferroptosis activation. A, Immunohistochemistry was used to show the expression of p-AXL in human liver transplantation samples (n = 5) and healthy liver samples (n = 3). B, The expressions of p-AXL and ferroptosis-related proteins, including TFR1, FTH1, ACSL4, GPX4, and SLC7A11 in liver transplantation grafts were detected by Western blotting (n = 30). C, Western blot analysis of the expression of p-AXL and ferroptosis-related proteins levels after 1h of liver ischemia and subsequent reperfusion for various times in mice (n = 4–6). D, Western blot analysis of the levels of p-AXL and ferroptosis-related proteins after 60 min hypoxia and reoxygenation for various times in primary hepatocytes (n = 4–6). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; FTH1, ferritin heavy chain 1; GPX4, glutathione peroxidase 4; I/R, ischemia/reperfusion; p-AXL, phosphorylation AXL; SLC7A11, solute carrier family 7 member 11; TFR1, transferrin receptor 1.

AXL Activation Ameliorates Hepatocyte Ferroptosis in H/R Injury

The expression of p-AXL was decreased in hepatocytes after H/R injury. When AXL was activated with rmGas6, the increase of p-AXL expression indicated that AXL was activated. rmGas6 treatment before H/R diminished the expression of TFR1 and ACSL4 and restored the expression levels of FTH1 and GPX4 compared with the H/R

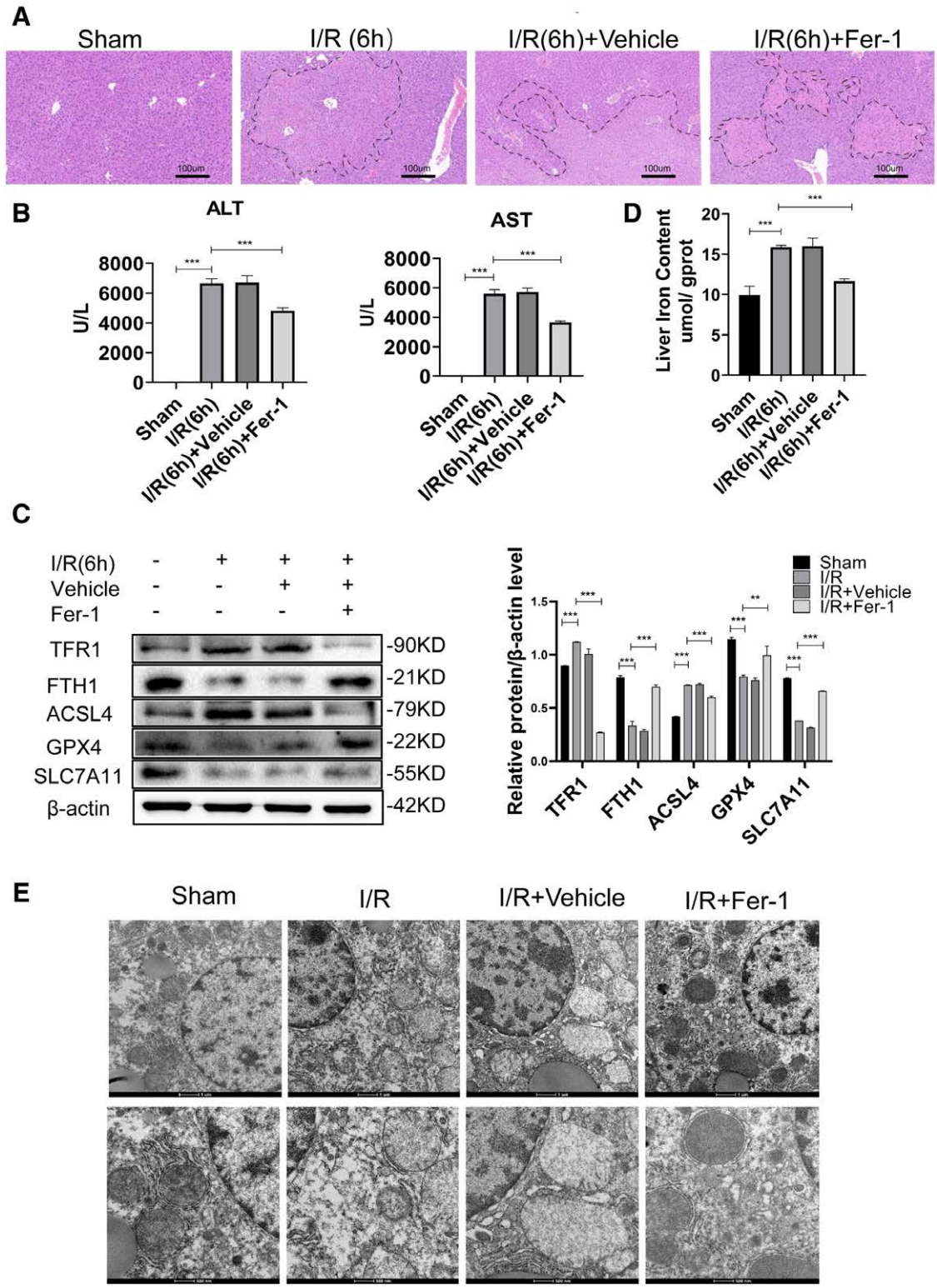

FIGURE 2. Ferroptosis is involved in hepatic I/R injury. Mice were pretreated with or without Fer-1 for 1 h by intraperitoneal injection and then subjected to 60 min of liver ischemia and reperfusion for 6 h. A, H&E staining showing liver damage after hepatic I/R injury with or without Fer-1 treatment. B, Serum levels of ALT and AST in livers of I/R mice with or without Fer-1 treatment. C, The expression of ferroptosis-related proteins (TFR1, FTH1, ACSL4, GPX4, and SLC7A11) in liver tissues was determined by Western blotting. D, Liver iron contents in mice were detected. E, Representative TEM images of mitochondria in the liver tissues of mice. A–D, Each group n = 4–6. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Fer-1, ferrostatin-1; FTH1, ferritin heavy chain 1; GPX4, glutathione peroxidase 4; H&E, hematoxylin and eosin; I/R, ischemia/reperfusion; SLC7A11, solute carrier family 7 member 11; TEM, transmission electron microscope; TFR1, transferrin receptor 1.

