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RESEARCH PAPER

Inhibition of Endogenous Hedgehog Signaling Protects Against Acute Liver Injury After Ischemia Reperfusion

Akshay Pratap • Ravikiran Panakanti • Ningning Yang • James D. Eason • Ram I. Mahato

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ABSTRACT

Purpose Although Hedgehog (Hh) signaling is required for endodermal commitment and hepatogenesis, the possibility that it regulates liver injury after ischemia reperfusion (I/R) has not been considered. Therefore, we determined the expression pattern of Hh signaling and its role in liver injury following I/R using Hh antagonist cyclopamine (CYA).

Methods Sprague-Dawley rats were randomly divided into three groups. Sham group underwent a sham operation with no liver I/R. Vehicle or CYA preconditioned I/R groups underwent liver ischemia for 90 min followed by reperfusion for 1 h. Liver tissue and blood were analyzed for gene expression, histological and biochemical evaluation.

Results Hedgehog ligands were upregulated after reperfusion injury. Serum levels of aspartate transaminase and alanine transaminase, inflammatory cytokines, neutrophil infiltration, and tissue damage were significantly less in CYA-pretreated rats compared with vehicle-pretreated rats. CYA also decreased the phosphorylated form of JNK and ERK.

Conclusions This study provides evidence that endogenous Hh signaling is an early mediator of liver injury and inflammation after I/R. CYA abrogates normothermic I/R injury in rats by inhibiting the MAPK pathway and decreasing the acute inflammatory response. This novel strategy of preconditioning livers with Hh antagonist may have effective therapeutic potential in preventing acute liver injury.

KEY WORDS Hedgehog signaling · liver ischemia reperfusion · cyclopamine

ABBREVIATIONS

CYA	cyclopamine
ERK	extracellular signal regulated kinase
Hh	Hedgehog
HBCD	2-hydroxypropyl-b-cyclodextrin
ICAM-1	inter-cellular adhesion molecule
IL-6	interleukin-6
I/R	ischemia reperfusion
JNK	c-Jun NH_2 terminal kinase
MAPK	Mitogen activated protein kinase
Ptch-1	Patched-1
ROS	reactive oxygen species
sALT	serum alanine transaminase
sAST	serum aspartate transaminase
Shh	Sonic Hedgehog
TNF-α	tumor necrosis factor α

INTRODUCTION

Hepatic ischemia reperfusion (I/R) injury is a phenomenon whereby restoration of blood flow accentuates tissue damage in an ischemic organ. Ischemia reperfusion injury to the liver occurs in several clinical circumstances, including liver transplantation, trauma, hemorrhagic and endotoxic shock (1–3), leading to life-threatening liver failure and, more importantly, primary non-function of liver allografts (4,5). The process of I/R injury is associated

A. Pratap · J. D. Eason
Division of Solid Organ Transplantation, Methodist University Hospital Transplant Institute
Memphis, Tennessee 38104, USA

R. Panakanti · N. Yang · R. I. Mahato (✉)
Department of Pharmaceutical Sciences, University of Tennessee Health Sciences Center
19 South Manassas, CRB RM224,
Memphis, Tennessee 38103-3303, USA
e-mail: rmahato@uthsc.edu
URL: http://www.uthsc.edu/pharmacy/rmahato

with Kupffer cell activation, release of pro-inflammatory cytokines, and generation of reactive oxygen species (ROS) (6,7). Better understanding of the pathogenesis of I/R injury and the availability of an agent that could attenuate I/R injury would have important clinical implications.

There is growing proof of data suggesting that the genetic pathways that initially direct organ morphogenesis during development are recruited later to regulate cell proliferation and tissue repair following injury (8,9). Hedgehog (Hh) signaling is one such transcriptional pathway essential to embryonic development and mediates the patterning of a diverse range of vertebrate structures (10–12). In addition to its roles in embryonic development, there is abundant data suggesting that Hh signaling is involved in the regeneration of various tissues, including the lens, limbs, vasculature, prostate, retina, facial nerve, bile duct, lung, and bone after injury (13–15).

In this study, we determined the role of Hh pathway as an early mediator of inflammation and cell injury after liver I/R. We show that Hh ligands and transcription factors are up-regulated in the liver after warm ischemia reperfusion *in vivo*. Antagonism of Hh pathway prevented hepatocellular damage and suppressed the activation of inflammatory cytokines. In addition, we show that the Hh-MAP kinase interaction plays a key role in I/R injury.

MATERIALS AND METHODS

Materials

Cyclopamine (CYA) free base was purchased from LC Laboratories (Woburn, MA) and TNF-α ELISA kit from eBioscience (San Diego, CA), respectively. Lactated Ringer's solution and isoflurane were purchased from Baxter Healthcare Corp. (Deerfield, IL). Goat anti-rabbit Sonic Hedgehog, Gli-1, P-selectin, ICAM-1, IL-6, p-JNK and ERK primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, CA). Rabbit anti-goat Patched-1 was purchased from Novus Biologics (Littleton, CO), and donkey anti-rabbit phosphohistone 3 from Cell Signaling Technology, Inc. (Danvers, MA). Goat anti-rabbit Alexa Fluor 488 and Alexa Fluor 594 and rabbit anti-goat Alexa Fluor 488 secondary antibodies and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA). SYBR Green real-time PCR master mix and reverse transcription reagents were purchased from Applied Biosystems (Foster city, CA). Atraumatic vascular clip was procured from Harvard Apparatus (Holliston, MA). 2-Hydroxypropyl-b-cyclodextrin (HBCD) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Ischemia Liver Reperfusion Injury

Animal experiments were performed as per the NIH (http://grants1.nih.gov/grants/olaw/references/phspol.htm) and institutional animal care and use guidelines using the protocol approved by the University of Tennessee Animal Use and Care Committee. To induce ischemia liver reperfusion (I/R) injury rat model, Sprague-Dawley rats weighing between 200–250 g were anesthetized with isoflurane (16). An upper midline abdominal incision was made, the portal circulation to the median and left lateral lobes of the liver was carefully dissected, and an atraumatic vascular clip was placed on the vessels, interrupting the portal venous and hepatic arterial blood supply to these lobes. Evidence of ischemia during the clamping period was confirmed by tissue blanching. Two ml of sterile saline were applied over the abdominal viscera to keep the organs moist and to make up for the loss of body fluid. The abdomen was closed with sterile staple sutures to prevent dehydration and possible contamination. The animals were kept in the recovery room under close supervision. After 90 min of partial hepatic ischemia, the clamp was removed, and reperfusion was resumed. Evidence of reperfusion was confirmed by immediate color change of the ischemic lobes. Sham-operated mice underwent the same procedure without vascular clamping. The abdomen was closed in a double layer using 3–0 nylon, and 2 ml sterile lactated Ringer's solution was administered subcutaneously to compensate for operative fluid loss. During the reperfusion, the mice were kept in clean cages. After 1 h of reperfusion, blood was collected from cardiac puncture for biochemical analysis, and rats were euthanized. Serum was collected immediately after euthanasia by centrifuging the blood at 10,000 rpm for 7 min at room temperature and stored at −80°C for future use. Livers and kidneys were harvested for histological studies as described below.

