Hesperidin ameliorates liver ischemia/reperfusion injury via activation of the Akt pathway

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Abstract. Hesperidin (HDN) is a bioflavonoid that serves a role as an antioxidant in biological systems. However, although HDN has hydrogen radical- and hydrogen peroxide-removal activities, the role of HDN in liver ischemia/reperfusion (I/R) injury remains unknown. This study aimed to determine the role of HDN in liver I/R injury. Male C57BL/6J wild-type (WT) mice were subjected to warm partial liver I/R injury. Liver damage was evaluated by measuring serum alanine aminotransferase (ALT) levels, cytokine production, oxidative stress indicators, tissue hematoxylin-eosin staining and cell death. The Akt signaling pathway was examined to elucidate the underlying mechanisms. HDN had no effect on ALT levels and tissue damage in WT mice without liver I/R injury. However, HDN significantly ameliorated liver I/R injury as measured by serum ALT levels and necrotic tissue areas. HDN decreased malondialdehyde content, but increased the levels of superoxide dismutase, catalase, glutathione peroxidase and glutathione. In addition, HDN significantly attenuated the mRNA expression levels of TNF-α, IL-6 and IL-1β after liver I/R injury. Furthermore, HDN protected the liver against apoptosis in liver I/R injury by increasing the levels of Bcl-2 and decreasing the levels of cleaved-caspase 3. Mechanistically, the levels of phosphorylated Akt were elevated by HDN during liver I/R injury. In addition, HDN could induce Akt activation in hepatocytes *in vitro*. Most importantly, treatment with the Akt inhibitor LY294002 in WT mice blocked the hepatoprotective effects of HDN in liver I/R injury. In summary, the results of the present study suggested that HDN may protect against liver I/R injury through activating the Akt pathway by ameliorating liver oxidative stress, suppressing inflammation and preventing hepatocyte apoptosis. HDN may be a useful factor for liver injury protection and a potential therapeutic treatment for liver I/R injury in the future.

Introduction

Ischemia-reperfusion (IR) injury remains a key medical challenge because it relates to organ transplantation and alterations in vascular perfusion (1-3). Liver I/R injury frequently occurs as a result of liver transplantation, partial hepatectomy, hemorrhagic shock, trauma, severe infection and metabolic disorder (1,4). I/R injury contributes to 10% of early graft dysfunction following organ transplantation and accounts for acute and chronic rejection (5,6). Much effort has been made to elucidate the underlying mechanisms of I/R injury, but so far little is understood (7,8). Oxidative stress, innate and adaptive immune responses, excessive inflammation, anaerobic metabolism and acidosis are considered to be the main molecular and cellular events that contribute to liver injury following I/R (7-9). However, no approved clinical intervention is currently available for I/R-related liver injury (10). Our latest study indicated that thymic stromal lymphopoietin protects against liver I/R injury via activation of the PI3K/Akt pathway (11). Akt is thought to be a potential therapeutic target in liver I/R injury (12). Thus, seeking factors that can activate Akt pathway may be a promising protection strategy for liver I/R injury.

Hesperidin (HDN) is a bioflavonoid, abundant in citrus fruits, such as oranges, grapefruits and lemons, that serves a role as an antioxidant in biological systems (13). It has been confirmed that HDN has hydrogen radical- and hydrogen peroxide-removal activities (13,14). Additionally, it has been reported that HDN has numerous beneficial effects, such as anti-inflammatory, antibacterial, anticancer and anti-edema properties (13-16). Thus, HDN has a protective effect on sterile organ injuries, including ischemic stroke and arthritis, and infectious diseases such as lipopolysaccharide-induced inflammation (17-20). A number of studies reveal that the activation of PI3K/Akt pathway is one of the most important mechanisms for the biological effects of HDN (16,18,21,22).

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Recently, Park *et al* (23) observed that HDN ameliorates hepatic injury in Sprague-Dawley rats. However, the underlying mechanisms remain unknown.

The present study hypothesized that HDN ameliorates liver I/R injury through the Akt pathway. Wild-type (WT) mice were subjected to liver I/R and administered HDN. HDN ameliorated liver I/R injury, but the hepatoprotective effects of HDN were prevented by an Akt inhibitor. The results of the present study demonstrated that HDN ameliorated liver oxidative stress, suppressed inflammatory responses and prevented hepatocyte apoptosis during liver I/R injury. Thus, HDN administration may be useful to prevent injury after liver I/R.

Materials and methods

Animals and model. A total of 65 male C57BL/6J WT mice (8-10 weeks old, 22-30 g) were purchased and housed in the Guangxi Medical University Laboratory Animal Center (Guangxi, China). The mice were bred in a specific pathogen-free animal facility under controlled conditions at 19-23°C and 40-60% humidity with a 12-h dark/light cycle and had free access to food and water. A warm partial liver I/R injury model with occlusion of 70% segments of the liver blood supply was established as previously described (24). Briefly, the mice were injected intraperitoneally (i.p.) with ketamine (100 mg/kg)-xylazine (5 mg/kg). The left and middle hepatic lobe blood supply was occluded with an atraumatic clamp (Fine Science Tools, Inc.). The clamp was removed after 60 min of ischemia, and the reperfusion procedure lasted for 6 h. HDN was administered orally to the mice by gavage for 3 days before surgery. To ascertain the optimal dose for the 3-day HDN administration, a fixed-dose study was designed with doses of 100, 200 and 400 mg/kg. LY294002 (0.5 mg/kg, i.p.) was administered 30 min before surgery. Sham (treated without HDN) and control (treated with HDN at the dose of 200 mg/kg for 3 days) mice underwent anesthesia, laparotomy and exposure of the portal triad but without vasculature occlusion. Mice were euthanized by exsanguination under anesthesia following reperfusion for 6 h. Mice were anaesthetized with 1.5% isoflurane, 1 ml of blood was harvested by cardiac puncture and liver samples were collected and stored as detailed below. Following blood and tissue collection, euthanasia was confirmed by bilateral thoracotomy and subsequently by the absence of any respiratory movement and heartbeat.

The whole blood of the mice was collected in a covered test tube and allowed to clot by leaving it undisturbed for 20 min at room temperature. The clot was removed by centrifuging at 2,000 x g for 10 min in a refrigerated (4°C) centrifuge. The resulting supernatant was designated serum and was immediately transferred into a clean polypropylene tube using a pipette. The serum was stored at -80°C for further analysis. Part of the liver samples were embedded in 2% paraformaldehyde for histological analysis, and the remainder were frozen in liquid nitrogen and stored at -80°C for further analysis.

The mice were divided into the following groups: i) Sham group (n=8); ii) control group (n=8); iii) I/R group (n=8); iv) I/R + HDN group (n=8/group, at three different concentrations: 100, 200 and 400 mg/kg); v) I/R+LY294002 group (n=8); and vi) I/R+HDN (200 mg/kg) +LY294002 group (n=8).

Reagents. Antibodies for western blotting were as follows: the GAPDH antibody (cat. no. ab8245) was purchased from Abcam, and the Bcl-2 (cat. no. 3498), pro-caspase 3 (cat. no. 9662), cleaved caspase 3 (cat. no. 9661), phosphorylated (p)-Akt (S473) (cat. no. 9271) and Akt antibodies (cat. no. 9272) were purchased from Cell Signaling Technology, Inc. Goat anti-mouse secondary antibody (cat. no. 31430) and goat anti-rabbit secondary antibody (cat. no. 31460, both at 1:20,000 dilution) were from Thermo Fisher Scientific, Inc. HDN (HPLC>98%; cat. no. XW05202631) was obtained from Sinopharm Chemical Reagent Co., Ltd. LY294002 (cat. no. L9908) was purchased from Sigma-Aldrich (Merck KGaA). Malondialdehyde assay kit (MDA; cat. no. A003-1), superoxide dismutase assay kit (SOD; cat. no. A001-1), catalase assay kit (CAT; cat. no. A007-2), glutathione peroxidase assay kit (GPx; cat. no. A006-1) and glutathione assay kit (GSH; cat. no. A006-1) were purchased from Nanjing Jiancheng Bioengineering Institute.