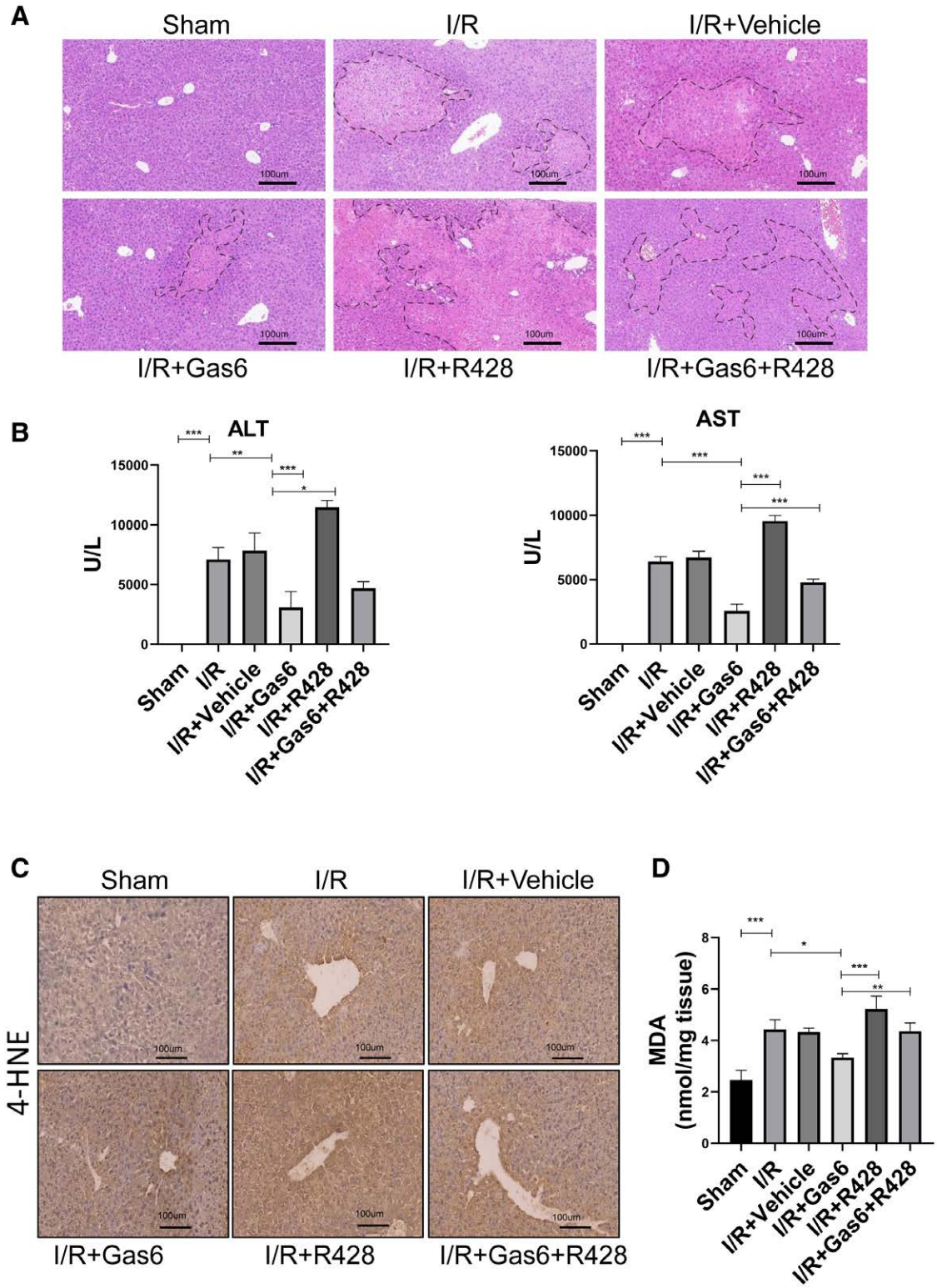

FIGURE 3. AXL activation attenuates lipid peroxidation in hepatic I/R injury in vivo. The mice underwent 1 h of liver ischemia followed by 6 h of reperfusion. AXL activator rmGas6 (5 μg), AXL inhibitor R428 (125 mg/kg), and PBS (vehicle) were intraperitoneally injected 2 h before liver ischemia. Mice were euthanized, and liver tissues and serums were collected for analysis. A, The area of liver necrosis was measured by H&E staining in the Sham and treatment mice after liver I/R injury. B, Serum levels of ALT and AST in Sham and I/R mice with or without rmGas6 or R428 treatment. C, Immunohistochemical analysis showing the expression of 4-HNE in liver tissues after I/R. D, MDA levels in liver tissues were determined by an assay kit. A–D, Each group n = 4–6. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. 4-HNE, 4-hydroxynonenal; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AXL, AXL receptor tyrosine kinase; Gas6, growth arrest-specific protein 6; I/R, ischemia/reperfusion, MDA, malondialdehyde.