Experimental Groups

Rats were allocated randomly into the following groups. (1) Sham-group rats were treated with saline and subjected to the surgical procedure alone, except that the blood vessels were not occluded, and the rats were maintained under anesthesia for the duration of the experiment ($n=10$). (2) I/R-group rats were subjected to I/R injury ($n=10$). (3) The Cyclopamine group was identical to the I/R group, but rats were administered Cyclopamine (CYA) at a dose of 10 mg/kg daily intraperitoneally (i.p.) daily for three consecutive days and 1 h before reperfusion, ($n=10$) to stimulate a protective effect against an ischemic insult. CYA was formulated in 10% 2-hydroxypropyl-β-cyclodextrin (HBCD) solution and stored at −40°C till further use. (4)

The vehicle group was identical to CYA group, but rats were administered only 10% HBCD for three consecutive days and 1 h before reperfusion ($n=10$). The dose of CYA used in the study was based on previous *in vivo* studies (17). Each experimental group consisted of at least three animals, and the experiments were performed in triplicates.

Serum Transaminase Levels

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as markers of liver injury. ALT and AST concentrations were measured using IDTox™ Alanine Transaminase color endpoint assay kit and IDTox™ Aspartate Transaminase (AST) Enzyme Assay Kit (ID Labs™ Inc, London, ON, Canada) according to the manufactures instructions and absorbance was measured using a spectrophotometer.

Histological Assessment

Liver specimens were fixed in 10% neutral buffered formalin. The tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

ELISA of TNF-α

Serum levels of tumor necrosis factor-alpha (TNF-α) were determined by using an ELISA kit according to the manufacturer's specifications (eBiosciences, San Diego, CA). Data were then presented as picograms per milliliter of serum TNF-α.

Real Time Polymerase Chain Reaction

To determine Shh, Patch-1, Gli1 gene and Interleukin-6 (IL-6) expression in rat liver at the mRNA level, total liver RNA was extracted using RNeasy extraction kit (Qiagen, Valencia, CA). Total RNA (385 ng) was reverse transcribed to cDNA templates using MultiScribe reverse transcriptase and random hexamers by incubation at 25°C for 10 min, followed by reverse transcription at 48°C for 30 min and enzyme inactivation at 95°C for 5 min. In all, 100 ng of cDNA was amplified by real-time PCR using SYBR Green dye universal master mix on an LightCycler®480 (LC 480) (Applied Biosystems, Inc., Foster City, CA) using the primers (Table 1) for Sonic Hedgehog (NCBI Accession#; NM_017221), Patched-1(NCBI Accession#; NM_053566), Gli-1 (NCBI Accession#; XM_345832) and IL-6 (NCBI Accession#; M26745). The PCR products were subjected to a melting curve analysis, and crossing point (Cp) was used for calculating the relative amount of mRNA compared to the housekeeping gene, hypoxanthinephosphoribosyltransferase (HPRT), and then scaled relative to controls, where control samples were set at a value of 1. Thus, results for all experimental samples were graphed as relative expression compared with the control.

Immunofluorescent Staining

Immunofluorescent staining was performed on snap frozen liver tissue. Briefly, 5 μm cryosections were cut on lysine coated slides and fixed in 95% cold ethanol. Slides were air dried and stored at −80°C until further use. The sections were blocked with 10% goat serum with 1% BSA in TBS for 2 h at room temperature. Cryosections then incubated with the following primary antibodies overnight at 4°C: Shh, Ptch-1, Gli-1, P-selectin, phosphohistone 3, ICAM-1 and IL-6. The following secondary antibodies were used: anti-rabbit Alexa Fluor 488, anti-rabbit Alexa Fluor 594, anti-goat Alexa Fluor 488. Nuclear staining was performed using 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized on a Zeiss Apoplan Microscopy system.

Western Blotting

Total protein extracts from tissues were lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN). The protein concentration was determined using a Bio-Rad RC DC protein assay kit (Hercules, CA). Proteins were resolved on 4–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred to Immobilon polyvinylidene fluoride (PVDF) membrane using iBlot™ Dry Blotting System (Invitrogen). After blocking with 5% nonfat dry milk in 1×PBST (PBS containing 0.05% Tween-20) for 1 h at room temperature, the membranes were incubated with rabbit anti-goat Patched 1, goat anti-rabbit p-JNK and ERK primary antibodies for 16 h at 4°C as described (18). To correct for equal loading and blotting, all

Table I List of Primer Sequences and Product Sizes for Real Time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size
Shh	GACCGCGACGAAGGTGCCAA	GTGGGCCCGGAGTCGTTGTG	122
Patch-1	CCCGTCTTTGCCCGGTCCAC	TTCGAGGCTGCTGGCCTTGC	127
Gli-1	CCCAGCTCGCTCCGCAAACA	CATGGTGCCCTCCGGCACAG	183
IL-6	GATGCCTGAGTGGATCGGGGG	AGGGGGTTCCGTAAGGAAGGCTG	169

blots were re-probed with anti-β-actin antibody. Membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-goat or anti-rabbit secondary antibody for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Pittsburgh, PA).

DNA Fragmentation Assay for Apoptosis

The liver tissues from sham, vehicle and CYA-treated rats were kept on ice for 30 min after adding 0.5 ml of the lysis buffer (10 mM Tris-Cl, pH 8.0; 25 mM EDTA; 0.25% Triton X-100) per 20 mg liver tissues. The samples were then homogenized, washed twice with PBS and centrifuged for 15 min at 13,800 g at 4°C. The supernatant was mixed with 1 µl RNase A (10 mg/ml) at 37°C for 30 min. Then, 5 µl Protease K (20 mg/ml) was added and incubated overnight at 56°C. The DNA was extracted using phenol and chloroform, 5 M NaCl was added and mixed well, and the fragmented DNA was precipitated with 1.5 volume of 100% ethanol. Fragmented DNA was dissolved in 20 µl TE, and then 600 µl nuclei lysis buffer (100 mM Tris, pH 8.0; 5 mM EDTA; 0.2 M NaCl; 0.2% SDS; 0.2 mg/ml Protease K) was added. After incubation overnight at 37°C, NaCl was added to a final concentration of 1.5 M. Then, 1 volume of 100% ethanol was added and centrifuged for 20 min. The pellet was washed with 70% ethanol and dissolved in 200 µl TE with RNase A at 37°C overnight. Fragmented DNA (5 µl) and genomic DNA (3 µl) were applied to 1.5% agarose gel and subjected to electrophoresis for 65 min under 120 V at room temperature.

Statistical Analysis

All values in the figures and text are expressed as the mean ± SD. The results were analyzed by ANOVA. P value <0.05 was considered significant, and individual group means were then compared with Student's unpaired t-test.