Hepatocyte isolation and culture. Hepatocytes (HCs) were isolated as described previously (25). Mice were euthanized with 5% isoflurane for 5 minutes in a plexiglass chamber, and bilateral thoracotomy was performed for a secondary confirmation of death. The mice were perfused following euthanasia *in vivo* by using an *in situ* collagenase (type VI, Worthington Biochemical Corporation) technique. Next, HCs were separated from nonparenchymal cells (NPCs) by two cycles of differential centrifugation (50 x g for 2 min, 4°C) to ensure that the purification exceeded 99%. Finally, trypan blue exclusion was used to test the viability to ensure that it was greater than 95%. HCs were cultured as described previously (26). Briefly, hepatocytes (1.5x105 cells/ml) were plated on gelatin-coated culture plates with collagen I (BD Pharmingen; BD Biosciences) in Williams medium E (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% calf serum (Thermo Fisher Scientific, Inc.), 15 mM HEPES (Thermo Fisher Scientific, Inc.), 1 μM insulin (Eli Lilly and Company), 2 mM L-glutamine (Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 U/ml; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C. Cells were allowed to attach to plates overnight, and the culture media was replaced with fresh culture media before the cells were treated for experiments.

Immunofluorescence staining. After hepatocytes were cultured for 24 h at 37°C, cells were washed by phosphate-buffered saline (PBS; pH 7.4) once, and then were fixed with 4% paraformaldehyde (Rich Joint) for 30 min at 4°C. Cells were then permeabilized by 0.1% Triton-X 100 (Rich Joint) for 15 min at room temperature, washed twice with PBS, incubated with 5% fetal bovine serum (HyClone; Thermo Fisher Scientific, Inc.) for 15 min at room temperature and then incubated with hepatocyte-specific marker Cytokeratin-18 (CK18) primary antibody (1:1,000 dilution) (cat. no. 10830-1-AP; Wuhan Sanying Biotechnology) overnight at 4°C. Cells were then washed with PBS three times (5 min each) and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:100 dilution; cat. no. SA00003-2; Wuhan Sanying Biotechnology) at 37°C for 1 h, followed by Hoechst 33258 (cat. no. C1017; Beyotime Institute of Biotechnology) for nuclear staining for 15 min at room temperature. Slides
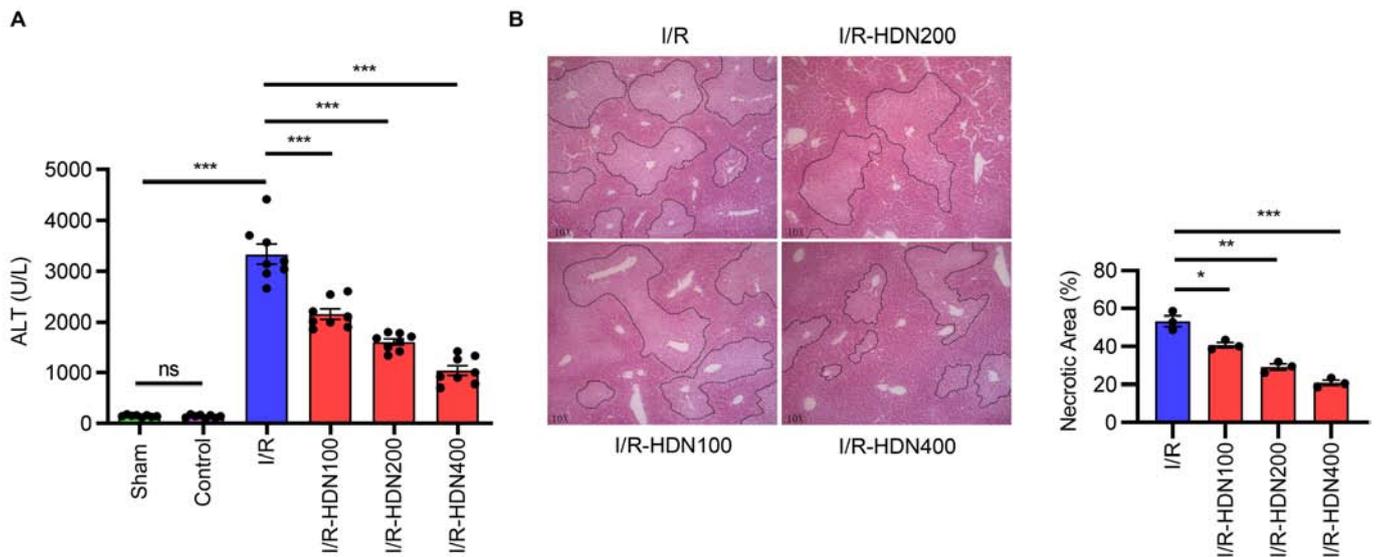
Figure 1. HDN ameliorates liver I/R injury. (A) Serum ALT levels in the livers of mice after sham surgery, control, I/R injury or I/R injury with 100, 200 or 400 mg/kg HDN treatment; n=8 mice/group. (B) Representative hematoxylin and eosin staining images and necrotic areas of ischemic liver lobes of mice at 6 h post-reperfusion with or without HDN treatment; magnification, x10. Dotted lines indicate measured areas of necrosis, quantified on bar graph. n=8 mice/group. *P<0.05, **P<0.01 and ***P<0.001. ALT, alanine aminotransferase; HDN, hesperidin; I/R, ischemia/reperfusion; ns, not significant.
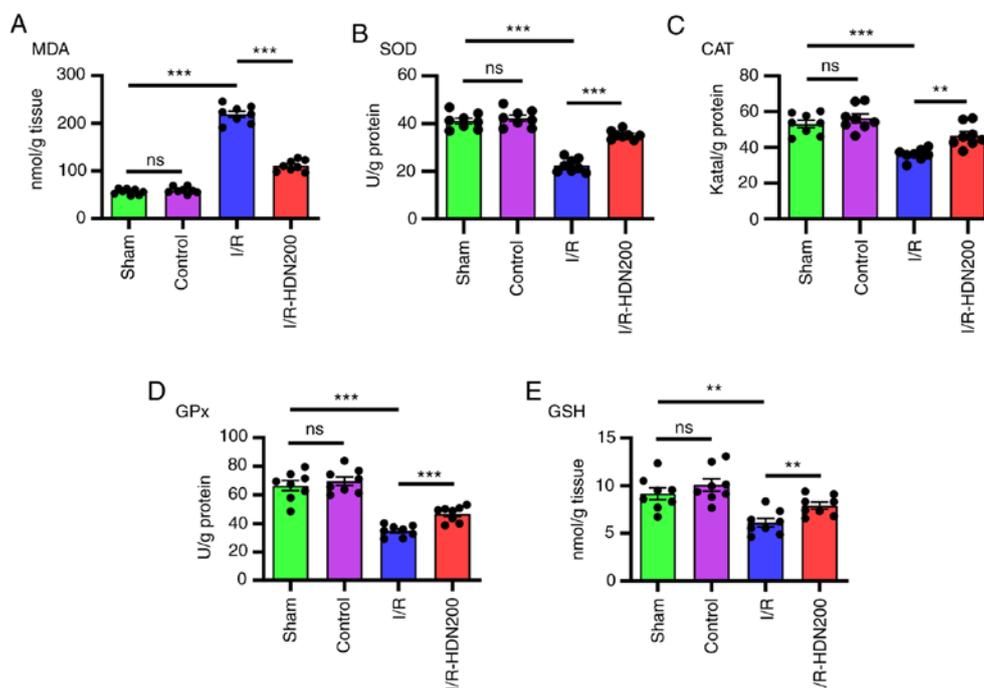

Figure 2. HDN ameliorates liver oxidative stress in liver I/R injury. The levels of (A) MDA, (B) SOD, (C) CAT, (D) GPx and (E) GSH of liver lobes in sham, control, I/R or I/R with HDN treatment (200 mg/kg) groups. n=8 mice/group; **P<0.01 and ***P<0.001. CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; HDN, hesperidin; I/R, ischemia/reperfusion; MDA, malondialdehyde; ns, not significant; SOD, superoxide dismutase.

were viewed using an Olympus IX71 fluorescence microscope (Olympus Corporation).