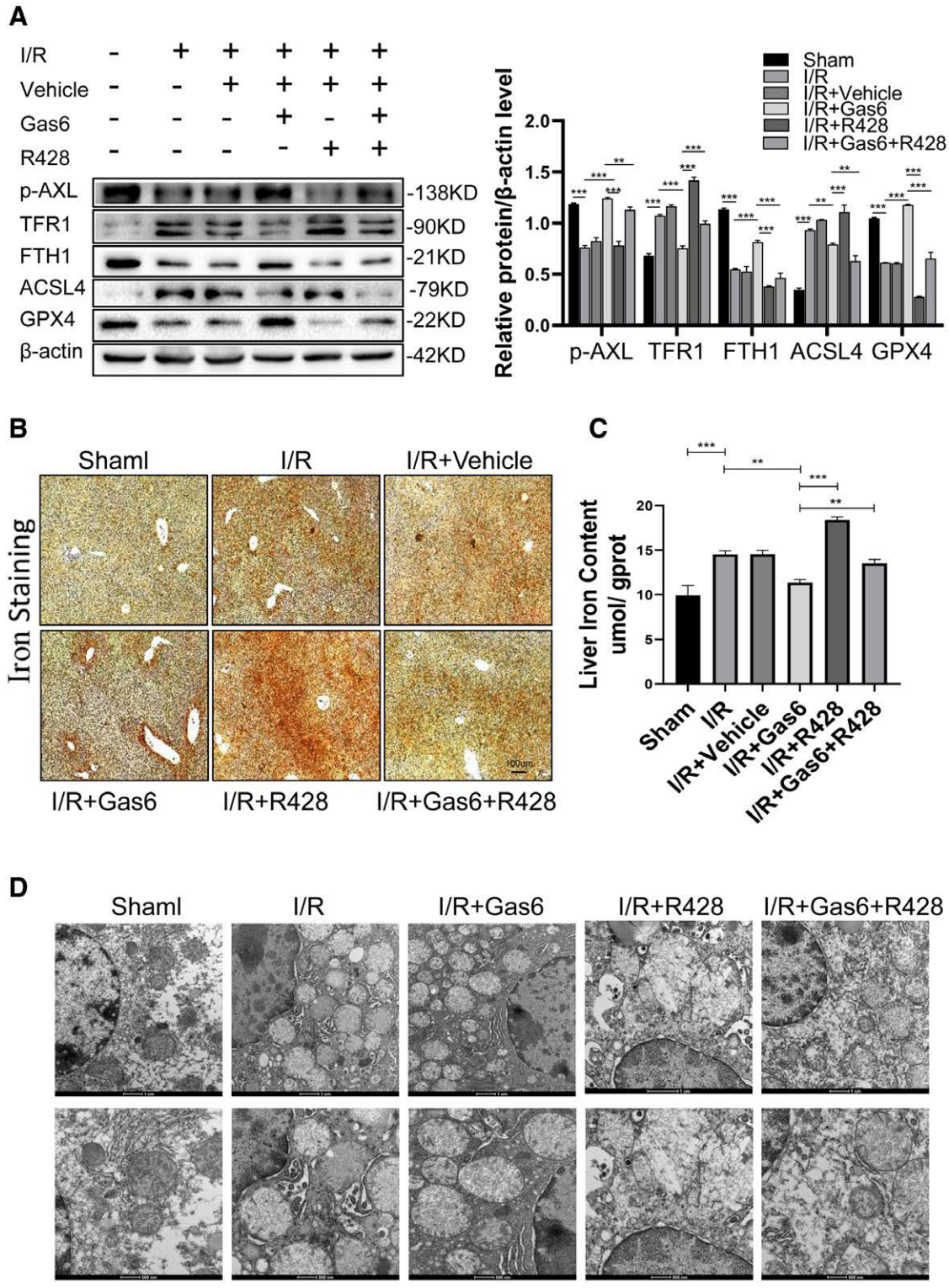

FIGURE 4. AXL activation alleviates iron accumulation to protect against hepatic I/R injury in vivo. A, Western blotting was used to assess the levels of the ferroptosis proteins TFR1, FTH1, ACSL4, and GPX4 after hepatic I/R injury in Sham and treatment groups. B and C, Iron stain and liver iron levels were examined by an assay kit after hepatic I/R injury in the Sham and rmGas6-treated group. D, TEM was used to detect the levels of mitochondrial damage after hepatic I/R injury in the Sham group and the treated group. A–D, Each group n = 4–6. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; AXL, AXL receptor tyrosine kinase; FTH1, ferritin heavy chain 1; Gas6, growth arrest-specific protein 6; GPX4, glutathione peroxidase 4; I/R, ischemia/reperfusion; p-AXL, phosphorylation AXL; rmGas6, recombinant mouse growth arrest-specific protein 6; TEM, transmission electron microscope; TFR1, transferrin receptor 1.

group. Conversely, inhibition of AXL activation by R428, a specific inhibitor of AXL, significantly reversed these effects (Figure 5A). Iron-catalyzed production of ROS is a crucial factor induced liver injury after I/R.[32] ROS production in hepatocytes were significantly increased after H/R, activation of AXL by rmGas6 significantly decreased

