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Original Paper

Galangin Alleviates Liver Ischemia-Reperfusion Injury in a Rat Model by Mediating the PI3K/AKT Pathway

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Key Words
Liver ischemia-reperfusion injury • Galangin • PI3K/AKT • Cell apoptosis

Abstract
Background/Aims: Liver ischemia-reperfusion (I/R) injury is a pathological process that often occurs during liver and trauma surgery. There are numerous causes of liver I/R injury, but the mechanism is unknown. Galangin (GA) is a flavonoid, a polyphenolic compound widely distributed in medicinal herbs that has anti-inflammatory, antioxidant, and antitumor activity. This study evaluated the protective effect of GA on hepatic I/R injury. ***Methods:*** An I/R model was created in male Wistar rats by clamping the hepatoportal vein, hepatic artery and hepatic duct for 30 min followed by reperfusion for 2 h. A hypoxia/restoration (H/R) model was established in buffalo rat liver (BRL) cells by hypoxia for 4 h followed by normoxic conditions for 10 h. The extent of liver injury was assayed by serum ALT/AST, hepatic histology, and MPO activity. Oxidative stress was assayed by serum superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA). Expression of apoptosis-related proteins in BRL cells was assayed in western blots. Expression of AKT and p-AKT proteins *in vivo* and vitro were assayed in western blots. ***Results:*** GA significantly decreased ALT/AST expression, reversed changes in oxidative stress markers induced by I/R, and mediated caspase-3 activity expression of apoptosis-related proteins *in vivo* and *in vitro*. Methylthiazol tetrazolium (MTT) assay, flow cytometry, and Hoechst 33258 staining confirmed that GA inhibited apoptosis of BRL cells. GA also increased the expression of phosphorylated AKT after H/R. ***Conclusion:*** GA reduced liver I/R injury both *in vivo* and vitro and inhibited BRL cell apoptosis. PI3K/AKT signaling have been involved. GA may protect against liver I/R and be a potential therapeutic candidate.

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Introduction

Liver ischemia-reperfusion (I/R) injury is a pathophysiological process common to liver dysfunction and failure after hepatic trauma, resection, transplantation, and circulatory shock [1]. Since the introduction of vascular control techniques in hepatic surgery, liver I/R has been recognized as one of the key elements that lead to postoperative higher morbidity and mortality [2]. I/R-induced liver injury involves multiple complex mechanisms, including anaerobic metabolism, mitochondrial damage, oxidative stress, endoplasmic reticulum stress, intracellular calcium overload, Kupffer cell activation, neutrophil infiltration, and production of cytokines and chemokines [1, 3, 4]. Ischemic preconditioning (IPC) has been considered to be a powerful endogenous intervention mechanism for protection against I/R [5]. However, I/R-induced liver injury still remains an important clinical problem despite the recent improvements in treatment conditions and surgical techniques.

Liver I/R mainly includes the following dynamic processes [6]. Adenosine triphosphate (ATP) depletion interferes with the cellular energy-dependent metabolic and transport processes [6, 7]. The reperfusion process consists of two phases: in the initial phase, activated resident macrophages of the liver, the Kupffer cells, induce oxidative stress mainly by reactive oxygen species (ROS) generation and, in the later phase, recruited neutrophils release inflammatory mediators which can cause cell apoptosis or death [4, 8]. So inhibition of cell apoptosis may be of clinical importance in managing I/R injury.

Specific morphological characteristics and energy-dependent biochemical mechanisms have been associated with programmed cell death or apoptosis [9], and apoptotic pathways are often induced by upstream activators including PI3K/AKT/mTOR signaling, proapoptotic proteins in the Bcl-2 family, cellular stress stimuli, and hypoxia [10]. PI3K/AKT signaling is a classic anti-apoptosis pathway and its involvement in the process of liver I/R injury has been reported [11-13].