RESULTS

Upregulation of Hedgehog Ligands and Transcription Factors after I/R Injury

The growth of immature bile ductular cells in culture is regulated by Hh ligands, and Hh pathway activity increases after bile duct ligation (19). Therefore, we evaluated our model of partial liver I/R injury to determine whether I/R injury was associated with Hh pathway activity. Shh, Ptch-1 and Gli-1 gene expression was determined by quantitative real-time PCR. Expression of Shh, Ptch-1 and Gli-1 was significantly increased in I/R injury rats at 1 h of reperfusion compared with sham-operated group (Fig. 1a-c). Similarly, immunofluorescence analysis demonstrated weak immunostaining for Shh, Ptch-1 and Gli-1 in sham-operated liver tissue (Fig. 2a, c and e) but strong immunostaining in bile ductular cells and periportal region within 4 hours of IR injury (Fig. 2b, d and f). Thus, expression of these factors identifies cells with Hh activity. Furthermore, these results demonstrate that cells in the ischemic liver tissue produce Shh and express Ptch-1 gene, indicating that the endogenous Hh pathway is physiologically active during I/R injury.

Cyclopamine Attenuates Ischemia Reperfusion Injury

To further address the role of Hh signaling during I/R injury, we attempted to inhibit Hh signaling by treating rats with CYA. To determine if endogenous Hh contributed to organ damage after liver I/R, CYA formulated in 10% HBCD or vehicle alone was administered daily for three

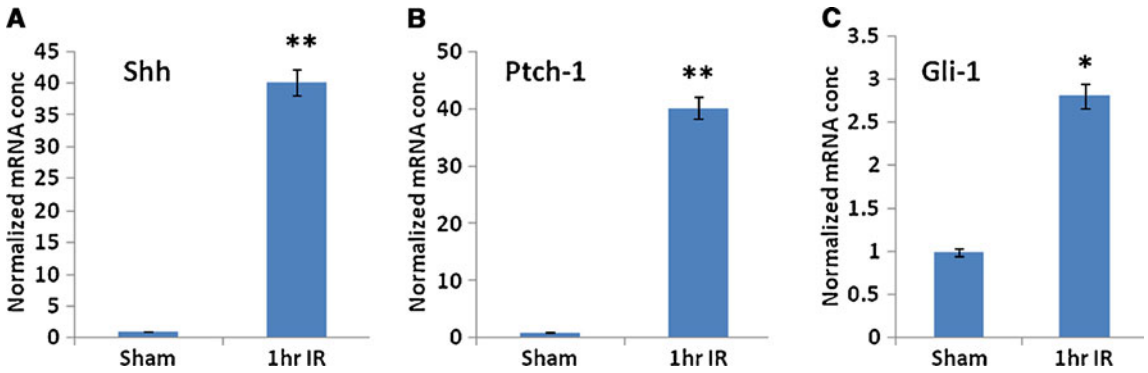

Fig. 1 Gene expression of Hedgehog ligands and its transcription factors is upregulated after ischemia reperfusion injury. Real time PCR data showing upregulation of (**A**) sonic hedgehog (Shh), (**B**) Patched 1(Ptch-1) and (**C**) Gli-1 after warm ischemia of 90 min followed by reperfusion for 1 h. RNAs from five rats were used for real time PCR, and each sample was analyzed in triplicates. Results are expressed as the mean ± SD of mRNA concentration relative to sham rats. *$P<0.05$, ** $P<0.005$.

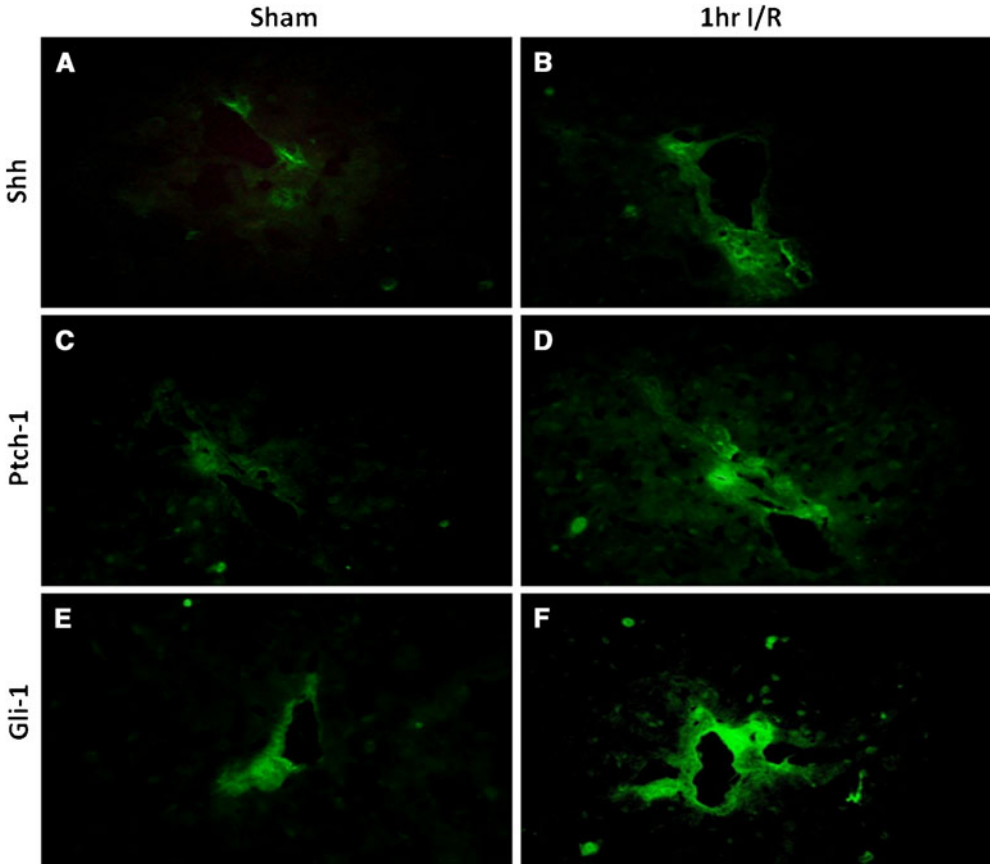

Fig. 2 Immunoflorescence expression and localization of hedgehog proteins in snap frozen sections of liver tissue in sham operated and I/R injury animals at 1 h of reperfusion. Expression and localization of Hh proteins in the liver after 1 h of I/R injury. Compared to sham rats (**A**, **C** and **E**), strong expression of Shh, Ptch-1 and Gli-1 proteins are detected in the epithelium of bile ducts and portal vein after I/R injury (**B**, **D** and **F**).

consecutive days and 1 h before reperfusion after 90 min of warm ischemia. The efficacy of Hh pathway inhibition was confirmed by studying the expression pattern of Ptch-1 by qRT-PCR, Western blot and immunoflorescence. Preconditioning with CYA dramatically downregulated Ptch-1 gene expression (Fig. 3a) and protein expression (Fig. 3b), which is indicative of Hh pathway inhibition. To eliminate the unpredictable variations seen with CYA bioavailability, we also analyzed Patched-1 protein expression by immunofluorescence on kidneys of vehicle-treated and CYA-treated rats. Efficient blockade of Hh pathway by CYA is represented as absent to weak staining in the kidneys (Fig. 3c-e).

Ninety minutes of warm liver ischemia followed by 1 h of reperfusion significantly increased serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) levels in the vehicle-treated rats that were subjected to I/R. In contrast, treatment with CYA resulted in significant protection from hepatic injury (Fig. 4a and b). This protection was not evident at 6 h after reperfusion in CYA-treated rats (data not shown).