Serum sample assays. Serum alanine aminotransferase (ALT) levels were measured by alanine aminotransferase assay kit using standard spectrophotometric procedures with 5 µl serum according to the manufacturer's instructions (cat. no. C009-2-1, Nanjing Jiancheng Institute of Biotechnology). The levels of ALT were expressed as units/L serum (U/L).

Histological analysis. Liver tissues were harvested as described in the Animals and model subsection, embedded in 2% paraformaldehyde for 2 h at 4°C and then removed for preservation at 4°C with 70% ethanol. Liver sections (5 µm) were stained with hematoxylin and eosin (H&E) to assess histopathology. Images of five randomly selected fields were acquired with a light microscope (magnification, x10). Necrotic areas were analyzed by ImageJ software (version 1.51w, National Institutes of Health).
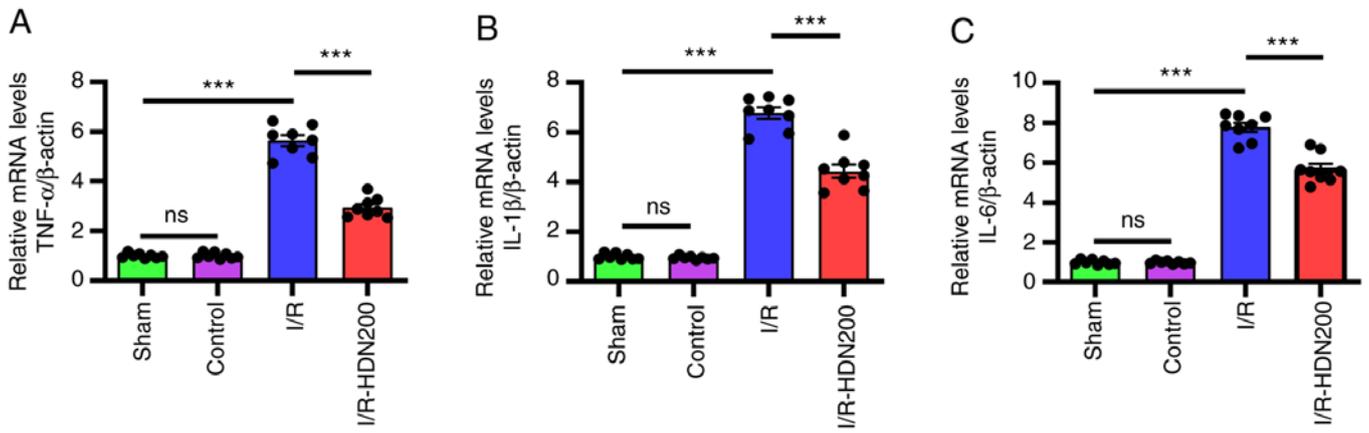
Figure 3. HDN suppresses inflammation during liver I/R injury. mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 assessed by reverse transcription-quantitative PCR in liver tissues from mice in sham, control, I/R or I/R with HDN treatment (200 mg/kg) groups; β-Actin served as a control. n=8 mice/group. Results are expressed as the relative fold increase from three experiments. ***P<0.001. HDN, hesperidin; I/R, ischemia/reperfusion; ns, not significant.
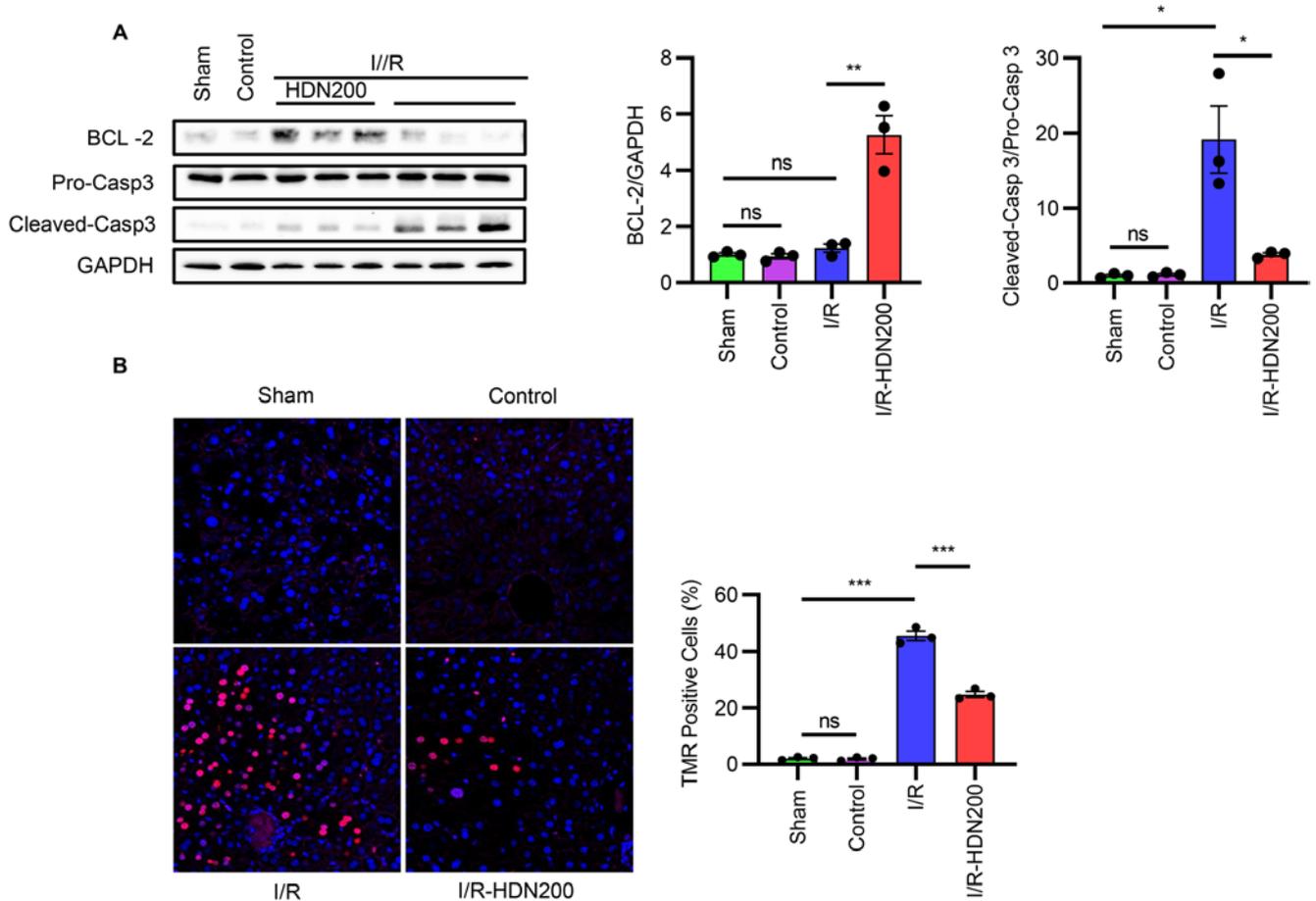

Figure 4. HDN protects the liver against apoptosis in liver I/R injury. (A) Western blotting analysis of Bcl-2 and cleaved-casp3 protein expression in the liver of mice after I/R injury with or without HDN treatment. (B) Confocal images from mouse livers stained with TMR red (red) and Hoechst nuclear stain (blue) after sham surgery or liver I/R injury with or without HDN (200 mg/kg) treatment. Magnification, x40. Percentage of TMR-positive cells was quantified using ImageJ software and represented in a bar graph. n=8 mice/group; *P<0.05, **P<0.01 and ***P<0.001. Casp3, caspase 3; HDN, hesperidin; I/R, ischemia/reperfusion.; ns, not significant.