Galangin (GA), is a naturally occurring flavonoid compound that can be isolated from the rhizome of Alpinia galangal. It is used for cooking in Asian countries and as an herbal treatment of diabetes, cough, colds, stomach ache, and diarrhea. GA was found to reduce oxidative damage and inflammatory changes in fructose-fed rat liver [14]. The anticancer effects of GA have been shown in melanoma, leukemia, colon cancer cell lines [15-17]. Considering its antioxidant and anti-inflammatory activities, we hypothesized that GA can protect against liver I/R injury, possibly by regulating PI3K/AKT signaling.

Materials and Methods

Hepatic I/R injury model

Male Wistar rats weighing 200–240 g from the Animal Research Center at Harbin Medical University, Harbin, China were used. The study was approved by the Experimental Animal Ethics Committee of Harbin Medical University. Hepatic ischemia was induced as previously described [18]. Briefly, Wistar rats were anesthetized with sodium pentobarbital combined with methoxyflurane (inhalation) anesthesia, and a midline laparotomy was performed to isolate and clamp the portal vein, hepatic arterial, and hepatic duct. After 30 min, the clip was removed to initiate hepatic reperfusion, which was maintained for 2 h. Experimental animals were randomly allocated to three groups of ten rats each, controls (laparotomy only), laparotomy with I/R, and I/R with intraperitoneal injection of 100 μmol/kg GA (Sigma) (I/R+GA). GA was intraperitoneally injected 1h before ischemia. When the experimental procedures were completed, blood was collected by cardiac puncture for serum analysis and liver tissue was harvested; both were stored at −80ºC until used.

Blood and tissue analysis

Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed by an automated biochemical analyzer (Toshiba, Tokyo, Japan). Serum SOD and CAT activity (U/mg) and liver tissue GSH (mg/g) and MDA (nmol/mg) were assayed using commercial kits (Jiancheng Biotechnology, Nanjing, China) following the manufacturer's instructions. The activity of myeloperoxidase (MPO), an enzyme specific to polymorphonuclear neutrophils, was assayed in liver tissue using a commercial kit (Jiancheng Biotechnology, Nanjing, China) following the manufacturer's instructions. Caspase-3 activity was assayed in liver tissue using a commercial kit (Beyotime, Shanghai, China) following the manufacturer's instructions.

Histopathology

Liver sections were fixed in 4% paraformaldehyde overnight, dehydrated, cleared, and embedded in paraffin. The tissues were then sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for examination by light microscopy.

Cell culture

The Buffalo rat liver (BRL) cell line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum and 1% penicillin and streptomycin at 37ºC and 5% CO2. To model hypoxia/restoration (H/R), cells were incubated in hypoxic conditions with a gas mixture of 92% N2, 5% CO2, and 3% O_2 for 4 h and then in normal culture conditions for 10 h as previously described [19]. Before use, BRL cells were rested for 24 h in DMEM without serum and then divided into four treatment groups. These were controls, H/R, GA added at the beginning of H/R treatment (H/R + GA), and GA plus 25 mM LY294002, a phosphatidylinositol 3-kinase inhibitor [20], added 2h before the addition of GA at the beginning of H/R treatment (H/R + GA + LY).

Apoptosis assay

BRL cells apoptosis can be quantitatively analyzed by detection of DNA fragmentation via a fluorescence assay based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (Beyotime Institute of Biotechnology, China) as previously described [21].

Methylthiazol tetrazolium (MTT) assay

BRL cells were seeded into a 96-well plate and incubated with GA of different doses.

Next, BRL cells were cultured in hypoxia for 4 h and then in normal culture conditions for 10 h. Medium containing 0.5% MTT was added, the supernatant was removed after culture at 37ºC 4 h, and 150 μl/well DMSO was added at room temperature for 10 min. Absorbance at 490 nm was then read using a standard microplate reader.

Hoechst 33258 staining

Hoechst 33258 staining was used to detect morphological evidence of cell apoptosis. After treatment, BRL cells were stained with Hoechst 33258 (1 μg/mL) for 30 min in the dark. Nuclear morphology was evaluated by fluorescence microscopy at 350 nm and 460 nm.