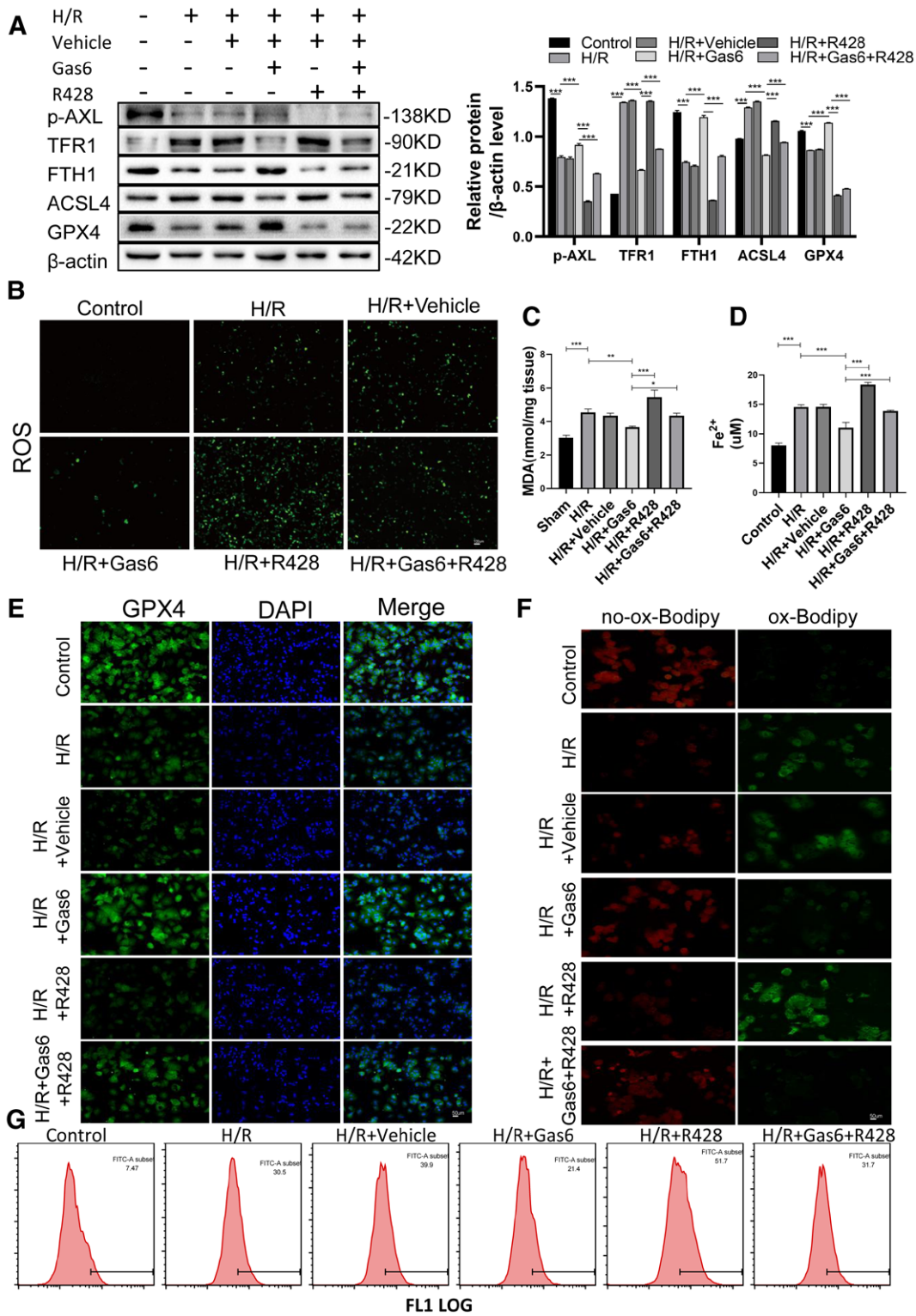

FIGURE 5. AXL activation ameliorates hepatocyte ferroptosis in H/R injury. Mouse primary hepatocytes were exposed to hypoxia for 1 h followed by reoxygenation for 6 h and pretreated with the AXL activator rmGas6 or the AXL inhibitor R428 for 2 h before H/R. A, Western blotting was used to assess the levels of the ferroptosis factors TFR1, FTH1, ACSL4, and GPX4 after H/R in control and treated hepatocytes. B, ROS levels in hepatocytes were measured by upright fluorescence microscope. C, The levels of MDA in hepatocytes were assessed by an assay kit. D, The levels of Fe^{2+} in hepatocytes. E, Representative immunofluorescence images of the GPX4 expression in hepatocytes exposed to H/R. F and G, The accumulation of lipid peroxidation in hepatocytes was analyzed by BODIPY staining, followed by immunofluorescence imaging and flow cytometry analysis, respectively. A–G, Each group n = 4–6. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; AXL, AXL receptor tyrosine kinase; BODIPY, boron dipyrromethene; DAPI, 4′,6-diamidino-2-phenylindole; FITC-A, fluorescein isothiocyanate-A; FTH1, ferritin heavy chain 1; Gas6, growth arrest-specific protein 6; GPX4, glutathione peroxidase 4; H/R, hypoxia/reoxygenation; MDA, malondialdehyde; p-AXL, phosphorylation AXL; rmGas6, recombinant mouse growth arrest-specific protein 6; ROS, reactive oxygen species; TFR1, transferrin receptor 1.