Reverse transcription-quantitative PCR (RT-qPCR). The ischemic lobe of the liver was frozen in liquid nitrogen and stored at -80°C for comparative PCR analysis. Total RNA was extracted with the RNeasy Mini Kit (Qiagen China Co., Ltd.) according to the manufacturer's instructions. cDNA was synthesized using 1 μg of RNA and with 2 μM oligodT primers (Qiagen China Co., Ltd.), 4 U Omniscript™ reverse transcriptase (Qiagen China Co., Ltd.), 4 μl of 5X RT buffer, and 16 μl RNA in RNase-free water. The synthesis of cDNA was performed at 37°C for 60 min. SYBR Green PCR Master Mix
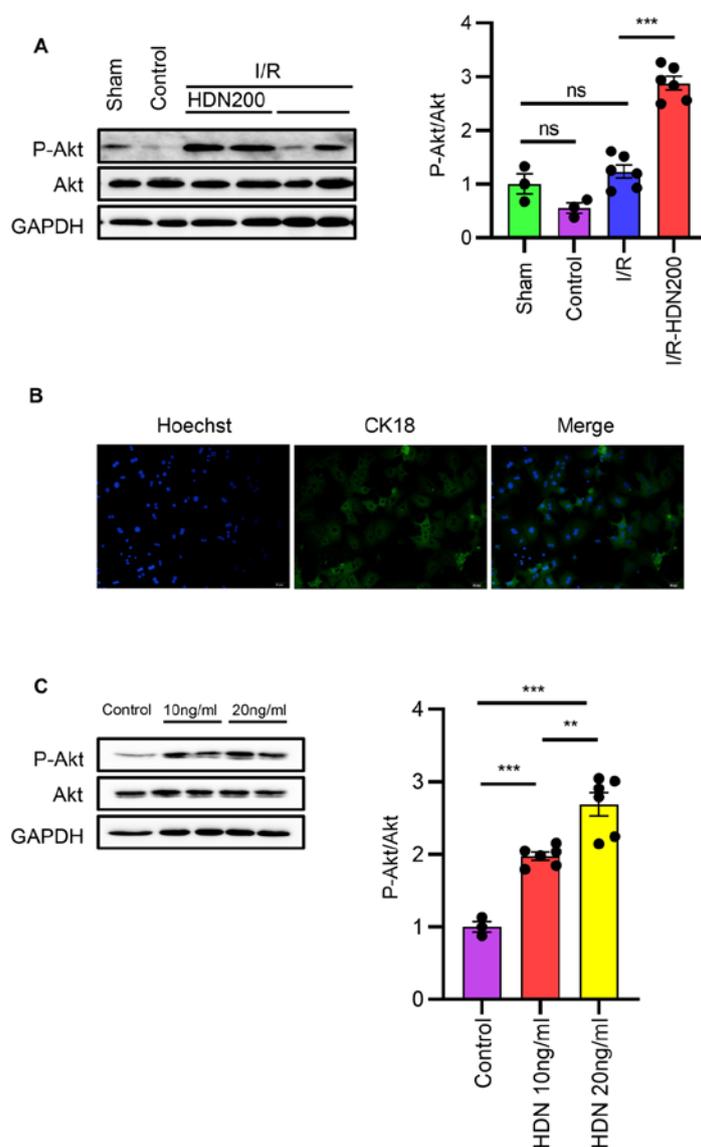
Figure 5. HDN activates the Akt pathway in liver I/R injury *in vivo* and in hepatocytes *in vitro*. (A) Western blotting showing the levels of total and p-Akt in liver of mice after liver I/R injury with or without HDN treatment. Each lane represents a separate animal. (B) Immunofluorescence staining showing Hoechst (blue) and CK18 (green) positive cells. Magnification, x200 (C) Western blotting showing the levels of total and p-Akt in primary hepatocytes after exposed to different doses of HDN (10 and 20 ng/ml; 6 h) *in vitro*. The blots shown are representative of three experiments with similar results. **P<0.01 and ***P<0.001. CK18, cytokeratin-18; HDN, hesperidin; I/R, ischemia/reperfusion; p-, phosphorylated; ns, not significant.

(Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to prepare the PCR reaction mixes. qPCR was performed with sense and antisense primer pre-validated and specific for β-actin, IL-1β, IL-6, and TNF-α (Qiagen China Co., Ltd.) with the following thermocycling parameters: Initial denaturation at 94°C for 5 min; followed by 40 cycles of 94°C for 15 sec, 60°C for 20 sec and 72°C for 30 sec. The reaction cocktail used for test contained 10 μl iTaq SYBR Green PCR Master Mix, 8 μl RNase-free water, 0.5 μl sense primer, 0.5 μl antisense primer and 1 μl cDNA. All samples were assayed in duplicate and normalized to β-actin mRNA abundance. Gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (27). The primers used for qPCR were as follows: β-actin, forward 5'-TGTGATGGTGGGAATGGGTCAG-3', reverse 5'-TTT GATGTCACGCACGATTTCC-3'; IL-1β, forward 5'-CCA GCTTCAAATCTCACAGCAG-3', reverse 5'-CTTCTTTGG GTATTGCTTGGGATC-3'; IL-6, forward 5'-TCCAGTTGC

CTTCTTGGGAC-3', reverse 5'-GTACTCCAGAAGACC AGAGG-3'; and TNF-α, forward 5'-CACAGAAAGCATGAT CCGCGACGT-3', reverse 5'-CGGCAGAGAGGAGGTTGA CTTTCT-3'.