Annexin V-FITC apoptosis assay

An Annexin V-FITC apoptosis detection kit (Beyotime) was used to determine apoptotic ratios. Briefly, BRL cells were collected and washed three times with cold PBS, resuspended in 195 μL of Annexin V-FITC binding buffer, incubated with 5 μL Annexin V-FITC and 10 μL propidium iodide for 15 min at room temperature in the dark. The percentage of apoptotic cells was assayed immediately by flow cytometry.

Western blot assay

Liver samples were homogenized in a micro-tissue grinder in ice cold storage buffer (Tris 50 mM, pH 7. 4, NaCl 150 mM, Triton X-100%, EDTA 1 mM, and PMSF 2 mM) and then centrifuged at 13, 500 g at 4ºC for 15 min. The supernatants were collected and used for western blot assays. BRL cells were suspended in lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100%, EDTA 1 mM, and PMSF 2 mM) containing phosphatase inhibitor. The lysates were sonicated, centrifuged at 13, 500 g at 4ºC for 15 min, the insoluble fraction was discarded and the supernatants were collected and used in the western blot assays. The protein concentration was determined by bicinchoninic acid assay and 10 μL aliquots containing 20 μg total cell proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes as previously described [22]. The membranes were blocked at room temperature for 2 h in TBST and 0.1% Tween 20 containing 5% nonfat dry milk. The membranes were

incubated overnight at 4ºC with antibodies against Cleaved Caspase-3 (1:200), Bax (1:100), Bcl-2 (1:200, all from Boster, Wuhan, China), Akt (1:500), phosphorylated Akt (Ser473, 1:500, both from Beyotime), NLRP3 (1:1000, Abcam) and β-actin (1:1000, Boster). After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1h. Membranes were developed with enhanced chemiluminescence reagents (ECL; Applygen Technology Inc. Beijing, China) and the final results were obtained by X-ray exposure.

Statistical analysis

The composite data were expressed as mean ± SD. Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences were considered to be significant at $P \leq 0.05$.

Results

GA decreased I/R-induced liver injury

We treated I/R groups with different concentrations of GA and detected the ALT and AST level (Fig. 1A-B). Finally, we chose 100 μmol/kg as the most suitable concentration. As shown in Fig. 2A and B, ALT and AST levels were higher in the I/R than in the control group after 2 h reperfusion. GA significantly decreased the I/R-induced peaks in ALT and AST level. The arrangement of liver plates was disturbed and sinusoid tissue congestion was observed in liver tissue from the I/R group but not in tissue from sham operated controls at 10× magnification. Vacuole formation, cellular swelling, inflammatory cells, and lymphocyte infiltration were evident in I/R but not control tissue. The I/R-related changes were reduced in tissue from I/R+GA treated animals (Fig. 2C). MPO activity, which indicates neutrophil activity, was increased after reperfusion in I/R group compared with controls. GA reduced MPO activity (Fig. 2D).

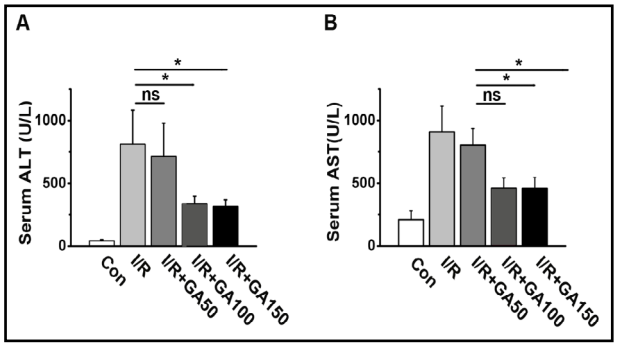


Fig.1. Serum ALT (A) and AST (B) levels in I/R rats. Values are means ± SD (n=6). *P <0.05.

