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Cyclopamine Attenuates Acute Warm Ischemia Reperfusion Injury in Cholestatic Rat Liver: Hope for Marginal Livers

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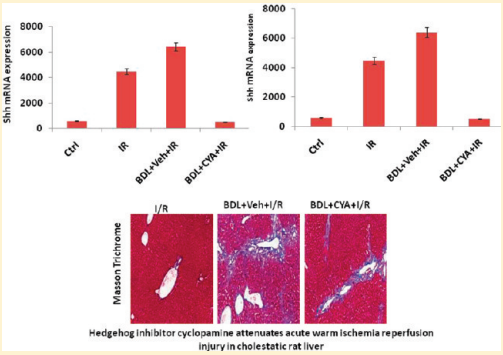
ABSTRACT: Cholestasis is a significant risk factor for immediate hepatic failure due to ischemia reperfusion (I/R) injury in patients undergoing liver surgery or transplantation. We recently demonstrated that inhibition of Hedgehog (Hh) signaling with cyclopamine (CYA) before I/R prevents liver injury. In this study we hypothesized that Hh signaling may modulate I/R injury in cholestatic rat liver. Cholestasis was induced by bile duct ligation (BDL). Seven days after BDL, rats were exposed to either CYA or vehicle for 7 days daily before being subjected to 30 min of ischemia and 4 h of reperfusion. Expression of Hh ligands (Sonic Hedgehog, Patched-1 and Glioblastoma-1), assessment of liver injury, neutrophil infiltration, cytokines, lipid peroxidation, cell proliferation and apoptosis were determined. Significant upregulation of Hh ligands was seen in vehicle treated BDL rats. I/R injury superimposed on these animals resulted in markedly elevated serum alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin accompanied with increased neutrophil recruitment and lipid peroxidation. Preconditioning with CYA reduced the histological damage and serum liver injury markers. CYA also reduced neutrophil infiltration, proinflammatory cytokines such as TNF-α and IL-1β expression of α-smooth muscle actin and type 1 collagen resulting in reduced fibrosis. Furthermore CYA treated animals showed reduced cholangiocyte proliferation, and apoptosis. Hepatoprotection by CYA was conferred by reduced activation of protein kinase B (Akt) and extracellular signal regulated kinase (ERK). Endogenous Hh signaling in cholestasis exacerbates inflammatory injury during liver I/R. Blockade of Hh pathway represents a clinically relevant novel approach to limit I/R injury in cholestatic marginal liver.



KEYWORDS: preconditioning, bile duct ligation, partial ischemia reperfusion, hepatocellular injury

INTRODUCTION

The number of cadaveric organ donors has remained static while the candidate waiting list for organ transplants has grown exponentially. This growing disparity in supply and demand has catalyzed the use of marginal grafts which are either steatotic or cholestatic.[1,2] Marginal livers have an increased susceptibility to cold and warm ischemia reperfusion (I/R) injury resulting in poor graft outcomes.[3−5] Cholestasis is a major risk factor for oxidative stress and complications of I/R injury. Recent studies have clearly established a critical role for neutrophils in the mechanism of hepatocyte injury during cholestasis.[6] The mechanism by which cholestasis triggers the production of proinflammatory mediators that promote accumulation and activation of neutrophils in the liver, however, remains unknown. Elucidation of this pathway could provide clinically relevant insight into ways to prevent inflammation from occurring in cholestatic liver during I/R. One potential regulator of inflammation in the cholestatic liver may be the Hedgehog (Hh) signaling pathway. Hh ligands are soluble factors that interact with the plasma membrane-spanning receptor, Patched (Ptch), to derepress the downstream intracellular signaling intermediate, Smoothened (Smo). Smo-initiated signals, in turn, activate Gli-family transcription factors to induce expression of Hh-responsive genes that regulate target cell proliferation, viability and differentiation.[7] The mechanisms by which Gli genes are regulated in response to Hh signaling are still not completely understood. Unlike Gli-1, which represents a direct transcriptional Hh target gene, Gli-2 and also Gli-3 are considered latent transcriptional regulators activated by Hh signaling. In fact, induction of Gli-1 mRNA expression by Hh signaling is a reliable marker for pathway.[8,9] During embryogenesis, Hh ligands function as morphogens by modulating both mesendodermal fate and epithelial−mesenchymal transitions (EMT).[10,11] Consistent with these observations there is a growing evidence that Hh signaling regulates remodeling of various adult tissues, including the nervous system, skin, heart, lung, and gastrointestinal tract. Excessive Hh activity has also been noted in cancers arising in these tissues.[12−14] We have recently shown that