Western blotting analysis. Protein expression was determined in frozen liver tissues using a previously described western blotting protocol (11). Briefly, snap-frozen liver was homogenized in cell lysis buffer (Cell Signaling Technology, Inc.) and centrifuged (16,000 x g for 15 min, 4°C), after which the supernatant was collected. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Loading buffer was added to the samples (30 μg/well), which were then resolved by 10 or 15% SDS-PAGE. Samples were then transferred to a polyvinylidene difluoride membrane at 250 mA for 2 h. The membrane was blocked in 5% milk for
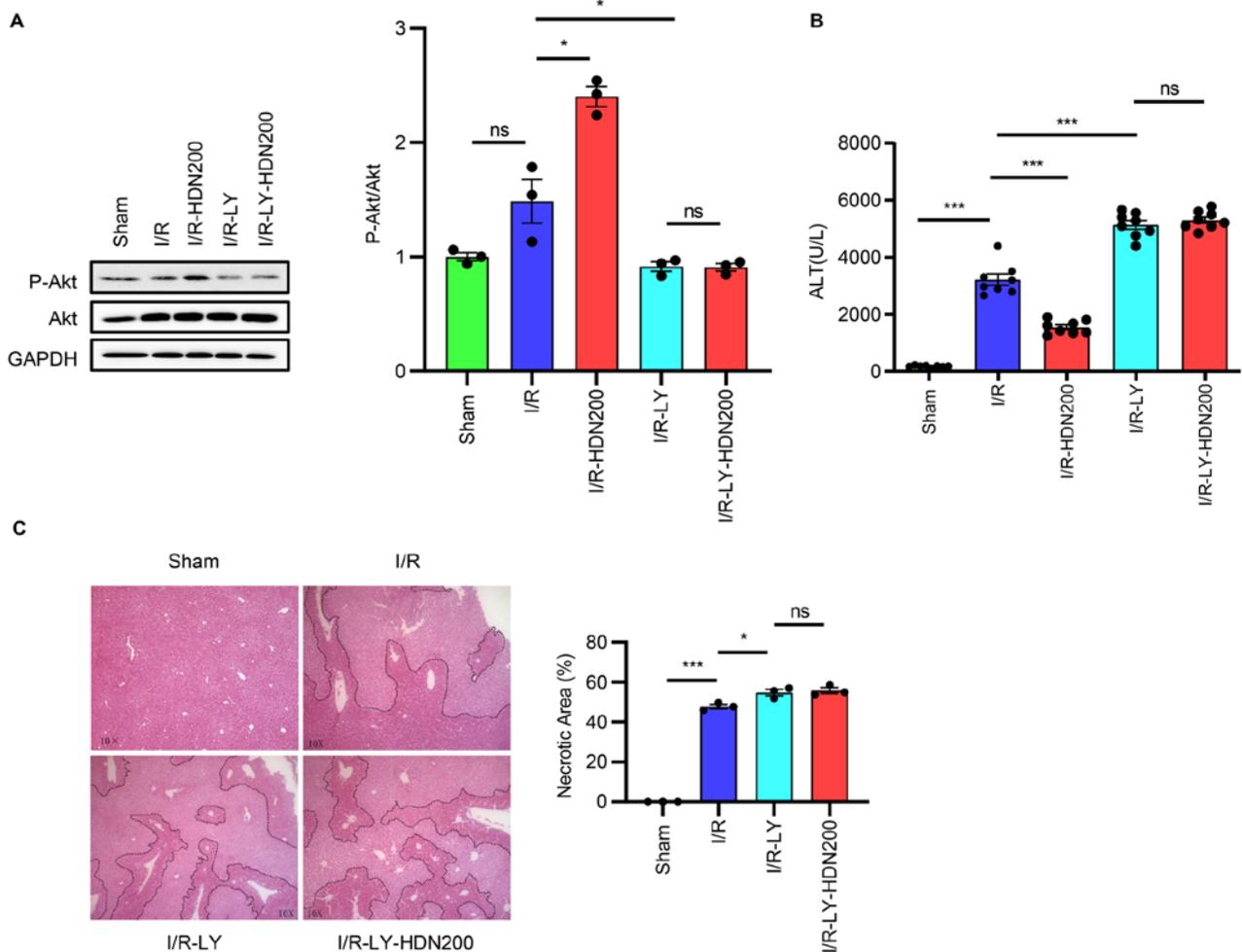
Figure 6. HDN ameliorates liver I/R injury via the Akt signaling pathway. (A) Western blotting showing the levels of total and p-Akt in liver of mice after I/R injury with HDN (200 mg/kg) or Akt inhibitor (LY294002) or LY294002 plus HDN treatment. Each lane represents a separate animal. The blots shown are representative of three experiments with similar results. (B) Serum ALT levels of mice after liver I/R injury treated with HDN (200 mg/kg), LY294002 or LY294002 plus HDN treatment. (C) Representative hematoxylin and eosin staining images and necrotic areas of ischemic liver lobes of mice at 6 h post-reperfusion with LY294002 or LY294002 plus HDN treatment; dotted lines indicate measured areas of necrosis, quantified on bar graph. Magnification, x10. n=8 mice/group; *P<0.05 and ***P<0.001. ALT, alanine aminotransferase; HDN, hesperidin; I/R, ischemia/reperfusion; LY, LY294002; ns, not significant; p-, phosphorylated.

1 h at room temperature and then incubated with primary antibody (1:1,000 dilution) in 1% milk overnight at 4°C. Membranes were washed in Tris-buffered saline-0.05% Tween-20 (TBST) for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution) for 1 h and then washed for 1 h in TBST at room temperature, before being developed by an enhanced chemiluminescence kit (Thermo Fisher Scientific Inc.). The signal was acquired and quantified with a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc.).

Cell death detection assay. Cell death was detected as described previously (25). Briefly, liver tissues were incubated with the *In-Situ* Cell Death Detection TMR Red kit (cat. no. 12156792910; Roche Diagnostics) according to the manufacturer's instructions. Images (magnification, x40) were captured with a Nikon A1 confocal microscope (Nikon Instruments) from at least six randomly selected fields/sections. Dead cells were quantified by NIS Elements (version 4.1, Nikon Instruments).

Statistical analysis. All experiments were performed in triplicate. All data are presented as the mean ± standard error of the mean. Two-tailed unpaired Student's t-test was performed to compare the differences between two experimental groups. One-way ANOVA followed by a Bonferroni post hoc test was performed to compare the differences between multiple groups. GraphPad Prism (version 8.0.2; GraphPad Software, Inc.) was used to perform the data analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Hesperidin ameliorates liver I/R injury. To determine the role of HDN in liver I/R, WT mice were subjected to warm partial liver I/R injury with or without HDN treatment. Liver injury was assessed by detecting serum ALT levels and morphological indexes (H&E staining). Compared with that of the sham group the ALT levels indicated that liver damage was markedly increased after liver I/R (Fig. 1A). There was
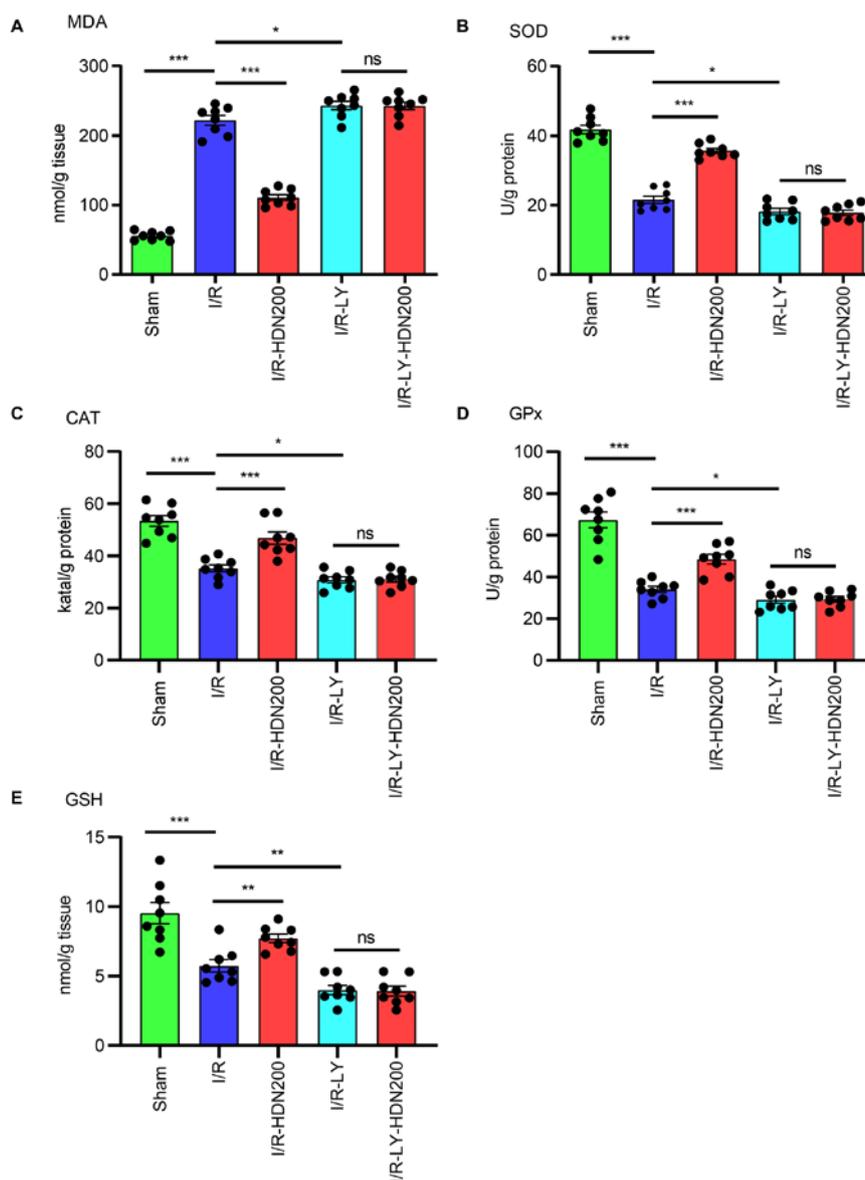
Figure 7. HDN ameliorates liver oxidative stress during I/R injury through the Akt signaling pathway. The levels of (A) MDA, (B) SOD, (C) CAT, (D) GPx and (E) GSH of livers in sham, I/R, I/R with HDN treatment (200 mg/kg), I/R with Akt inhibitor (LY294002) treatment or I/R with LY294002 plus HDN treatment groups. n=8 mice/group; *P<0.05, **P<0.01 and ***P<0.001. CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; HDN, hesperidin; I/R, ischemia/reperfusion; LY, LY294002; MDA, malondialdehyde; ns, not significant; SOD, superoxide dismutase.