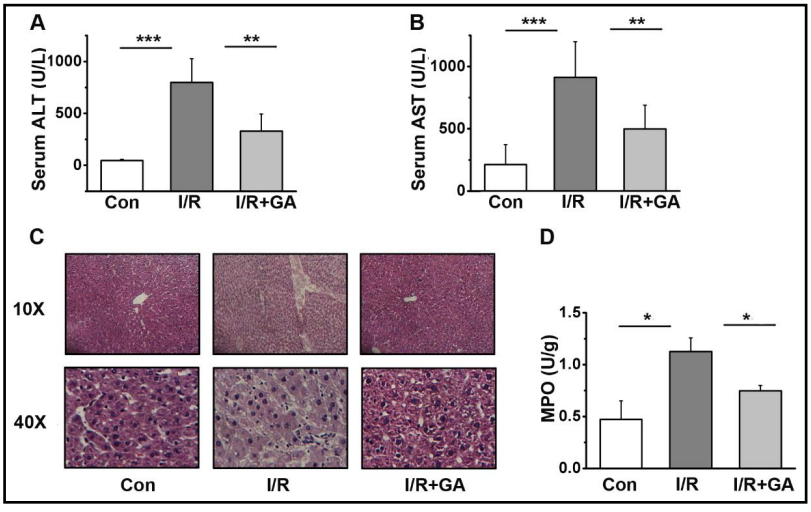


Fig. 2. GA decreased liver injury induced by I/R. Serum ALT (A) and AST (B) were assayed after liver ischemia and 2 h of reperfusion with or without intraperitoneal injection of GA. (C) Representative hematoxylin–eosin (H&E) stained liver sections from Controls, I/R, and I/R+GA groups. Upper panel, ×10; lower panel, ×40. (D) MPO activity after 2 h reperfusion. Values are means ± SD (n = 6). *P <0.05; **P <0.01.

GA reduced oxidative stress in I/R rats

As shown in Fig. 3A, I/R injury appreciably reduced SOD activity in the I/R group compared with the controls. SOD activity was significantly increased by GA treatment. Similarly, CAT activity was significantly decreased after reperfusion compared with the controls, but activity was not as strongly affected in the I/R+GA group. (Fig. 3B). GSH concentration was significantly lower in the I/R than in the control group and was significantly higher in the I/R+GA than in the I/R group (Fig. 3C). The MDA concentration was significantly higher in the I/R than in the control group and was significantly lower in the I/R+GA than in the I/R group (Fig. 3D).

In vivo, GA has an antiapoptotic effect on liver cells

To determine effect of GA on hepatocellular apoptosis induced by IR, we assayed caspase-3 activity in ischemic livers. Caspase-3 activity was markedly elevated in rats in the I/R group; however, GA treatment reduced Caspase-3 activity (Fig. 4A). We also assayed hepatocellular apoptosis in western blots, which showed that the expression of apoptosis-related proteins was affected by I/R, and that GA had an antiapoptotic effect (Fig. 4B-D). We also found that the expression of inflammasome complex NLRP3 was increased in the I/R group; however, GA treatment reduced the expression of NLRP3 (Fig. 4E). We used TUNEL assay to detect the BRL cells apoptosis. The number of apoptotic BRL cells increased in I/R compared with the normal group. However, GA inhibited BRL cells apoptosis induced by I/R (Fig. 4F). A similar result was obtained with Hoechst 33258 staining (Fig. 4G).

GA enhanced BRL survival after H/R treatment

Exposure to H/R conditions significantly decreased the viability of BRL cells compared with controls. However, when the cells were cultured with GA (0.5, 1, 2, 4, 8μM) before exposure to H/R conditions, viability was improved compared with H/R alone. As shown in Fig. 5, GA enhanced the viability of BRL cells at a concentration of 4μM.