Liver histology confirmed sALT and sAST estimation of liver damage. Severe sinusoidal congestion and hepatocellular necrosis was present in liver tissue from rats that were treated with vehicle alone, whereas minimal damage was noted in samples from CYA-treated rats (Fig. 4c-e). There was sparing of the periportal areas with progressively increased injury approaching the central vein. Liver samples from the vehicle animals exhibited ~40.5±7.2% necrotic hepatocytes compared with ~10.2±1.9% necrotic cells in the CYA-treated group ($P<0.05$).

Infiltration of Inflammatory Cells is Markedly Reduced by Cyclopamine

Reperfusion injury was associated with a marked number of neutrophils infiltrating the midzonal region of vehicle-treated rats, especially around the central vein, compared to CYA-treated rats (Fig. 5a and b). After 1 h of reperfusion, intense positive immunohistochemical staining for P-selectin and ICAM 1 was found in the liver tissue sections from I/R injury vehicle-treated rats. In contrast, immunostaining for P-selectin (Fig. 5c-e) and ICAM 1 (Fig. 5f-h) was decreased significantly in sections from rats treated with CYA.

Cyclopamine Suppresses Expression of Proinflammatory Cytokines after I/R Injury

TNF-α is an important proinflammatory cytokine and plays a key role in the pathophysiology of hepatic I/R injury (20).

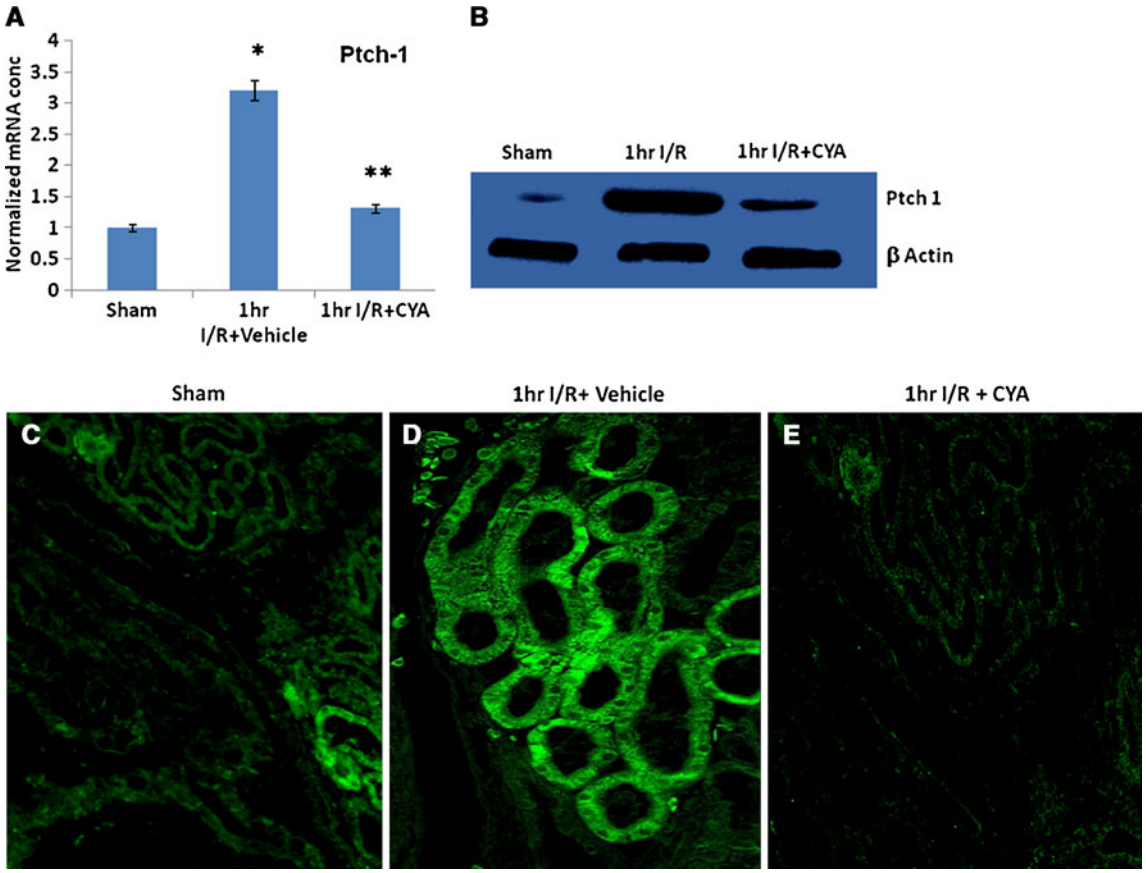

Fig. 3 Hedgehog expression in the liver is abrogated by treatment of rats with cyclopamine. (**A**) Quantitative real-time PCR of mRNAs from sham operated, vehicle and cyclopamine (CYA) (10 mg/kg) animals harvested after 1 h of reperfusion. CYA was formulated in 10% 2-hydroxypropyl-β-cylodextrin (HBCD). HBCD with no CYA was used as vehicle. Results are expressed as the mean ± SD of mRNA concentration relative to sham rats. *$P < 0.05$ *versus* sham; **$P < 0.05$ *versus* I/R + vehicle. (**B**) Western blot of liver homogenate for Ptch-1 expression in sham operated, vehicle treated and CYA treated animals after 1 h of reperfusion showing suppressed Ptch-1 expression by pretreatment with CYA. Beta actin was used as a loading control. (**C**-**E**) Kidneys sections of vehicle-treated and CYA-treated animals were analyzed for Ptch-1 Immunoflorescence to verify efficacy of CYA inhibition of Hh signaling. Note significantly decreased Ptch-1 expression in CYA-pretreated rats indicating efficient blockade of Hh pathway (**E**).

Therefore, we measured TNF-α expression by ELISA in the sham-treated I/R injury rats pretreated with the vehicle or CYA at 1 h of reperfusion. As shown in Fig. 6a, TNF-α concentration in the serum was significantly increased after 90 min of ischemia followed by 1 h of reperfusion. Although the sham operation induced a slight cytokine response, serum TNF-α in the sham-operated animals remained low compared to the corresponding I/R rats. Pretreatment with CYA significantly reduced serum levels of TNF-α at 1 h of reperfusion, suggesting CYA can inhibit inflammation induced by I/R injury. The gene expression of another cytokine IL-6 was also analyzed following preconditioning with CYA. Significant downregulation of IL-6 mRNA was seen with CYA pretreatment (Fig. 6b). On immunofloresecence, no IL-6 was seen in sham-operated rats (Fig. 6c), while intense IL-6 expression (Fig. 6d) was seen along the epithelium of bile ducts (arrows) and endothelium of portal vein (block arrows). Pretreatment with CYA blunted the expression of IL-6 (Fig. 6e).