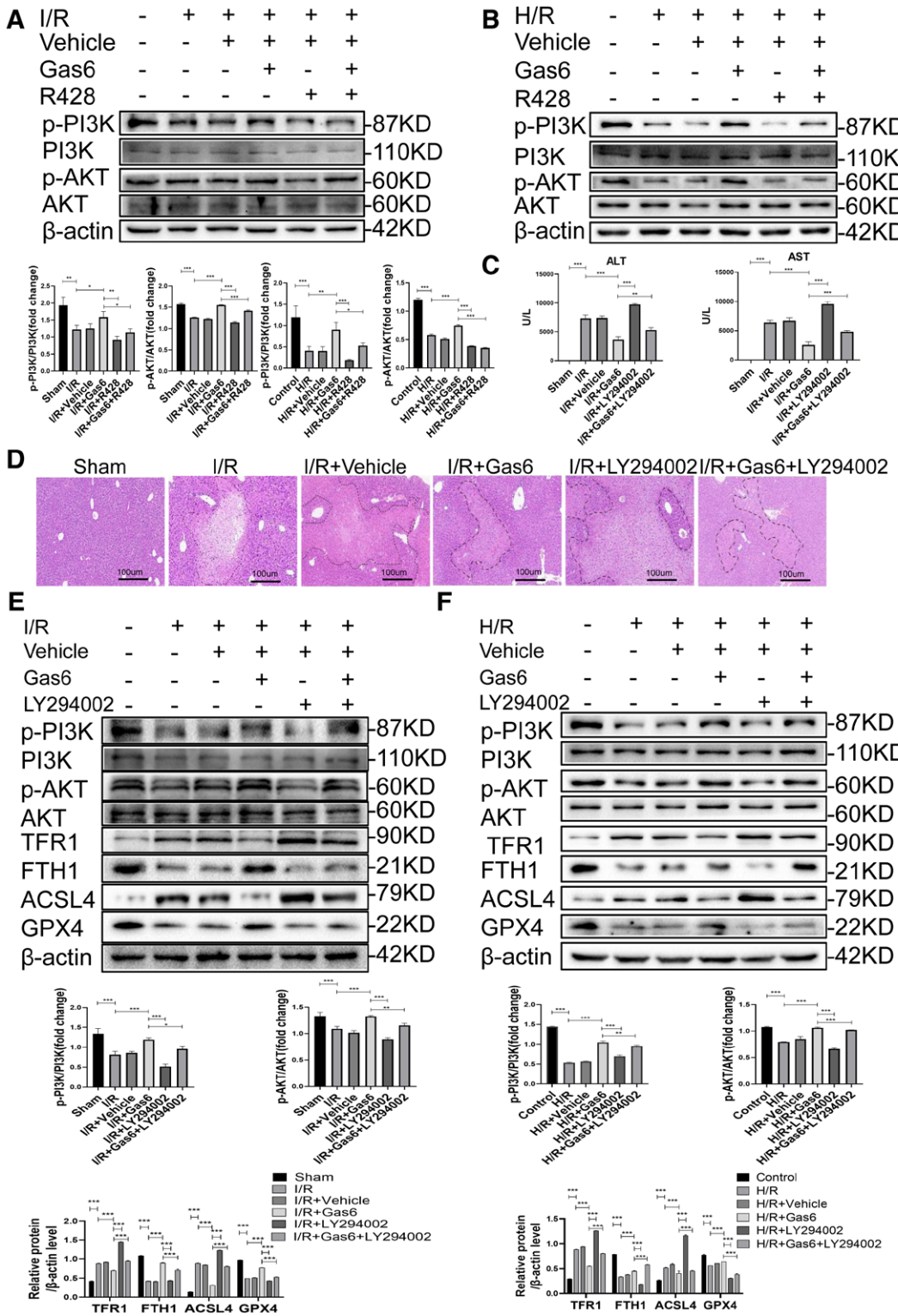

FIGURE 6. AXL inhibition exacerbates ferroptosis in hepatic I/R injury through the PI3K/AKT pathway. A, Western blot analysis of the protein expression of PI3K, p-PI3K, AKT, and p-AKT in liver tissue in the Sham group, the rmGas6-treated group and the R428 treatment group after liver I/R. B, Western blot analysis of the expression of PI3K, p-PI3K, AKT, and p-AKT in the control group, the rmGas6-treated and the R428-treated group after H/R. C, Serum levels of ALT and AST in hepatic I/R-subjected mice treated with or without the LY294002. D, H&E staining was used to detect liver injury in Gas6-treated or LY294002-treated mice after hepatic I/R injury. E, Western blot analysis of PI3K, p-PI3K, AKT, p-AKT, TFR1, FTH1, ACSL4, and GPX4 expression in Sham and treatment groups after hepatic I/R injury. F, Western blot analysis of PI3K, p-PI3K, AKT, p-AKT, TFR1, FTH1, ACSL4, and GPX4 expression in the control and treatment primary hepatocytes after H/R injury. A–F, Each group n = 4–6. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; AKT, the Ser and Thr kinase AKT; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AXL, AXL receptor tyrosine kinase; FTH1, ferritin heavy chain 1; Gas6, growth arrest-specific protein 6; GPX4, glutathione peroxidase 4; H&E, hematoxylin and eosin; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; p-AKT, phospho-AKT; PI3K, phosphatidylinositol 3-kinase; p-PI3K, phosphorylation phosphatidylinositol 3-kinase; rmGas6, recombinant mouse growth arrest-specific protein 6; TFR1, transferrin receptor 1.

ROS production (Figure 5B). MDA and Fe^{2+} levels were significantly increased due to H/R injury in hepatocytes but significantly decreased in rmGas6 treated hepatocytes (Figure 5C and D). Furthermore, immunofluorescence revealed that the expression of GPX4 was decreased in hepatocytes after H/R but that was significantly elevated in rmGas6-treated hepatocytes (Figure 5E). The imbalance between the generation and degradation of lipid ROS induced the occurrence of ferroptosis; therefore, we monitored hepatocyte lipid peroxidation with boron dipyrromethene C11. The accumulation of lipid peroxidation in hepatocyte was increased after H/R. AXL activation by rmGas6 could significantly reduce the product of hepatocyte lipid peroxidation induced by H/R. Inhibition of AXL activation by using R428 significantly reversed this result (Figure 5F and G). These data indicate that AXL activation ameliorates hepatocyte ferroptosis during H/R injury.