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Hh signaling is upregulated in normal liver after I/R injury and its inhibition confers hepatoprotection.[15] Whether Hh is an essential regulator of inflammation during cholestasis, however, remains to be investigated. The studies presented herein tested the hypothesis that Hh is instrumental in hepatic injury during I/R of cholestatic liver. To this end, expression of Hh ligands and effect of inhibition Hh pathway by preconditioning with cyclopamine (CYA) were evaluated in the rat liver with cholestasis.

MATERIAL AND METHODS

Materials. CYA free base was purchased from LC Laboratories (Woburn, MA). Goat anti-rabbit SHH, Gli-1, Patch-1, TNF-α, collagen Type 1 and IL-1β primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho ERK, total ERK, phospho Akt, and total Akt primary antibodies were purchased from Cell Signaling (Danvers, MA). Goat anti-mouse CK-7, beta actin and α-smooth muscle actin were purchased from Abcam (Cambridge, MA). Mouse proliferating cell nuclear antigen (PCNA) monoclonal antibody (Clone IPO-38) and TBARS assay kit were purchased from Cayman Chemical Company (Ann Arbor, MI). Goat anti-rabbit Alexa Fluor 488 and Alexa Fluor 594, rabbit anti-goat Alexa Fluor 488 and goat anti-mouse 594 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 purchases from Applied Biosystems (Foster City, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Animals. 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. Animals were anesthetized with isoflurane.

Bile Duct Ligation. Bile duct ligation (BDL) or sham operation was performed as described previously.[16] After a midline laparotomy the bile duct was doubly ligated with 4-0 silk and transected. The sham operation was performed similarly, with the exception of ligating and transecting the bile duct.

Ischemia Reperfusion. Ischemia reperfusion injury (I/R) injury was done 7 days after BDL as described earlier.[17,18] An atraumatic vascular clip was placed to interrupt the blood supply. 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 30 min of partial hepatic ischemia, the clamp was removed and reperfusion was resumed. Sham operated mice underwent the same procedure without vascular clamping. The abdomen was closed in a double layer using 4-0 nylon. During the reperfusion, the mice were kept in clean cages. After 4 h of reperfusion, animals were euthanized for blood and tissue collection.

Drug Administration and Sample Collection. Rats were allocated randomly into the following groups, 5 rats per group: sham controls; I/R injury; BDL with I/R injury, underwent daily administration of beta cyclodextrin solution (10 mg/kg/day daily); BDL with I/R injury, underwent daily administration of intraperitoneal CYA (10 mg/kg/day daily). Both vehicle and CYA treatment were started 3 days after BDL and continued once a day for 7 days. On the 14th day animals were subjected to I/R injury as described above and after 4 h of reperfusion were euthanized. Blood was collected via cardiac puncture, and serum was collected immediately after euthanasia. Liver samples were immediately frozen in liquid nitrogen, or fixed in 10% neutral buffered formalin (NBF) prior to embedment in paraffin.

Assessment of Liver Injury. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin were used as markers of liver injury. Their levels were measured using IDTox alanine transaminase color end point assay kit, IDTox aspartate transaminase (AST) enzyme assay kit (ID Laboratories Inc., London, ON, Canada) and IDTox. Total bilirubin levels were measured using a MaxDiscovery total bilirubin assay kit from Bio Scientific (Austin, TX) and absorbance at 560 nm was measured using a UV spectrophotometer.

Hematoxylin−Eosin (H&E) Staining. Liver specimens were fixed in 10% NBF and embedded in paraffin. Liver sections (5 μm) were stained with Hematoxylin−Eosin and then analyzed blindly. The histological severity of I/R injury was graded using Suzuki's criteria.[19] Liver sections were also analyzed to evaluate biliary proliferation. Ten portal tracts were examined. A score of 0 was given if there were no proliferating ducts, a score of 1 for greater than 0 but less than 10%, a score of 2 for proliferation between 10% and 25%, a score of 3 for ducts greater than 25% but less than 50% and a score of 4 for proliferating ducts greater than 50%.