Cyclopamine Inhibits Cellular Proliferation but Not Apoptosis after Liver I/R Injury

Whether the protective effect of CYA pretreatment was due to the inhibition of proliferation of cells and/or inhibition of apoptosis after I/R injury, we assessed the cell proliferation and DNA fragmentation as indices for proliferating cells and apoptotic cells, respectively. Immunoflorescence analysis using phosphohistone three staining demonstrated marked reduction in proliferating cells in CYA-pretreated rats compared with vehicle-pretreated animals at 1 h of reperfusion (Fig. 7a-c). DNA ladder was found in the sham-operated group, but was not found in vehicle- or CYA-pretreated groups after 1 h of reperfusion (Fig. 7d).

Influence of Cyclopamine on MAPK Signal Transduction

To identify the mechanism by which CYA preconditioning induces cytoprotection, we studied the activation of mitogen-

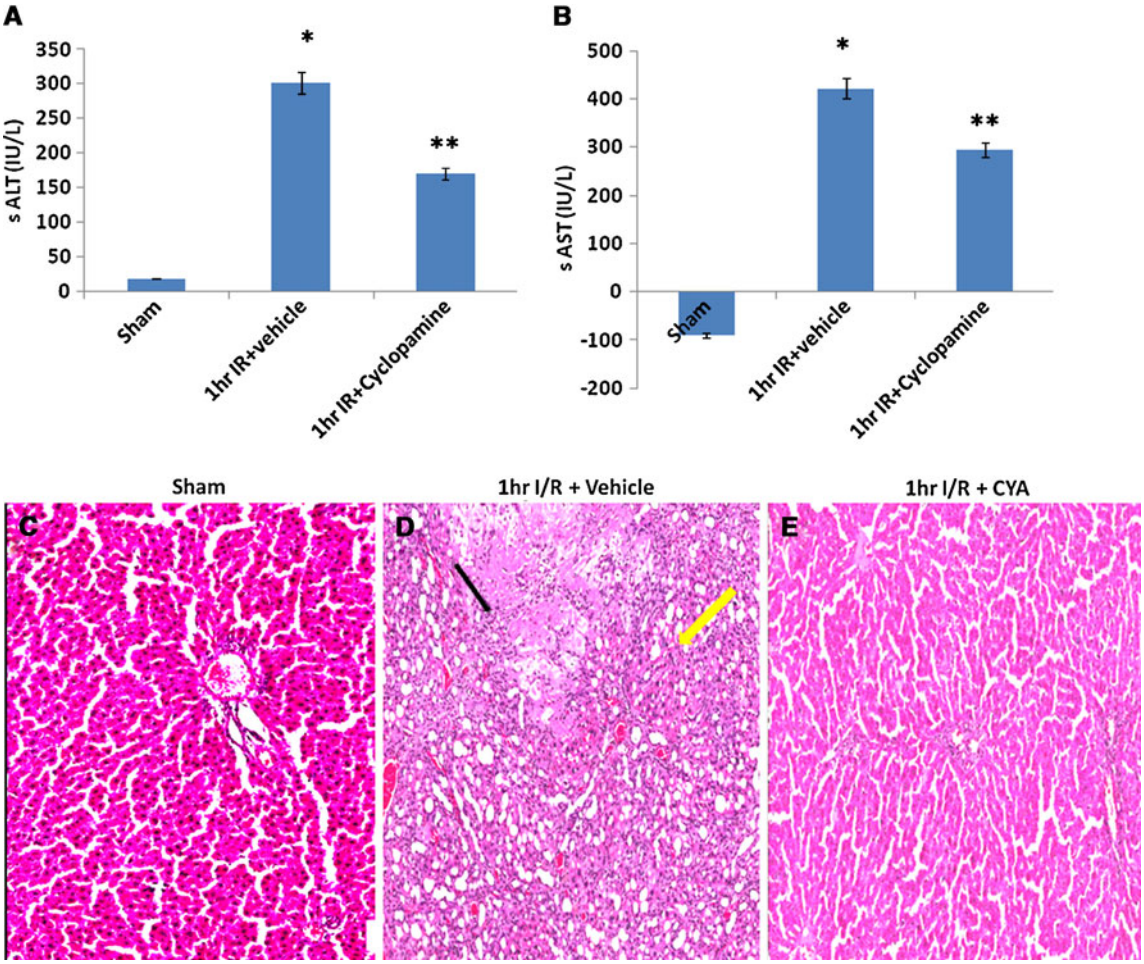

Fig. 4 Pretreatment with cyclopamine protects against liver I/R injury. Sham rats and rats that underwent ischemia and 1 h of reperfusion were pretreated with CYA or vehicle i.p. for three consecutive days and 1 h before reperfusion. (**A** and **B**) Serum ALT and AST levels were analyzed as a measure of hepatocellular injury. Data represent the means ± SD. *$P < 0.05$ *versus* sham; **$P < 0.05$ *versus* I/R vehicle treated rats. (**C**-**E**) Representative liver histology (hematoxylin-eosin staining; magnification, X 200) of ischemic (90 min) liver lobes reperfused for 1 h in vehicle-treated and CYA-treated rats. CYA was formulated in 10% 2-hydroxypropyl-β-cylodextrin (HBCD). HBCD with no CYA was used as vehicle. Note severe lobular edema, congestion, ballooning (yellow arrow) and hepatocellular necrosis (black arrows) in vehicle-treated animals. In contrast, CYA-treated animals showed good preservation of architecture and histological details.

activated protein (MAP) kinases. After 1 h of I/R, phosphorlyation of ERK and JNK was increased in vehicle-pretreated rats. In contrast, CYA-pretreated rats showed significantly decreased phosphorlytaion of ERK, and JNK (Fig. 8a and b).

DISCUSSION

The Hh pathway expression has been systematically characterized extensively during the embryogenesis of many organs. A vast majority of these studies have primarily focused on the role of Hh family members in the regulation of epithelial–mesenchymal associations related to organogenesis (21,22). In contrast, a role for the Hh signaling in the regulation of adult tissue regeneration following ischemic or hypoxic injury has received very limited interest (23,24). Repair of damaged livers involves a wound healing process resulting in the expansion and differentiation of progenitor cells residing in adult livers. This expansion of progenitor cell population is orchestrated by a complex network of paracrine and autocrine signals. Some of these signals appear to include injury-related pro-inflammatory cytokines. It has been demonstrated that some of these factors induce certain types of adult liver cells to produce Hh ligands (15,25).