GA inhibited H/R-induced apoptosis of BRL cells by activating the PI3K/AKT pathway

As shown in Fig. 6A, apoptotic BRL cells stained with Hoechst 33258 appeared bright blue with filaments in the cell nucleus. Most apoptotic BRL cells were in the H/R group. GA had a protective effect on cell apoptosis that was decreased by the AKT pathway inhibitor LY294002. As shown in Fig. 6B, flow cytometry quadrants Q2 and Q4 are associated with cell apoptosis. The percentage of apoptotic cells increased with H/R treatment. GA decreased the apoptosis percentage, but the effect of GA was decreased by LY294002. To determine whether the PI3K/AKT pathway was involved in GA-mediated apoptosis of H/R BRL cells, we assayed p-Akt protein and AKT protein expression. Compared with controls, p-Akt expression was decreased by H/R treatment, increased in the H/R+GA group compared with the H/R group,

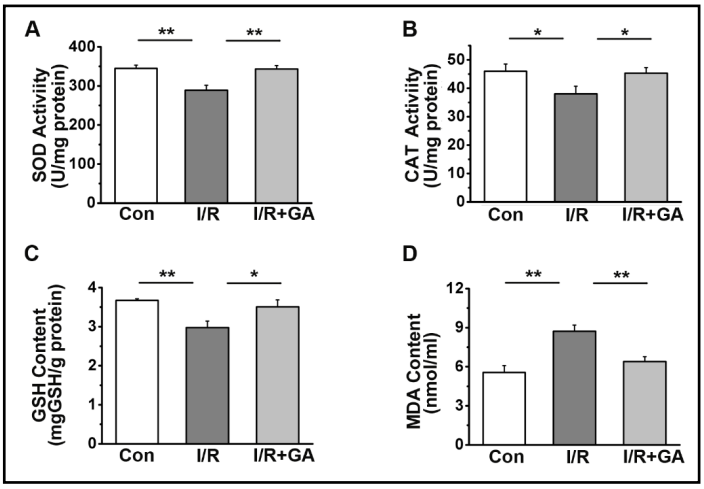


Fig. 3. Serum SOD (A), CAT (B), GSH (C), and MDA (D) levels. Values are means ± SD (n=6). *P <0.05; **P<0.01.