Hepatic Neutrophil Infiltration. To assess neutrophil accumulation in the livers, tissue sections were stained for chloroacetate esterase present on neutrophils, using a naphthol-ASD chloroacetate esterase kit (Sigma Aldrich, St. Louis, MO). The numbers of neutrophils present in the sinusoids and extravasated into the parenchymal tissue were counted in 20 high-power fields. All cell counts were performed in a blinded fashion.

Lipid Peroxidation. Oxidative stress in the cellular environment results in the formation of highly reactive and unstable lipid hydroperoxides.[20] Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBARS).[21] MDA assay from liver homogenates was performed using TBARS assay kit (Cayman Chemical Company, Ann Arbor, MI) using the manufacturer's instructions.

Proliferating Cell Nuclear Antigen Immunohistochemistry. PCNA immunoflorescence staining was performed on 5 μm fresh frozen sections using mouse monoclonal PCNA antibody.

TUNEL. Fresh frozen tissue sections (5 μm) were prepared, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using *in situ* cell death detection kit (Promega, Madison, WI). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive cells in 10 random microscopic fields (×40), as previously described.[22]

Real Time Polymerase Chain Reaction. Shh, Patch-1, Gli-1, TNF-α, and IL-1β gene expression in rat livers was determined by real time PCR as described before, Briefly, total liver RNA was extracted and reverse transcribed to cDNA templates, followed by reverse transcription. In all 100 ng of cDNA was amplified by real-time PCR using SYBR Green dye universal master mix on an LightCycler480 (LC 480) (Applied Biosystems, Inc., Foster City, CA) using the primers for Shh (NCBI Accession No. NM_017221), Patched-1 (NCBI Accession No. NM_053566), Gli-1 (NCBI Accession No. XM_345832), TNF-α (NCBI Accession No. NM_012675) and IL-1β (NCBI Accession No. NM_031512). Following melting curve analysis, crossing point (Cp) was used for calculating the relative amount of mRNA compared to the house keeping gene, hypoxanthine phosphoribosyltransferase (HPRT), and