AXL Inhibition Exacerbates Ferroptosis in Hepatic I/R Injury Through the PI3K/AKT Pathway

Previous studies have reported that the PI3K/AKT pathway plays a crucial role in ferroptosis.[33] It is also the downstream pathway of the Gas6/AXL axis.[34] rmGas6 treatment rescued the expression of p-PI3K and p-AKT, which were inhibited by hepatic I/R injury, suggesting that PI3K/AKT signal pathway is downstream of the AXL receptor (Figure 6A and B). To further investigate whether this pathway affects the regulatory function of activated AXL on ferroptosis, mice were pretreated with the PI3K inhibitor LY294002. The LY294002 group exhibited the higher transaminase (ALT and AST) level and the greater degree of liver damage compared with the I/R group. In spite of the use of rmGas6, liver damage in the LY294002 group was not relieved, suggesting that LY294002 abolished the protective effect of rmGas6 on liver I/R injury (Figure 6C and D). In addition, we detected the changes of the PI3K/AKT pathway and ferroptosis during hepatic I/R injury after LY294002 pretreatment in vivo and vitro. Notably, the LY294002 group exacerbated ferroptosis compared with the I/R group. However, activation of AXL after LY294002 pretreatment did not improve ferroptosis in hepatic I/R injury or inhibit the PI3K/AKT signaling pathway (Figure 6E and F). These results indicate that AXL activation attenuates ferroptosis by activating the PI3K/AKT signaling pathway (Figure 7).

DISCUSSION

Hepatic I/R injury is considered a major cause of liver dysfunction or failure after various liver surgeries, which severely affects the postoperative recovery of patients. Hepatocyte death caused by liver reperfusion injury is a feature of hepatic I/R injury.

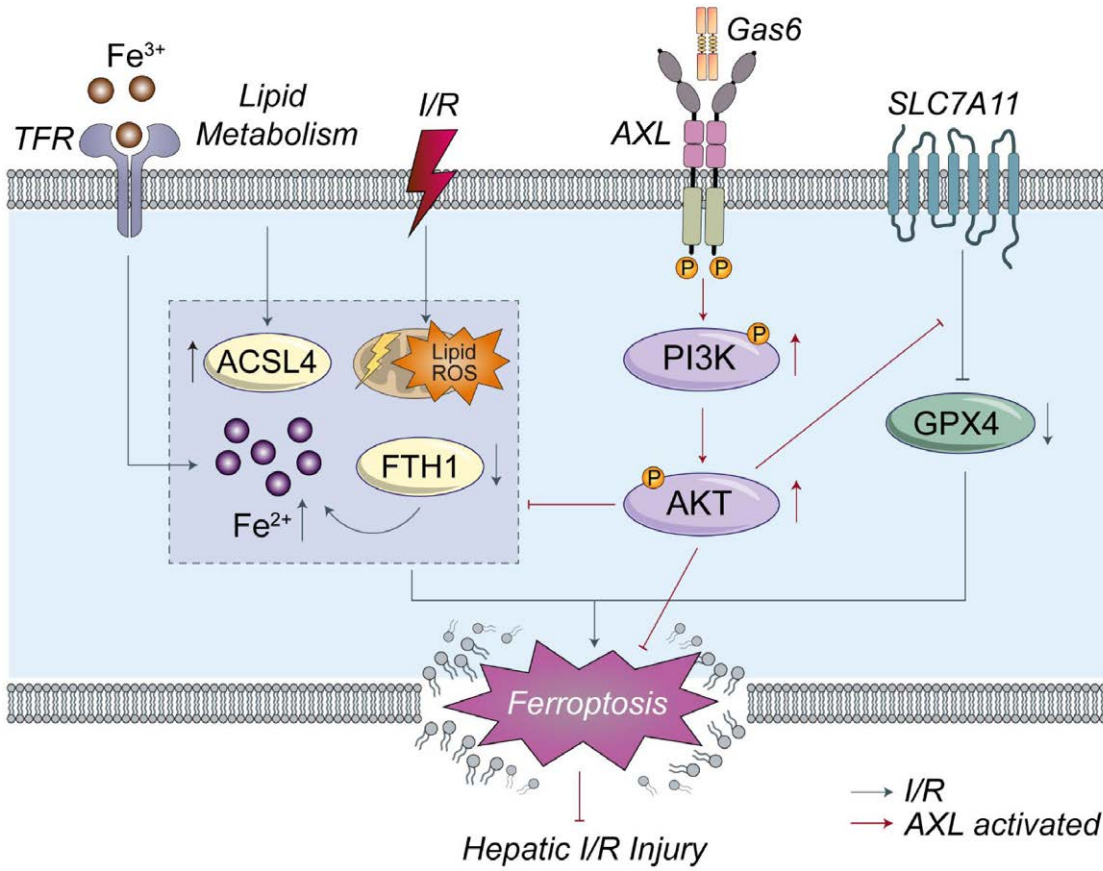

FIGURE 7. Gas6/AXL alleviates hepatic I/R injury by inhibiting ferroptosis via the PI3K/AKT pathway. Activated AXL attenuates iron overload and lipid peroxidation in hepatic I/R injury by activating the PI3K/AKT signaling pathway, thereby inhibiting ferroptosis. ACSL4, acyl-CoA synthetase long-chain family member 4 antibody; AKT, the Ser and Thr kinase AKT; AXL, AXL receptor tyrosine kinase; FTH1, ferritin heavy chain 1; Gas6, growth arrest-specific protein 6; GPX4, glutathione peroxidase 4; I/R, ischemia/reperfusion; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SLC7A11, solute carrier family 7 member 11; TFR1, transferrin receptor 1.