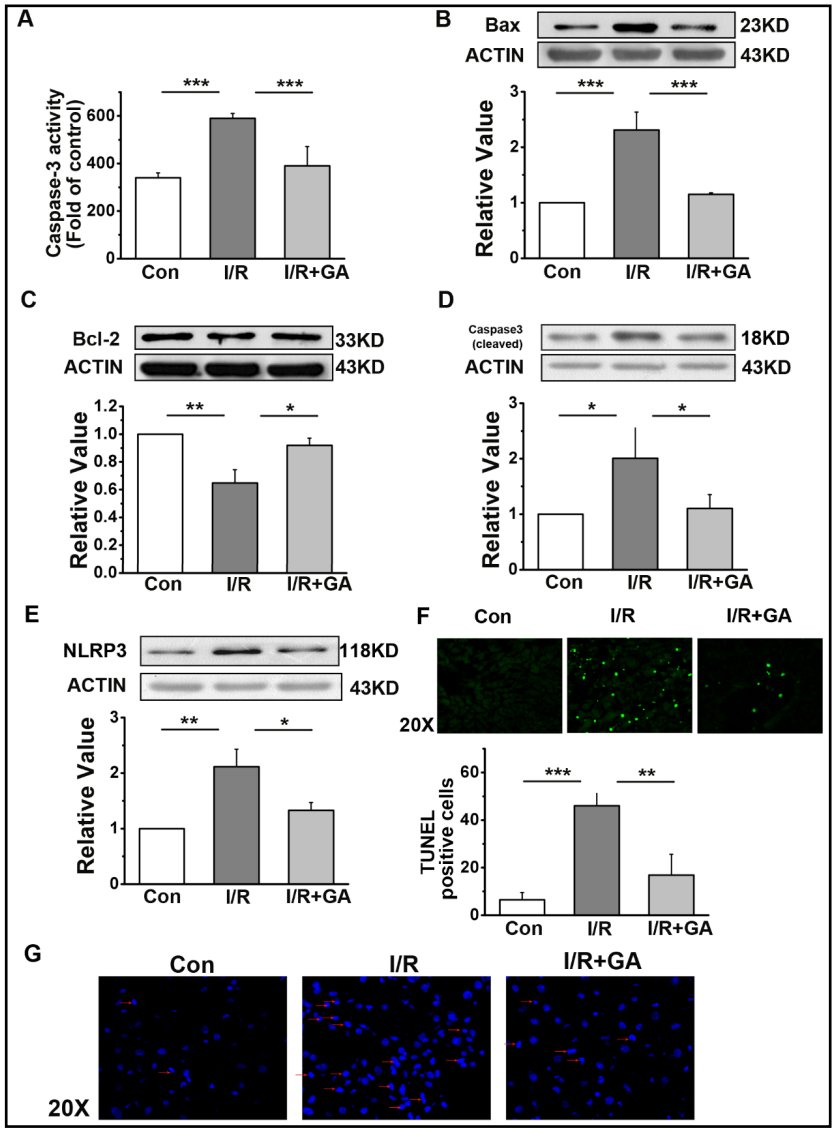


Fig. 4. In vivo, GA inhibited apoptosis induced by I/R. (A) Caspase-3 activity. (B–E) Western blot assay of Bax, Bcl-2, Cleaved Caspase-3, NLRP3 and β-actin. (F) Apoptosis of BRL cells evaluated by TUNEL assay. (G) Morphology of BRL cells evaluated by Hoechst 33258 staining. Values are means ± SD. (n=5). *P <0.05; **P <0.01; ***P <0.001.

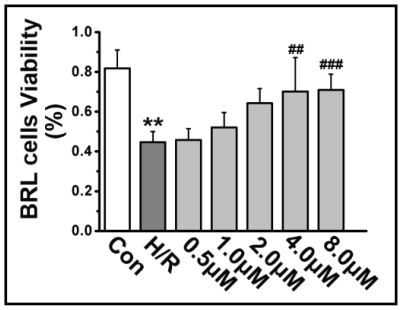


Fig. 5. MTT assay of cell viability after H/R. Normal BRL cells (control), BRL cells exposed to H/R, and BRL cells exposed to H/R and treated with GA (0.5, 1, 2, 4, or 8 μM). Values are means ± SD (n=5). **P <0.01 vs. the control group; ##P <0.01; ###P <0.001 vs. H/R group.

and decreased in the H/R+GA+LY group compared with the H/R+GA group. Akt expression did not change (Fig. 7A). Changes in the expression of apoptosis-related proteins were consist with the above results (Fig. 7B–D). We assayed NLRP3 protein expression *in vitro*. Compared with controls, NLRP3 expression was increased by H/R treatment, decreased in the H/R+GA group compared with the H/R group, and increased in the H/R+GA+LY group compared with the H/R+GA group. (Fig. 7E). We also detected the protein expression of p-Akt and AKT *in vivo* and found that liver I/R decreased the expression of p-Akt, while GA increased the expression of p-Akt (Fig. 8).