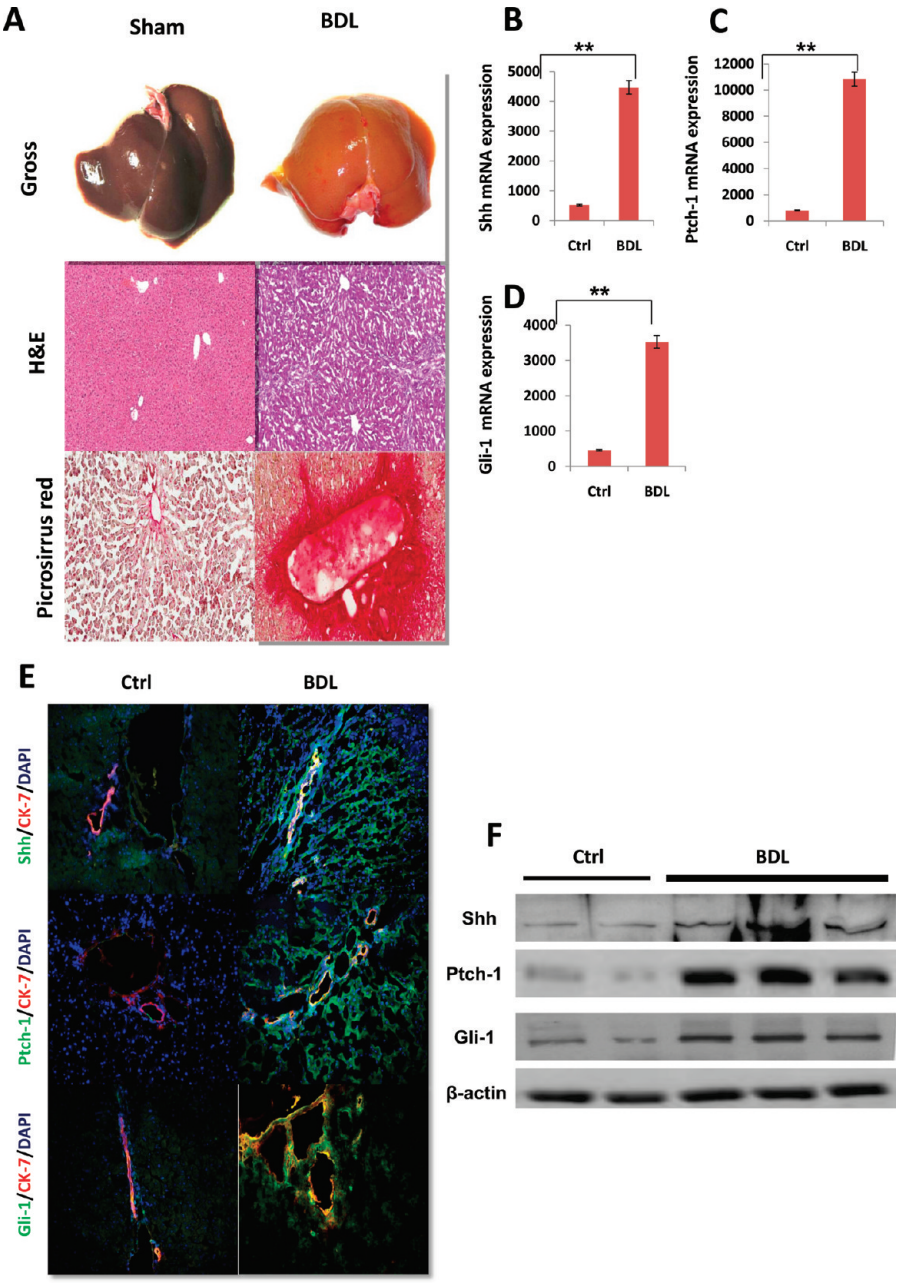

Figure 1. Histological evaluation and expression of Hedgehog (Hh) ligands in BDL rats. (A) Gross morphology, H&E and Picrosirrus red staining of liver specimens from sham controls and BDL rats are represented. Control livers were grossly normal, exhibiting normal architecture and normal deposition of collagen supporting structures. In contrast, BDL livers were enlarged and cholestatic and microscopy showed bile duct proliferation and extensive collagen deposition. (B−D) Shh, Ptch-1 and Gli-1 expression in normal and BDL rats quantified by real time polymerase chain reaction after correction for the housekeeping keeping gene HGPRT, $^{**}P < 0.01$ vs sham controls. (E) Total protein was isolated and Western blot analysis performed for Shh, Ptch-1 and Gli-1 normalized to β actin. (F) Fresh frozen sections were immunostained using anti-Shh, Ptch-1 and Gli-1 and CK-7 ($\times$100 original magnifications). Hh ligands (green) and CK-7 (red) colocalized in the biliary ductular epithelium (yellow).

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 were then incubated with the following primary antibodies overnight at 4 °C: Shh, Ptch-1, Gli-1, CK-7, α-smooth muscle actin, ICAM-1, and PCNA. 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). Immunoflorescence was visualized on a Zeiss Apoplan microscopy system.

Western Blotting. Total protein was extracted by homogenizing liver tissues and lyzing in RIPA buffer containing protease