In this study, we hypothesized that Hh signaling is probably involved in the pathogenesis of liver injury after warm ischemia reperfusion. The foundation of our hypothesis stems from evidence in the literature that Hh ligands coordinate remodeling of damaged epithelia in various adult organs (26–28). This possibility is also supported by recent reports showing that hepatic stellate cells produce Hh ligands (29), and, consequently, amplified levels Ptch-1 and Smo transcripts are seen in chronically injured livers (30). The present study is the first to demonstrate a striking

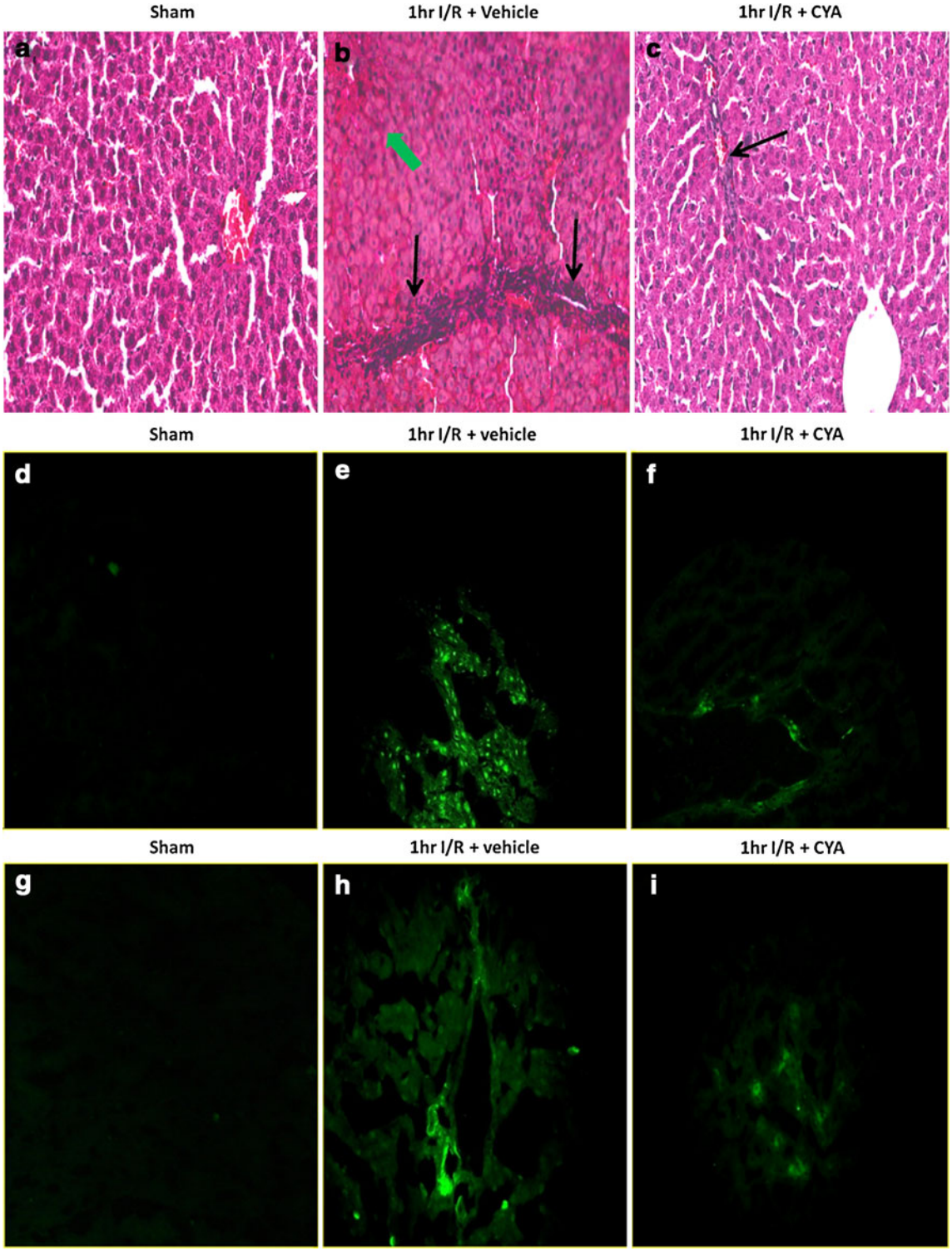

Fig. 5 Cyclopamine pretreatment attenuates neutrophil infiltration after 1 h of liver IR injury. (**a**-**b**) Representative liver histology (hematoxylin-eosin staining; magnification, X 400) of ischemic (90 min) liver lobes reperfused for 1 h in vehicle treated and CYA-treated rats. Note intense neutrophilic infiltration around portal tract and sinusoids (green arrows) in vehicle-treated rats compared with CYA-treated rats. (**c**-**e**) Intense positive staining for P-selectin is demonstrated along the endothelium of the vasculature and sinusoids in vehicle-treated animals. The degree of positive staining for P-selectin was reduced markedly in rats pretreated with CYA. (**f**-**h**) ICAM-1 immunoflorescence showing few hepatocytes positive for ICAM-1 in CYA-treated rats compared with vehicle-treated rats. Original magnification X200 for all figures. The figures are representative of at least three experiments.