no effect on ALT levels in control group but without liver I/R (Fig. 1A). However, HDN significantly ameliorated liver I/R injury in a dose-dependent way (Fig. 1A). H&E staining also indicated that the necrotic areas of the ischemic hepatic lobe were significantly attenuated by HDN treatment (Fig. 1B). In order to explore the underlying mechanisms, the median concentration of HDN (200 mg/kg) was used for the following experiments.

Hesperidin ameliorates liver oxidative stress in liver I/R injury. Oxidative stress is considered to serve a key role in liver I/R injury (9,28). It has been reported that HDN eliminates oxidative stress in a number of diseases, such as toxin-induced tissue damage and inflammation (13,14). To ascertain whether HDN ameliorates oxidative stress during liver I/R, the levels of MDA, SOD, CAT, GPx and GSH in ischemic liver lobes were

measured using commercial kits. Notably, compared with that of the sham group MDA content increased significantly after liver I/R, and this was significantly reversed by HDN treatment (Fig. 2A). In addition, compared with that of the sham group the antioxidant activities of SOD, CAT, GPx and GSH, decreased significantly after liver I/R, and these effects were reversed by HDN treatment (Fig. 2B-E).

Hesperidin suppresses inflammation during liver I/R injury. Inflammation serves important roles in liver I/R injury, and numerous cytokines are involved in liver injury (7,29). To determine the relationship between HDN and inflammation during liver I/R injury, the mRNA levels of TNF-α, IL-6 and IL-1β in ischemic liver lobes were assessed by RT-qPCR. Consistent with a previous study (5), the results demonstrated that compared with those of the sham group the mRNA levels
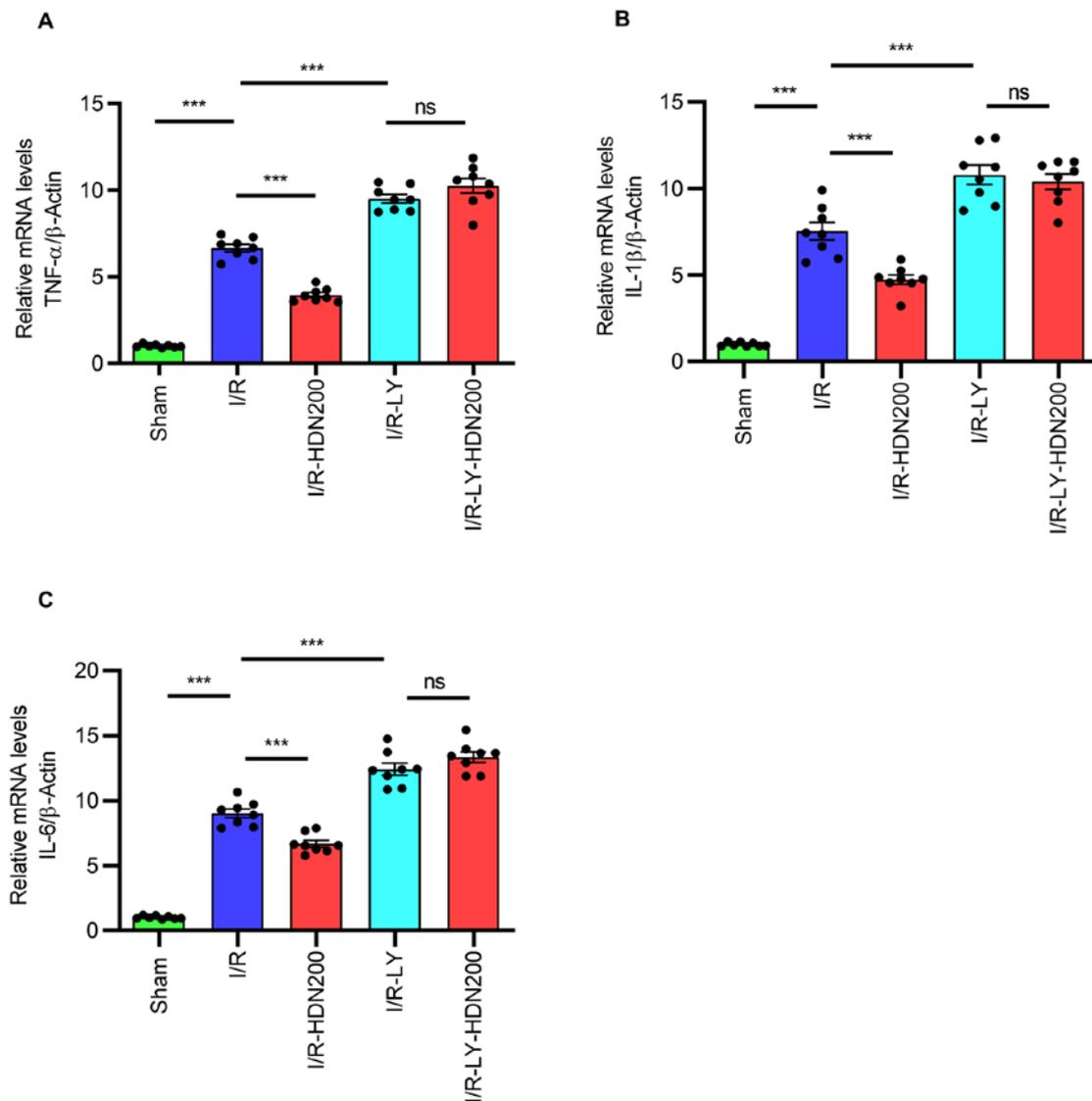
Figure 8. HDN suppresses liver inflammation during I/R injury through the Akt signaling pathway. mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 assessed by reverse transcription-quantitative PCR in liver tissues from mice in sham, I/R, I/R with HDN treatment (200 mg/kg), I/R with Akt inhibitor (LY294002) treatment or I/R with LY294002 plus HDN treatment groups; β-Actin served as a control. n=8 mice/group. Results are expressed as the relative fold increase from three experiments. ***P<0.001. HDN, hesperidin; I/R, ischemia/reperfusion; LY, LY294002; ns, not significant.

of TNF-α, IL-6 and IL-1β in liver tissues increased significantly after I/R and HDN significantly attenuated these effects (Fig. 3A-C).