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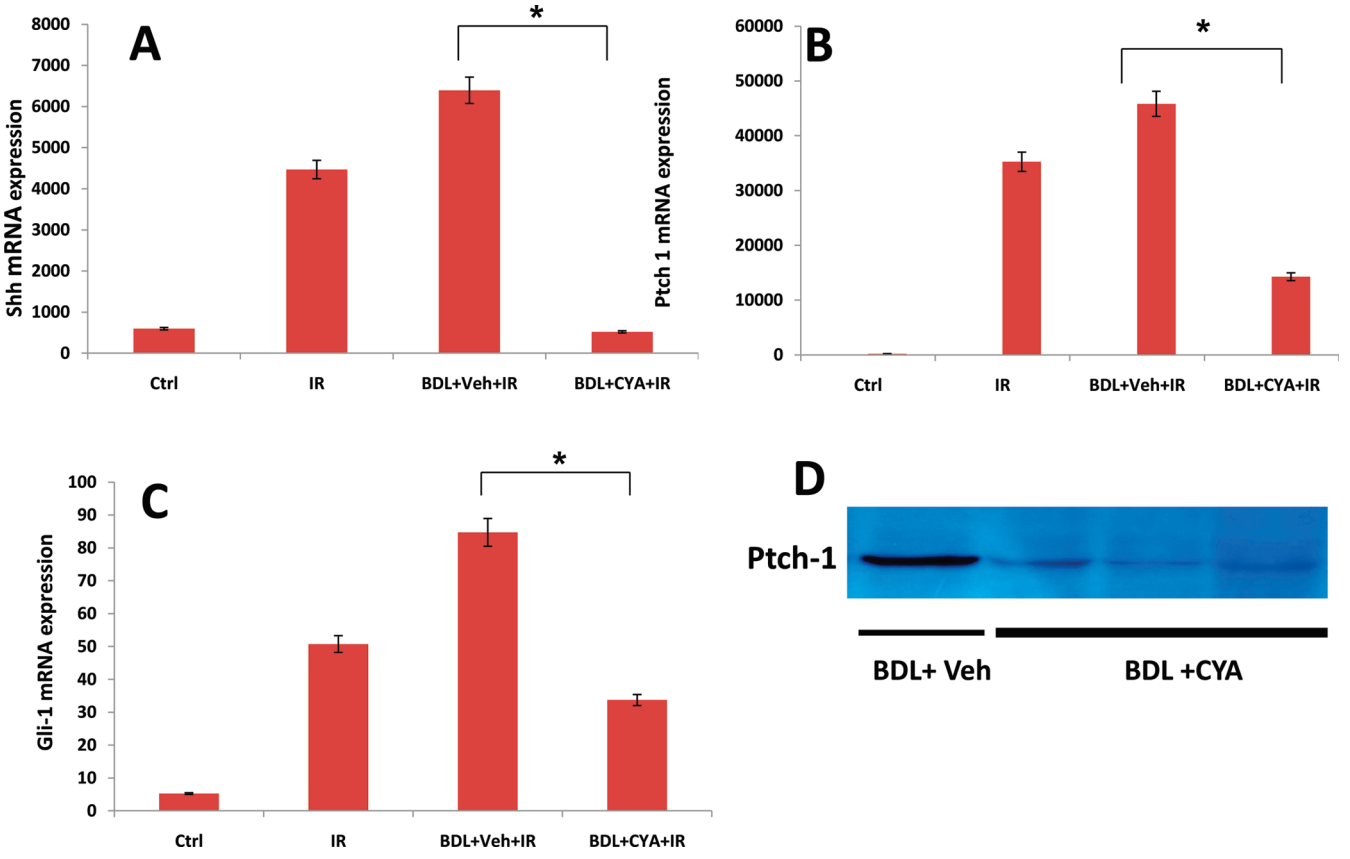

Figure 2. Hh pathway is efficiently inhibited by cyclopamine. (A−C) Real-time PCR showing downregulation of Hh signaling after treatment with CYA, *$P < 0.05$. (D) Western blot for Ptch-1 used to confirm protein expression after CYA treatment.

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, Carlsbad, CA). 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 Shh, Ptch-11, Gli-1, TNF-α, IL-1β, α-sma, type 1 collagen, phospho and total ERK, phospho and total Akt, beta actin primary antibodies for 16 h at 4 °C as described.[23] To correct for equal loading and blotting, all blots were reprobed with beta 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). Quantitation of Western blots was done using Gelscape software (http://www.gelscape.ualberta.ca:8080/htm/index.html).

Data Analysis. Data are presented as mean ± standard error of mean (SEM). Two sample comparisons were made and analyzed using two tailed unpaired *t* test. A *P* value <0.05 was considered statistically significant.

RESULTS

Bile Duct Ligation Is Associated with Cholestasis and Fibrosis. To confirm the establishment of cholestasis and fibrosis in our experimental group, 7 days after BDL three rats were euthanized and their livers harvested, fixed in 10% NBF and embedded in paraffin. In addition, livers from three sham operated animals were also harvested to serve as a control group. The livers of control animals showed normal gross morphology, histology and distribution of collagen (Figure 1A, upper panel). BDL animals showed enlarged pale yellow livers suggestive of cholestasis (Figure 1A, lower panel). Microscopic sections of BDL livers showed marked periportal ductal proliferation and inflammatory reaction. Picrosirrus red staining showed extensive collagen deposition around the ductular structures extending into the sinusoids and parenchyma (Figure 1A, lower panel).