Recent studies have shown that the ferroptosis inhibitor significantly attenuated hepatic I/R injury and renal I/R injury, suggesting an important pathogenic role for ferroptosis in I/R injury.[8] In addition, it has been recently documented that ferroptosis is a major contributor to postoperative liver injury and that inhibition of ferroptosis protects the liver from I/R injury.[17]

In our previous report, proteomic analysis showed that AXL expression was upregulated in hepatic I/R injury.[2] Our current study confirmed that AXL protein expression is low in hepatocytes under normal conditions. However, AXL expression is significantly upregulated after hypoxia for 1 h and reoxygenation for 6 h in hepatocytes (as shown in Figure 1D). Rankin et al[35] reported that hypoxia inducible factor-1 and hypoxia inducible factor-2 directly bind to the hypoxia-response element in the AXL proximal promoter, resulting in AXL activation, which may account for AXL expression in hepatic I/R injury. In addition, it has been proved that the expression of Gas6 was inhibited in liver I/R injury.[24] Therefore, the reason for this interesting phenomenon may be that the decreased expression of endogenous Gas6 in the AXL high-affinity ligand led to decreased activation of AXL in hepatic I/R injury, which also provides evidence for the use of rmGas6 to activate AXL in our subsequent study.

In this study, we found a correlation between the expression of p-AXL and ferroptosis-related proteins in transplanted liver samples. Then, we confirmed that ferroptosis is involved in and shed significant light on hepatic I/R injury. The expression of p-AXL was decreased in hepatic I/R injury; therefore, we used rmGas6, the endogenous AXL activators, to activate AXL. AXL activation by rmGas6 could improve hepatic I/R injury by reducing iron overload and lipid peroxidation (as characterized by the levels of 4-HNE and MDA). Furthermore, AXL activation could alleviate ferroptosis (downregulation of TFR1 and ACSL4 and upregulation of GPX4 and FTH1) during hepatic I/R injury. In addition, AXL activation attenuated iron accumulation (the expression of TFR1and FTH1) and lipid peroxidation (increased ROS and lipid peroxidation levels) in hepatocytes after H/R. Therefore, we concluded that AXL activation protected against hepatic I/R injury by mitigating ferroptosis. However, the mechanisms and regulator effects of AXL on ferroptosis have not been clearly now.

The PI3K signaling pathway is an important cellular signaling system that links multiple receptors to many important cellular functions. AKT is an important effector of PI3K and plays a key role in the PI3K signaling pathway.[36] It has been reported recently that activation of the PI3K-AKT-mechanistic target of rapamycin signaling pathway inhibits ferroptosis.[37,38] And LY294002, an inhibitor of PI3K, can block PI3K pathway in hepatic I/R injury.[39] In this study, we observed that AXL activation could activate the PI3K/AKT pathway. However, when using LY294002 (the PI3K/AKT inhibitor) to block PI3K pathway during hepatic I/R injury, hepatic I/R-induced ferroptosis and liver injury were not ameliorated even in the presence of AXL activation. These results indicate that AXL activation attenuates hepatocyte ferroptosis to prevent hepatic I/R injury by activating the PI3K/AKT pathway.

There were some limitations in our study. Although AXL activation could alleviate ferroptosis during hepatic I/R injury, the direct molecular mechanism between AXL and the key regulators of ferroptosis has not been explored. In addition, the expression of AXL in other liver cell types and its effects on ferroptosis was not quantified, and these may need to be explored in further studies.

In conclusion, our study provides evidence that AXL activation can suppress ferroptosis via the PI3K/AKT signaling pathway during hepatic I/R injury. The link between AXL receptors and ferroptosis has not been investigated in hepatic I/R injury. Here, this study is the first investigation on the AXL receptor and ferroptosis, and activation of AXL to reduce ferroptosis may be an innovative therapeutic strategy to combat hepatic I/R injury.