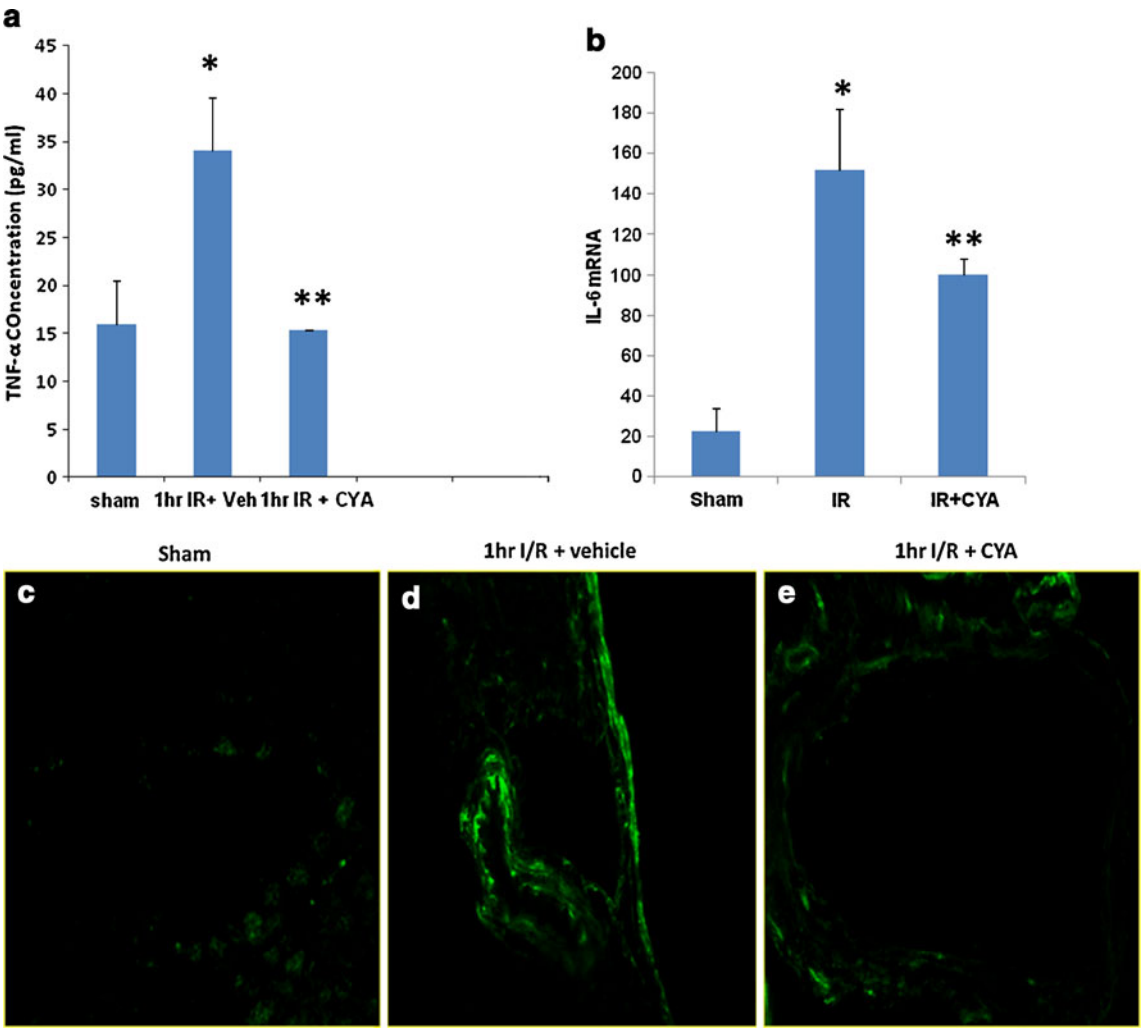

Fig. 6 Cyclopamine pretreatment reduces release of proinflammatory cytokines. (**a**) Serum levels of TNF- α after 1 h of hepatic IR in vehicle-treated and CYA-treated rats. Serum levels of TNF- α are expressed as the mean ± SD. (**b**) Quantitative real-time PCR of IL-6 mRNAs from sham-operated, vehicle and cyclopamine (CYA) (10 mg/kg) animals harvested after 1 h of reperfusion. *$P < 0.05$ *versus* sham group, **$P < 0.05$ *versus* I/R vehicle treated group. (**c**-**e**) Immunoflorescence expression of IL-6 in snap frozen sections of liver tissues. **c**) sham-operated rats, **d**) rats with I/R injury, and **e**) rats with II/R injury and treated with CYA.

relationship between endogenous Hh signaling and I/R injury. At baseline, sham-operated rats did not demonstrate great expression of Hh ligands. However, strong upregulation of Shh, Ptch-1 and Gli-1 ligands in areas around the portal tracts were seen as early as 1 h after I/R injury. Our findings are in good accordance with the literature describing recruitment and engagement of embryonic signaling pathways following ischemia reperfusion (31).

Hepatic I/R injury is a multifaceted and complex process associated with massive neutrophil infiltration and production of inflammatory mediators (32). The involvements of specific intracellular signal transduction mechanisms have been long established to induce an inflammatory response in hepatic I/R injury (33,34). To investigate whether Hh signaling plays such a proinflammatory role in I/R injury, one approach is to study the effects of Hh inhibition on liver I/R injury process. To inhibit Hh signaling, we disengaged Hh message from its downstream receptor smoothened (SMO) by pre-treating with CYA, which is a small-molecule *Veratrum* alkaloid that inhibits Hh signaling by inhibiting SMO receptor and thus interferes with the downstream Hh signaling in hedgehog responsive cells (35).

Preconditioning with CYA for three consecutive days before warm ischemia and 1 h before reperfusion considerably reduced hepatocellular injury, as evidenced by significant decrease in sALT and sAST levels and necrosis on histopathological examination after 1 h of reperfusion (Figs. 4 and 5). Furthermore, inhibition of Hh signaling resulted in diminished local neutrophil infiltration and reduced expression of proinflammatory cytokines such as TNF-α and IL-6 (Fig. 6).

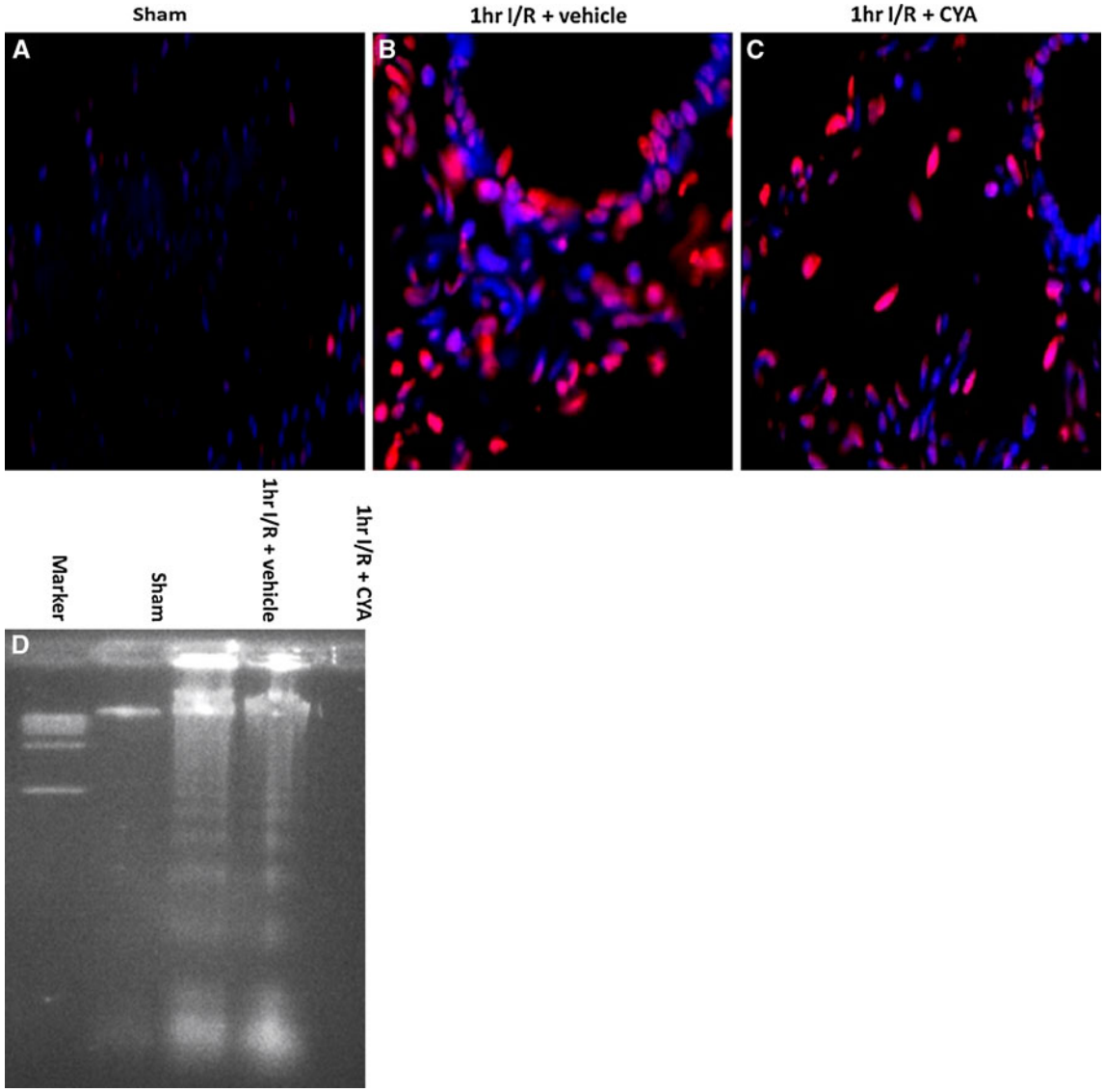

Fig. 7 Cyclopamine inhibits cellular proliferation, not apoptosis after liver IR injury. (**A**-**C**) Sham-operated rats, vehicle-pretreated and CYA-pretreated rats were analyzed for proliferating cells using anti-phosphohistone 3 antibody (red). Nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI, blue). Cyclopamine preferentially reduced the number of proliferating cells after liver I/R injury. All images were taken at magnification X 400. (**D**) Analysis of DNA ladder from liver cell extract, showing a DNA ladder in sham-operated group, but not found in vehicle or CYA-pretreated groups after 1 h of reperfusion.