Hesperidin protects the liver against apoptosis following I/R injury. Inhibition of apoptosis has been reported to have a modest protective effect on liver I/R injury (30). Thus, apoptotic markers in the livers of WT mice with or without HDN treatment after liver I/R injury was assessed. Western blotting results demonstrated that the expression levels of cleaved-caspase 3 increased markedly after liver I/R and were significantly reversed after HDN treatment (Fig. 4A). By contrast, no significant differences were observed in the levels of Bcl-2 in the liver after I/R compared with those of the sham group, but HDN treatment significantly increased the levels of Bcl-2 compared with those of the I/R group (Fig. 4A). In addition, TMR assay results demonstrated that there were significantly fewer TMR-positive apoptotic cells in livers in

the I/R group treated with HDN compared with the number of apoptotic cells in the untreated I/R group (Fig. 4B).

Hesperidin activates the Akt pathway in liver I/R injury in vivo and in hepatocytes in vitro. Having determined that HDN ameliorates liver I/R injury, the mechanisms underlying the modulation of cellular functions by HDN were next examined. Akt serves a key role in protecting against liver I/R injury (11,12), and several previous studies indicated that HDN induces Akt phosphorylation and influences apoptosis (16,18,21,22). Thus, the relationship between HDN and the Akt pathway in liver I/R injury was investigated. No significant differences were observed in the levels of p-Akt in the liver after I/R compared with those of the sham group, but HDN increased the expression levels of p-Akt compared with those of the untreated I/R group (Fig. 5A). To ascertain the role of HDN in phosphorylating Akt in HCs, HCs were isolated and cultured *in vitro* (Fig. 5B). Cultured HCs were
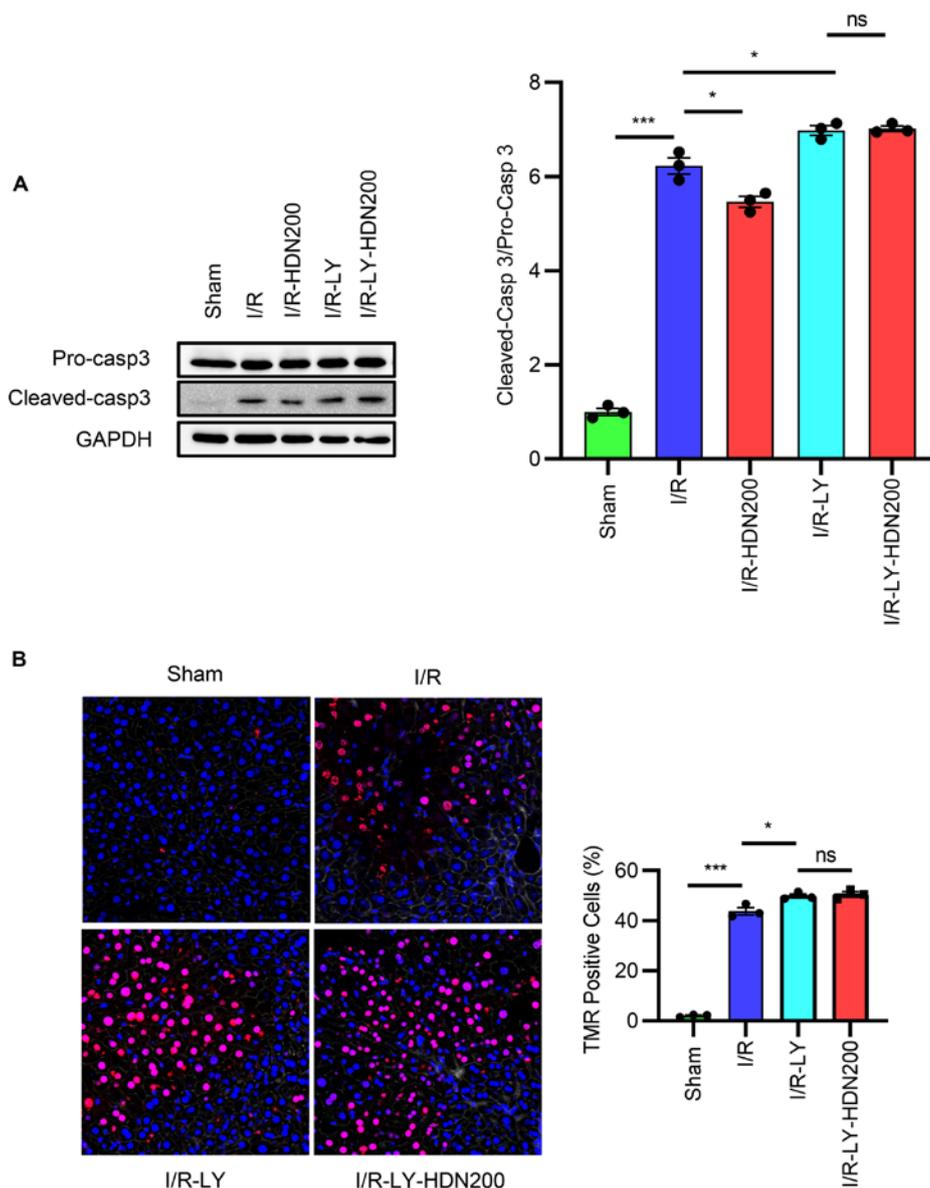
Figure 9. HDN protects the liver against apoptosis during I/R injury through the Akt signaling pathway. (A) Western blotting analysis of cleaved-casp3 in the liver of mice in the sham, I/R, I/R with HDN treatment (200 mg/kg), I/R with Akt inhibitor (LY294002) treatment or I/R with LY294002 plus HDN treatment. Each lane represents a separate animal. The blots shown are representative of three experiments with similar results. (B) Confocal images from mice liver stained with TMR red (red) and Hoechst nuclear stain (blue) after liver I/R injury with or without HDN treatment in the presence of LY294002. Magnification, x40. n=8 mice/group. Percentage of TMR-positive cells was quantified using ImageJ software and represented in a bar graph. *P<0.05, ***P<0.001. Casp3, caspase 3; HDN, hesperidin; I/R, ischemia/reperfusion;LY: LY294002; ns; not significant.

exposed to HDN for 6 h, and the results demonstrated that 10 and 20 ng/ml HDN increased the levels of phosphorylated Akt in HCs (Fig. 5C).

Hesperidin ameliorates liver I/R injury through the Akt pathway. To elucidate whether activation of the Akt pathway is necessary for HDN to ameliorate liver I/R injury, LY294002, which inhibits Akt activation and exacerbates liver injury after I/R, was used (11,31). The results demonstrated that compared with those of the I/R group the expression levels of p-Akt were significantly decreased after administration of LY294002 (Fig. 6A). Notably, these effects were not rescued by the administration of HDN during liver I/R (Fig. 6A). In addition, consistent with the results of p-Akt, the levels of serum ALT increased significantly after administration of

LY294002 compared with those of the I/R group (Fig. 6B). Furthermore, the protective effect of HDN was eliminated after administration of LY294002 (Fig. 6B). The elimination of the protective effect of HDN in the presence of LY294002 was also confirmed by the H&E staining index (Fig. 6C).

Having determined that HDN ameliorated liver oxidative stress during liver I/R injury, whether the hepatoprotective effects were eliminated by the Akt inhibitor LY294002 was investigated. The results demonstrated that LY294002 significantly increased MDA levels and significantly decreased the levels of SOD, CAT, GPx and GSH compared with those of the respective I/R group, and these effects were unaffected by HDN in the presence of LY294002 (Fig. 7).