Hepatic Expression of Hedgehog Related Molecules in Cholestatic Liver. To investigate the expression of Hh signaling in cholestatic liver, we performed real time PCR for mRNA expression of Sonic Hedgehog (Shh), Patched-1 (Ptch-1) and Glioblastoma-1 (Gli-1) genes in sham operated and BDL rats. Weak expression of Shh was detected in healthy control rats. In contrast, significant upregulation of Shh, Ptch-1 and Gli-1 was seen at both mRNA (Figure 1B−D) and protein levels (Figure 1E). Dual immunoflorescence of Hh molecules with CK-7, a marker of mature and immature bile ductular cells, showed that Hh ligands colocalized with CK-7 in ductular cells of bile ducts and sinusoids (Figures 1F).

Hedgehog Pathway Is Abrogated by Cyclopamine. The major focus of this study was to inhibit the Hh pathway by preconditioning BDL rats with CYA before subjecting them to I/R injury. Efficiency of Hh blockade was determined by mRNA

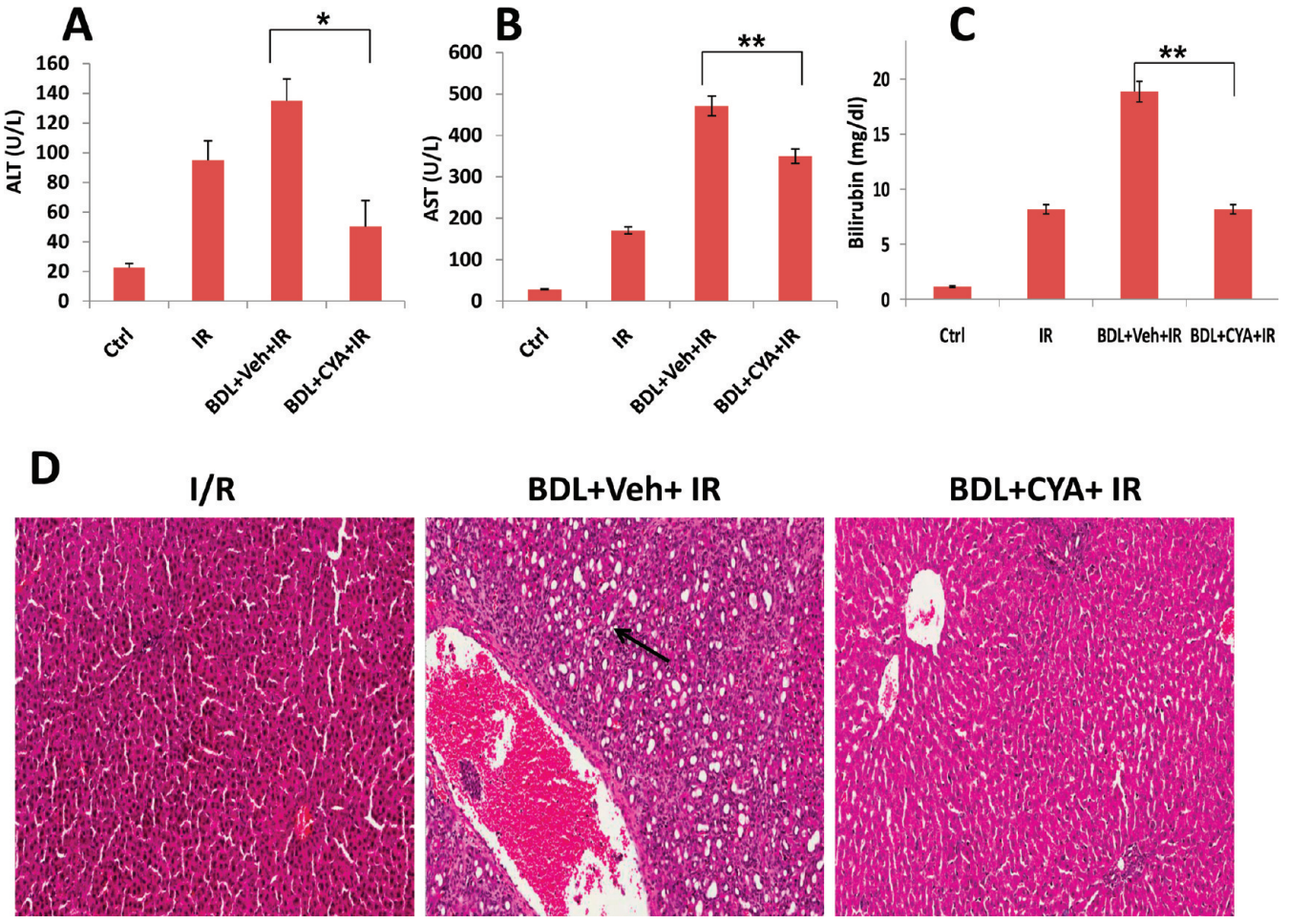

Figure 3. Cyclopamine markedly preserves liver functions and reduces histological liver injury. (A−C) Serum AST, ALT and bilirubin in normal, I/R, vehicle treated BDL and CYA treated BDL rats after 30 min ischemia and 4 h reperfusion were quantified. Liver injury markers in the CYA treated group were significantly lower than those in the vehicle treated group, $^{*}P < 0.05$ and $^{**}P < 0.01$. (D) H&E staining of representative liver sections from rats as described above. Necrosis and infiltration of inflammatory cells were observed in vehicle BDL rats (arrow indicates vacuolization and sinusoidal congestion). These effects were attenuated in the CYA treated group.

expression of Shh, Ptch-1 and Gli-1 by real time PCR (Figure 2A−C) and protein expression of Ptch-1 (Figure 2D).

Cyclopamine Markedly Blunted the Increase of Serum ALT, AST and Bilirubin. Thirty minutes of ischemia and 4 h of reperfusion significantly increased serum AST, ALT and bilirubin levels in BDL vehicle treated rats compared to control rats I/R rats ($^{**}P < 0.01$ and $^{*}P < 0.05$, Figure 3A−C). CYA resulted in a 28-fold, 4-fold and 10-fold decrease in serum ALT, AST and bilirubin levels. These results indicate that cholestatic livers are more susceptible to hepatocellular injury following ischemia reperfusion and inactivation of Hh signaling significantly attenuates this injury.