Theoretically, inflammatory pathology should not be predominant within 1 hour of I/R injury, which is supposed to consist of initial and subsequent phases (36). However, in a study by Yoshiyuki *et al.*, neutrophil accumulation began at 30 min to 45 min of reperfusion and peaked at 6 h of reperfusion (37). Experimental procedures could account for such varied onset of neutrophilic infiltration reported in the literature. To further clarify the important role of neutrophils in reperfusion injury, we studied the activation of neutrophil adhesion molecules such as P-selectin and ICAM-1 (38–40). We observed strong induction of P-selectin and ICAM-1 along the vascular endothelium after I/R injury in vehicle-pretreated rats. Pretreatment with CYA blunted the expression of these adhesion molecules (Fig. 5c-f). Taken collectively, these results demonstrate that the inhibition of Hh pathway disrupts the interaction between neutrophils and endothelium by downregulating the expression of adhesion molecules during early phase of I/R injury.

In the context of liver I/R injury, hepatocytes undergo temporally regulated proliferation in a bimodal fashion, as evident from peaks in proliferating cell nuclear antigen (PCNA, a marker for cellular proliferation) expression during the acute and subacute phases of reperfusion injury (41). Studies have shown that I/R injury promotes the activation of JNK1, as well as the induction of AP-1 DNA binding

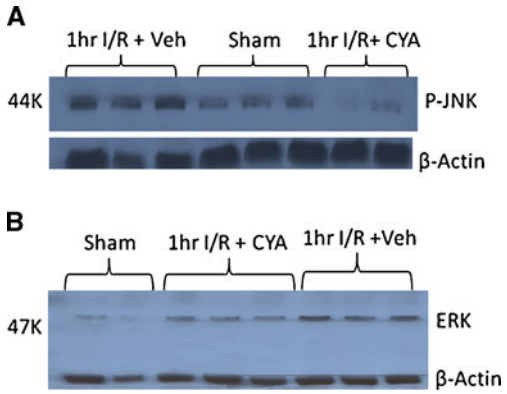

Fig. 8 Western blots showing the effects of cyclopamine on MAPK signal transduction pathway after liver I/R injury. (**A**) Levels of pJNK in the liver homogenates from vehicle-pretreatment group were increased at 1 h after reperfusion compared with sham-operated rats. Cyclopamine (CYA) pretreatment significantly reduced the levels of pJNK. (**B**) Levels of ERK in vehicle-pretreated group were similarly increased at 1 h after reperfusion compared to CYA-pretreated group.

complexes composed of Jun heterodimers during the reperfusion phases of injury(42,43). Given the activation of Hh ligands and its downstream transcription factors occurring in the ischemic reperfused liver, we next sought to examine whether Hh activation was responsible for the proliferation of cells during acute liver injury. Immunoflorescence with antiphosphohistone 3 revealed marked reduction in proliferating cells after pretreatment with CYA compared with vehicle-pretreated animals at 1 h of I/R injury (Fig. 7). Our results showing antiproliferative effects of CYA are in good agreement with the literature showing inhibition of cancer cells by this Hh antagonist (44,45).

To make clear the principal mechanism of CYA hepatoprotection after liver I/R injury, we studied the MAPK transduction pathway. The MAPK family consists of JNK, ERK, and p38 MAPKs, and is reported to play an important role in intracellular signal transduction in response to extracellular stimuli (46,47). Several studies showed that silencing p38 MAPK attenuated ischemia reperfusion-induced apoptosis in the liver during warm ischemia and reperfusion (48,49). The ischemia-reperfusion process differentially activates MAPKs. In renal ischemia-reperfusion, p38 MAPK is activated by ischemia, whereas JNK is not activated by ischemia alone but is activated by reperfusion after ischemia (50). Our results show a significant increase in phosphorylation of JNK and ERK in liver tissues from I/R vehicle-treated rats compared with sham-operated rats. Pretreatment with CYA decreased these phosphorylation levels significantly (Fig. 8a and b), suggesting that CYA accounts, in part, for MAPK activation following I/R injury. Interestingly, the inhibition of JNK phosphorylation should lead to a decrease in apoptosis in hepatocytes (51), a finding not seen in our study. A possible hypothesis is that although phosphorylation of JNK is proapoptotic, its effects on other substrates mediate antiapoptotic effects possibly by altering turnover, or the stability of other proteins. Hh expression has been reported to be upregulated particularly in neural progenitor cells (NPCs) and to modulate their neuroprotective proliferative response to ischemia/hypoxia (52).

Expression of Shh is also upregulated in cardiomyocytes and plays a critical role in protection of the heart after myocardial infarction by increasing angiogenic cytokine production (53). In the context of liver, Hh activity has been shown to be present in resident hepatic progenitors throughout life, suggesting that liver progenitors maintain their viability via autocrine and/or paracrine Hh signaling throughout fetal and adult life (54). In the background of this large pool of evidence that Hh signaling is essential to maintain the viability of progenitor cells, our results implicating that Hh signaling contributes to early liver injury come as a surprise. Although reasons for the disagreeing roles of Hh signaling are not clear at present, we believe that the regulating role of Hh signaling is likely to be versatile, species, *in vivo versus in vitro* and circumstance dependent. The exact mechanism of Hh-induced liver injury remains unclear. Nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are crucial transcription factors that coordinate the expression of many genes implicated in inflammation, and apoptosis (55,56). NF-κB and AP-1 activities are induced by a large number of physiological and environmental stimuli (57,58). Moreover, in many cases, JNK and NF-κB are activated by the same signaling pathways, and a close cross talk between these two pathways has been reported (59). Further, there is recent evidence that Hh acts as a direct transcriptional target of NF-κB (60). On the basis of these collective findings, one could contemplate, therefore, an appealing possibility that Hh activates and modulates the activity of NF-kB/AP-1/JNK pathways.

In summary, we have established for the first time an association between Hh expression and proinflammatory signals after warm liver I/R injury. These findings have significant clinical implications. At present, various ingenious protective attempts have been employed to reduce the burden of I/R injury following major liver surgeries. These include surgical maneuvers, gene therapy, and the use of pharmacological preconditioning (61–64). Among these, pharmacological preconditioning appears to be most appealing technique, as it is non-invasive and can be implemented preceding surgery. We provide comprehensive and compelling evidence that endogenous Hh signaling regulates acute liver injury following warm I/R. Preconditioning with Hh inhibitor significantly ameliorates the overwhelming effects of hepatocellular damage. This identifies novel and attractive pharmacological preconditioning strategy to confer hepatoprotection and reduce morbidity and mortality in patients undergoing major liver surgeries.

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