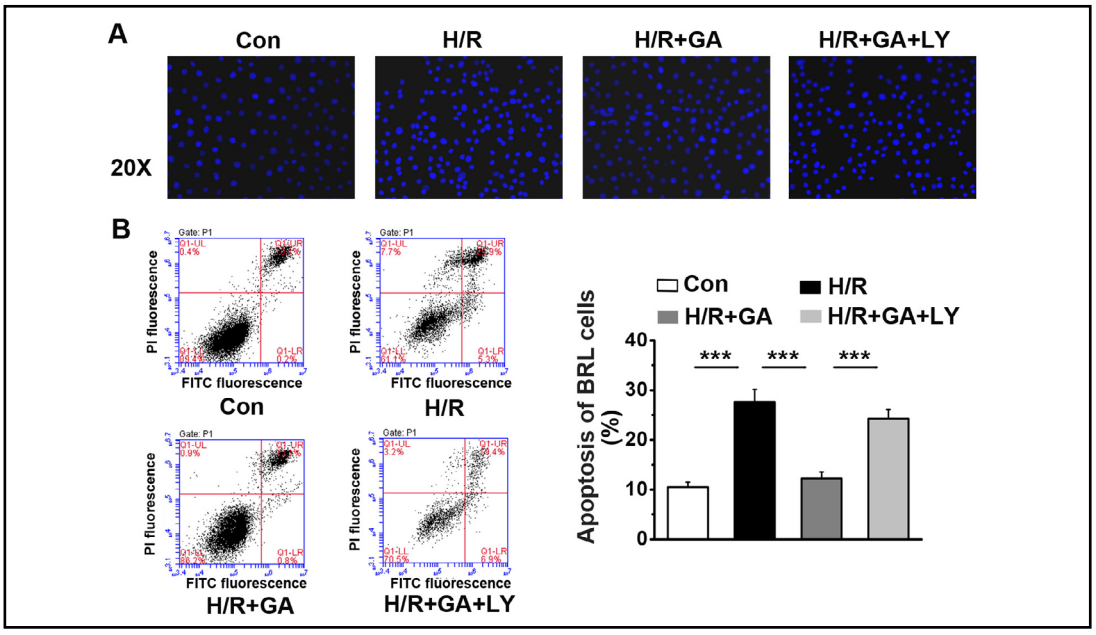
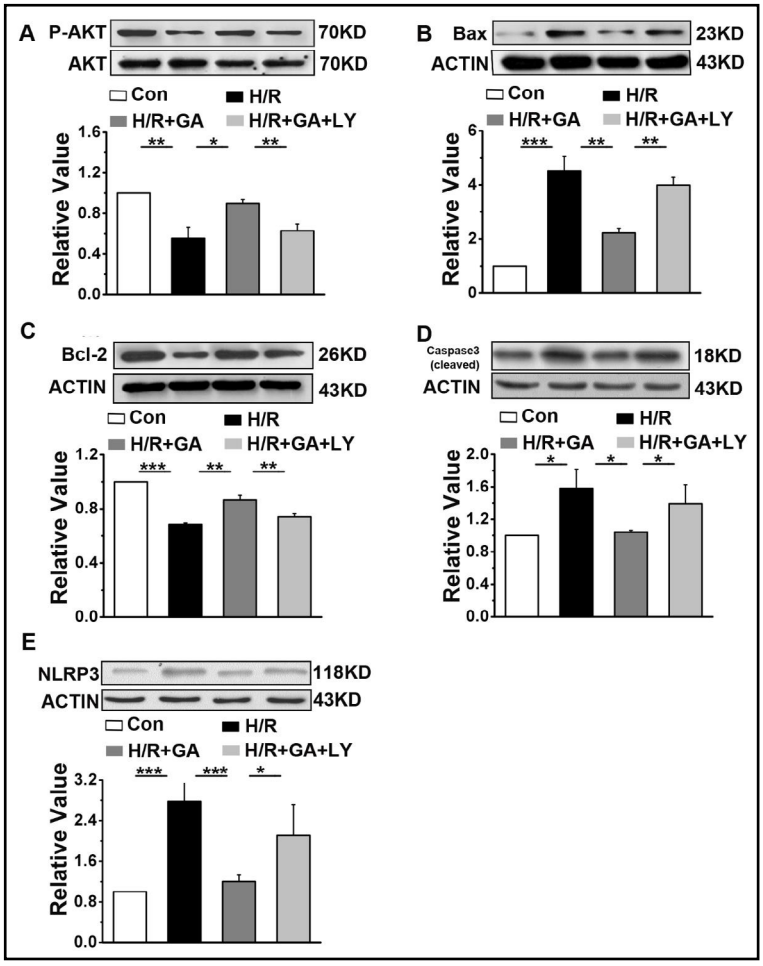


Fig. 6. In vitro, GA inhibited apoptosis induced by I/R. (A) Morphology of BRL cells evaluated by Hoechst 33258 staining. (B) Apoptosis of BRL cells assayed by flow cytometry. Values are means ± SD (n=5). ***P <0.001.

Fig. 7. Expression of AKT, P-AKT (A), Bax (B), Bcl2 (C), Cleaved Caspase-3 (D) and NLRP3 (E) by western blot assay. Values are means ± SD (n=5). *P <0.05; **P <0.01. Con, normal; H/R, BRL cells exposed to hypoxia; H/R+GA, BRL cells exposed to hypoxia and treated with GA; H/R+GA+LY, BRL cells exposed to hypoxia and treated with GA+LY.