Having determined that the inflammatory response increased significantly after liver I/R and decreased markedly

with HDN treatment, whether these effects were regulated by the Akt pathway was investigated. Cytokine expression levels after LY294002 administration were assessed; the results demonstrated that LY294002 significantly increased the mRNA expression levels of TNF-α, IL-6 and IL-1β compared with those of the untreated I/R group and that these effects were unaffected by co-treatment with HDN (Fig. 8).

It has been reported that the Akt inhibitor LY294002 promotes apoptosis during liver I/R (12,32); thus, the effects of HDN and the Akt pathway in liver I/R were investigated. The results demonstrated that compared with those of the I/R group the levels of cleaved-caspase 3 significantly increased after treatment with LY294002 and that these effects were unaffected by HDN treatment in the presence of LY294002 (Fig. 9A). In addition, TMR assays demonstrated that there were a similar number of TMR-positive apoptotic cells in livers co-treated with LY294002 and HDN or LY294002 alone (Fig. 9B).

Discussion

HDN is a compound that is extracted from citrus fruits (13), that has been demonstrated to have potent pharmacological effects against oxidative stress, inflammation, microbes and cancer (13-16). However, the exact role and mechanisms of HDN in liver I/R injury remains unknown. The results of the present study demonstrated that HDN ameliorated liver I/R injury by decreasing liver oxidative stress, suppressing inflammatory responses and preventing hepatocyte apoptosis through the activation of the Akt pathway. Thus, HDN may be a useful factor for protection against liver injury. These findings raise the possibility that HDN may be used to ameliorate liver injury during I/R.

During the ischemia phase, blood flow is blocked and hepatocytes suffer from anaerobic metabolism and oxidative stress, which are considered to be the initiation of ischemic injury (9). Factors involved in oxidative stress, such as reactive oxygen species and nitric oxide, have been reported to have important roles in liver I/R injury (9). Oxygen radicals cause lipid peroxidation of cellular membranes, resulting in inflammatory cell infiltration, neutrophil activation and hepatic cell injury (7-9,28). MDA, which is an oxidative damage indicator, is an essential product of lipid peroxidation (33). SOD, CAT, GPx and GSH, which can eliminate free radicals, are antioxidant indicators (34,35). To determine the oxidative stress in liver I/R, the oxidative damage and antioxidant parameters were evaluated in the present study. The results demonstrated that MDA content increased markedly after liver I/R and was reversed by HDN treatment. The antioxidant indicators, SOD, CAT, GPx and GSH, decreased significantly after liver I/R; however, these levels were increased by HDN treatment. These data suggested that HDN may ameliorate liver oxidative stress during liver I/R injury and were consistent with previous studies (36-39). In a sepsis-induced lung injury model, Yuan *et al* (37) reported that oxidative stress was attenuated by HDN. In a sodium arsenite-induced nephrotoxicity and hepatotoxicity model, Turk *et al* (38) observed that HDN reduced oxidative stress and oxidative DNA damage depending on the dose. Similar results were observed in bilateral common carotid artery occlusion reperfusion injury (36) and in skeletal

muscle I/R injury (39). However, how HDN ameliorates oxidative stress remains unknown.

Oxidative stress and nutrient deprivation initiate liver damage, which releases damage-associated molecular patterns (DAMPs), such as high mobility group box 1 and histone/DNA complexes (8). DAMPs induce inflammatory cell infiltration and activate innate immune responses, which leads to the release of pro-inflammatory cytokines and chemokines (8,40,41). In the subsequent reperfusion phase, the inflammatory responses become more extensive and cause more inflammatory mediator release (8,40,41). The pro-inflammatory milieu recruits and activates more innate and adaptive immune cells into the inflamed liver and causes more hepatocyte death (8,40,41). Thus, the inflammatory response is both the result from and cause of liver damage. The results of the present study demonstrated that the mRNA expression levels of TNF-α, IL-6 and IL-1β in liver tissues increased significantly after I/R, and that HDN significantly attenuated these expression levels after liver I/R. These results suggested that HDN may attenuate inflammation in I/R. In a previous study, HDN was reported to reduce the secretion of inflammatory cytokines, differentiation and proliferation of cardiac fibroblasts by TGF-β through the Notch 1 signaling pathway (42). In another infectious disease study, Ye *et al* (43) reported that HDN pretreatment significantly attenuated lipopolysaccharide-induced pulmonary injury and total protein concentration and markedly decreased the number of neutrophils and the levels of the inflammatory cytokines TNF-α and IL-6 in an acute lung injury model *in vivo* and *in vitro*. Additionally, they also found that HDN binds directly with myeloid differentiation 2 (MD2) and inhibits MAPK activation, regulates Iκβ degradation, and blocks the interaction of MD2 and its co-receptor Toll-like receptor 4 TLR4 (43). Thus, HDN attenuates inflammation in liver I/R. However, the underlying mechanisms need to be further explored.

Several previous studies have indicated that apoptosis serves a key role in liver I/R (5,30,44) and that inhibiting apoptosis attenuates liver injury (30). Additionally, it is reported that HDN ameliorates apoptosis in many situations (22,45-47). The results of the present study demonstrated that HDN suppressed apoptosis in liver I/R injury, as indicated by increased Bcl-2 and decreased cleaved-caspase 3 protein expression levels. In addition, HDN induced decreases in apoptotic cells were confirmed by TMR assays. Taken together, these data suggested that HDN suppressed apoptosis in liver I/R injury. These results corroborate with the effects of HDN in other I/R models. For example, Li *et al* (22) observed that short-term pretreatment with HDN protected against myocardial I/R injury by suppressing myocardial apoptosis. He *et al* (46) also observed that hesperetin (an active metabolite of HDN) post-treatment significantly inhibited apoptosis by increasing the expression of Bcl-2 and decreasing the expression of Bax and cleaved-caspase 3, which diminished the apoptotic cardiomyocyte ratio in an *in vitro* cardiomyocyte model of hypoxia/reoxygenation injury. HDN also attenuated cerebral I/R injury through inhibiting the apoptotic pathway (47). However, studies have also reported that HDN induces apoptosis to reduce cancer cell prolif-

eration (15,48). These differences may be due to the acute nature of I/R injury compared with the chronic induction of carcinoma.

The PI3K/Akt pathway is a protective pathway in liver I/R injury (11,12,32,44,49). Akt is considered to be a potential therapeutic target for liver I/R (12). HDN activates the Akt pathway to protect cardiomyocytes *in vivo* and *in vitro* (22,46,50). Consistent with these previous studies (22,46,50), the present study demonstrated that HDN increased the levels of p-Akt after liver I/R injury and that treatment with the Akt inhibitor LY294002 diminished the protective effects of HDN in liver I/R injury. Thus, these results suggested that HDN supports Akt signaling to protect against liver I/R injury.

In conclusion, the present study revealed that HDN may protect against liver I/R injury by ameliorating oxidative stress, suppressing inflammatory responses and decreasing apoptosis through activating the Akt pathway. HDN is a potential therapeutic factor for treating liver I/R injury.

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Availability of data and materials

The datasets used and /or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

SL designed and performed the majority of experiments, established the model mice, collected and analyzed the experimental data, and drafted the manuscript. QQ and DL performed the serum analysis and the H&E staining. WP and YW isolated and cultured hepatocytes, and performed the immunofluorescence staining. YX performed the RT-qPCR and western blot analysis. JZ and LS supervised the project, provided technical advice, conducted analyses of the raw data, and reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal protocols were approved by The Animal Care and Use Committee of The First Affiliated Hospital of Guangxi Medical University (Nanjing, China), and the experiments were performed in adherence to the National Institutes of Health guidelines for the use of laboratory animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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