REFERENCES

1. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, et al. Factors in the pathophysiology of the liver ischemia-reperfusion injury. *J Surg Res*. 2008;147:153–159.
2. Cai H, Qi S, Yan Q, et al. Global proteome profiling of human livers upon ischemia/reperfusion treatment. *Clin Proteomics*. 2021;18:3.
3. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*. 2012;149:1060–1072.
4. Yu Y, Yan Y, Niu F, et al. Ferroptosis: a cell death connecting oxidative stress, inflammation and cardiovascular diseases. *Cell Death Discov*. 2021;7:193.
5. Bai T, Li M, Liu Y, et al. Inhibition of ferroptosis alleviates atherosclerosis through attenuating lipid peroxidation and endothelial dysfunction in mouse aortic endothelial cell. *Free Radic Biol Med*. 2020;160:92–102.
6. Zhang C, Liu X, Jin S, et al. Ferroptosis in cancer therapy: a novel approach to reversing drug resistance. *Mol Cancer*. 2022;21:47.
7. Fan Z, Cai L, Wang S, et al. Baicalin prevents myocardial ischemia/reperfusion injury through inhibiting ACSL4 mediated ferroptosis. *Front Pharmacol*. 2021;12:628988.
8. Friedmann Angeli JP, Schneider M, Proneth B, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol*. 2014;16:1180–1191.
9. Li Y, Feng D, Wang Z, et al. Ischemia-induced ACSL4 activation contributes to ferroptosis-mediated tissue injury in intestinal ischemia/reperfusion. *Cell Death Differ*. 2019;26:2284–2299.
10. Xie Y, Hou W, Song X, et al. Ferroptosis: process and function. *Cell Death Differ*. 2016;23:369–379.
11. Zhang Z, Zhang F, Guo X, et al. Ferroportin1 in hepatocytes and macrophages is required for the efficient mobilization of body iron stores in mice. *Hepatology*. 2012;56:961–971.
12. Gao M, Monian P, Quadri N, et al. Glutaminolysis and transferrin regulate ferroptosis. *Mol Cell*. 2015;59:298–308.
13. Yang WS, Stockwell BR. Ferroptosis: death by lipid peroxidation. *Trends Cell Biol*. 2016;26:165–176.
14. Jiang X, Stockwell BR, Conrad M. Ferroptosis: mechanisms, biology and role in disease. *Nat Rev Mol Cell Biol*. 2021;22:266–282.
15. Fang X, Wang H, Han D, et al. Ferroptosis as a target for protection against cardiomyopathy. *Proc Natl Acad Sci U S A*. 2019;116:2672–2680.
16. Li Y, Cao Y, Xiao J, et al. Inhibitor of apoptosis-stimulating protein of p53 inhibits ferroptosis and alleviates intestinal ischemia/reperfusion-induced acute lung injury. *Cell Death Differ*. 2020;27:2635–2650.
17. Wu S, Yang J, Sun G, et al. Macrophage extracellular traps aggravate iron overload-related liver ischaemia/reperfusion injury. *Br J Pharmacol*. 2021;178:3783–3796.
18. Yamada N, Karasawa T, Wakiya T, et al. Iron overload as a risk factor for hepatic ischemia-reperfusion injury in liver transplantation: potential role of ferroptosis. *Am J Transplant*. 2020;20:1606–1618.
19. Fang X, Zhang J, Li Y, et al. Malic enzyme 1 as a novel anti-ferroptotic regulator in hepatic ischemia/reperfusion injury. *Adv Sci (Weinh)*. 2023;10:e2205436.
20. Guo J, Song Z, Yu J, et al. Hepatocyte-specific TMEM16A deficiency alleviates hepatic ischemia/reperfusion injury via suppressing GPX4-mediated ferroptosis. *Cell Death Dis*. 2022;13:1072.
21. Linger RMA, Keating AK, Earp HS, et al. TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. *Adv Cancer Res*. 2008;100:35–83.

22. Di Stasi R, De Rosa L, D'Andrea LD. Therapeutic aspects of the Axl/Gas6 molecular system. *Drug Discov Today*. 2020;25:2130–2148.
23. Tanaka M, Siemann DW. Gas6/Axl signaling pathway in the tumor immune microenvironment. *Cancers (Basel)*. 2020;12:1850.
24. Wang Z, Liu D, Yan Q, et al. Activated AXL protects against hepatic ischemia-reperfusion injury by upregulating SOCS-1 expression. *Transplantation*. 2022;106:1351–1364.
25. Nyakas M, Fleten KG, Haugen MH, et al. AXL inhibition improves BRAF-targeted treatment in melanoma. *Sci Rep*. 2022;12:5076.
26. Abe Y, Hines IN, Zibari G, et al. Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo. *Free Radic Biol Med*. 2009;46:1–7.
27. Liu Y, Lu T, Zhang C, et al. Activation of YAP attenuates hepatic damage and fibrosis in liver ischemia-reperfusion injury. *J Hepatol*. 2019;71:719–730.
28. Fang X, Cai Z, Wang H, et al. Loss of cardiac ferritin H facilitates cardiomyopathy via SLC7A11-mediated ferroptosis. *Circ Res*. 2020;127:486–501.
29. Tang D, Kroemer G. Ferroptosis. *Curr Biol*. 2020;30:R1292–R1297.
30. Tang D, Chen X, Kang R, et al. Ferroptosis: molecular mechanisms and health implications. *Cell Res*. 2021;31:107–125.
31. Luo L, Mo G, Huang D. Ferroptosis in hepatic ischemiareperfusion injury: regulatory mechanisms and new methods for therapy (Review). *Mol Med Rep*. 2021;23:225.
32. Tacchini L, Poli DF, Bernelli-Zazzera A, et al. Transferrin receptor gene expression and transferrin-bound iron uptake are increased during postischemic rat liver reperfusion. *Hepatology*. 2002;36:103–111.
33. Sun L, Wang H, Xu D, et al. Lapatinib induces mitochondrial dysfunction to enhance oxidative stress and ferroptosis in doxorubicin-induced cardiomyocytes via inhibition of PI3K/AKT signaling pathway. *Bioengineered*. 2022;13:48–60.
34. Li M, Ye J, Zhao G, et al. Gas6 attenuates lipopolysaccharide-induced TNF-alpha expression and apoptosis in H9C2 cells through NF-kappaB and MAPK inhibition via the Axl/PI3K/Akt pathway. *Int J Mol Med*. 2019;44:982–994.
35. Rankin EB, Fuh KC, Castellini L, et al. Direct regulation of GAS6/AXL signaling by HIF promotes renal metastasis through SRC and MET. *Proc Natl Acad Sci U S A*. 2014;111:13373–13378.
36. Revathidevi S, Munirajan AK. Akt in cancer: mediator and more. *Semin Cancer Biol*. 2019;59:80–91.
37. Yi J, Zhu J, Wu J, et al. Oncogenic activation of PI3K-AKT-mTOR signaling suppresses ferroptosis via SREBP-mediated lipogenesis. *Proc Natl Acad Sci U S A*. 2020;117:31189–31197.
38. Huang J, Yue S, Ke B, et al. Nuclear factor erythroid 2–related factor 2 regulates toll-like receptor 4 innate responses in mouse liver ischemia-reperfusion injury through Akt-forkhead box protein O1 signaling network. *Transplantation*. 2014;98:721–728.
39. Chen K, Xue R, Geng Y, et al. Galangin inhibited ferroptosis through activation of the PI3K/AKT pathway in vitro and in vivo. *FASEB J*. 2022;36:e22569.