Discussion

GA is a potent free radical scavenger [23] and is known to have anti-inflammatory, antioxidant and anticancer effects in several tissues and organs [24, 25]. This study found that GA had a protective effect on liver I/R. GA significantly decreased high ALT/AST levels induced by I/R and mediated oxidative stress in an I/R rat model. GA also inhibited liver damage in I/R rats. *In vitro*, the MTT assay, Hoechst33258 staining, annexin V-FITC apoptosis assay and western blots consistently showed that GA decreased apoptosis of BRL cells. The results also showed that the PI3K/AKT pathway was involved in GA inhibition of liver I/R injury. Overall, the study supports the potential of GA for clinical prevention of liver I/R injury.

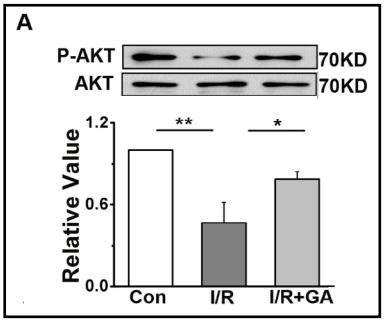


Fig. 8. Expression of AKT, P-AKT (A) by western blot assay. Values are means ± SD (n=6). *P <0.05; **P <0.01.

I/R injury is caused by a complex series of events including cell death resulting from oxygen deprivation and adenosine triphosphate depletion during ischemia and subsequent activation of cells of the innate immune system by inflammatory responses during reperfusion [26]. In the process of I/R injury, redox balance is dysregulated and the normal functions and integrity of tissues are damaged, resulting in the accumulation of reactive oxygen species (ROS). The accumulation of ROS and oxidative stress are the most common type of liver ischemia-reperfusion injury mechanism [27]. Antioxidants such as SOD, CAT, and glutathione (GSH) peroxidase are the first line of defense against oxidative stress. These enzymes catalyze the oxidation of oxygen into inactive substances [28]. Normally, the negative effects of superoxides are suppressed by SOD, which transforms them into hydrogen peroxide [29, 30]. Under the conditions associated with I/R, these defense mechanisms are impaired, and hydrogen peroxide is split into hydrogen and oxygen free radicals that damage many types of biological molecules, including amino acids, membrane transport proteins and nucleic acids [31]. GSH is a key antioxidant, and the increase of intracellular GSH concentration contributes to endothelial cell resistance to oxidative damage. CAT is an oxidoreductase that catalyzes the reduction of hydrogen peroxide into water and oxygen and protects cells against damage caused by ROS induced by I/R [32, 33]. This study showed that GA mediated the above mentioned oxidative stress markers associated with I/R and had a protective effect against the injury caused by oxidative stress.

Apoptosis of hepatocytes and sinusoidal endothelial cells is an important cause of liver I/R injury [34]. Many factors activate apoptotic cascade proteins and extensive evidence indicates that caspase 3 apoptosis "killers" are involved in I/R injury [35]. The Bcl-2 protein family also plays an important role in intrinsic hepatocyte apoptosis, especially Bcl-2 and Bax. The Bcl-2/Bax ratio determines cell fate in response to an apoptotic stimulus [36, 37]. This study confirmed that *in vivo* or *in vitro*, GA regulated the expression of apoptosis-related proteins after I/R. These data indicate that GA has a protective effect on mitochondrial apoptosis caused by liver I/R. Whether GA plays a role in apoptosis caused by death receptors awaits further investigation.

Conclusion

In this study, we used the PI3K/AKT pathway inhibitor LY294002 to treat BRL cells, and consistent with the previous results, p-AKT expression was decreased after H/R treatment. GA thus protected liver I/R partly by PI3K/AKT signaling, but the specific regulation mechanism needs further investigation.

Disclosure Statement

The authors declare to have no competing interests.

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