Preconditioning with Cyclopamine Reduces Histological Liver Injury. Histological architecture of control I/R rats after 4 h of reperfusion showed minimal damage (Figure 3D). In contrast, vehicle treated BDL demonstrated confluent areas of necrosis, and intense inflammatory neutrophilic infiltrates (black arrow, Figure 3D). CYA treated animals, however, showed preservation of liver architecture. The degree of tissue injury (based on Suzuki criteria) was 1.1 ± 0.02, 4.2 ± 0.01 and 2.1 ± 0.5 for I/R, BDL vehicle I/R and BDL CYA I/R treated rats respectively ($P < 0.05$).

Cyclopamine Blunts Neutrophil Infiltration and Lipid Peroxidation. To evaluate the role of Hh inhibition on the neutrophil infiltration, liver sections were stained with naphthol AS-D chloroacetate esterase and intercellular adhesion molecule, ICAM-1 (Figure 4A). Control I/R rats subjected to 4 h of reperfusion showed minimal neutrophilic infiltration in the sinusoids (4 ± 2 per 10HPF). The number of infiltrating neutrophils increased markedly in BDL vehicle treated animals (45 ± 10 per 10HPF). CYA markedly reduced the neutrophilic infiltration by 33% (15 ± 3 per 10HPF, $^{**}P < 0.01$, Figure 4B). Weak staining for ICAM-1 was seen along the endothelium of the portal vein in control I/R rats. Vehicle treated BDL rats showed more intense ICAM-1 expression along sinusoidal lining and endothelium of larger vessels. CYA treatment significantly attenuated the expression of ICAM-1. The rate of lipid peroxidation doubled in BDL vehicle treated group compared to control I/R rats (Figure 4C). In animals treated with CYA, tissue MDA level was markedly reduced.

Cyclopamine Attenuates Expression of Proinflammatory Cytokines. The production of TNF α and IL-1β in liver tissue was assessed by qRT-PCR and Western blot analysis. Vehicle treated BDL rats showed marked upregulation of TNF-α and IL-1β mRNA

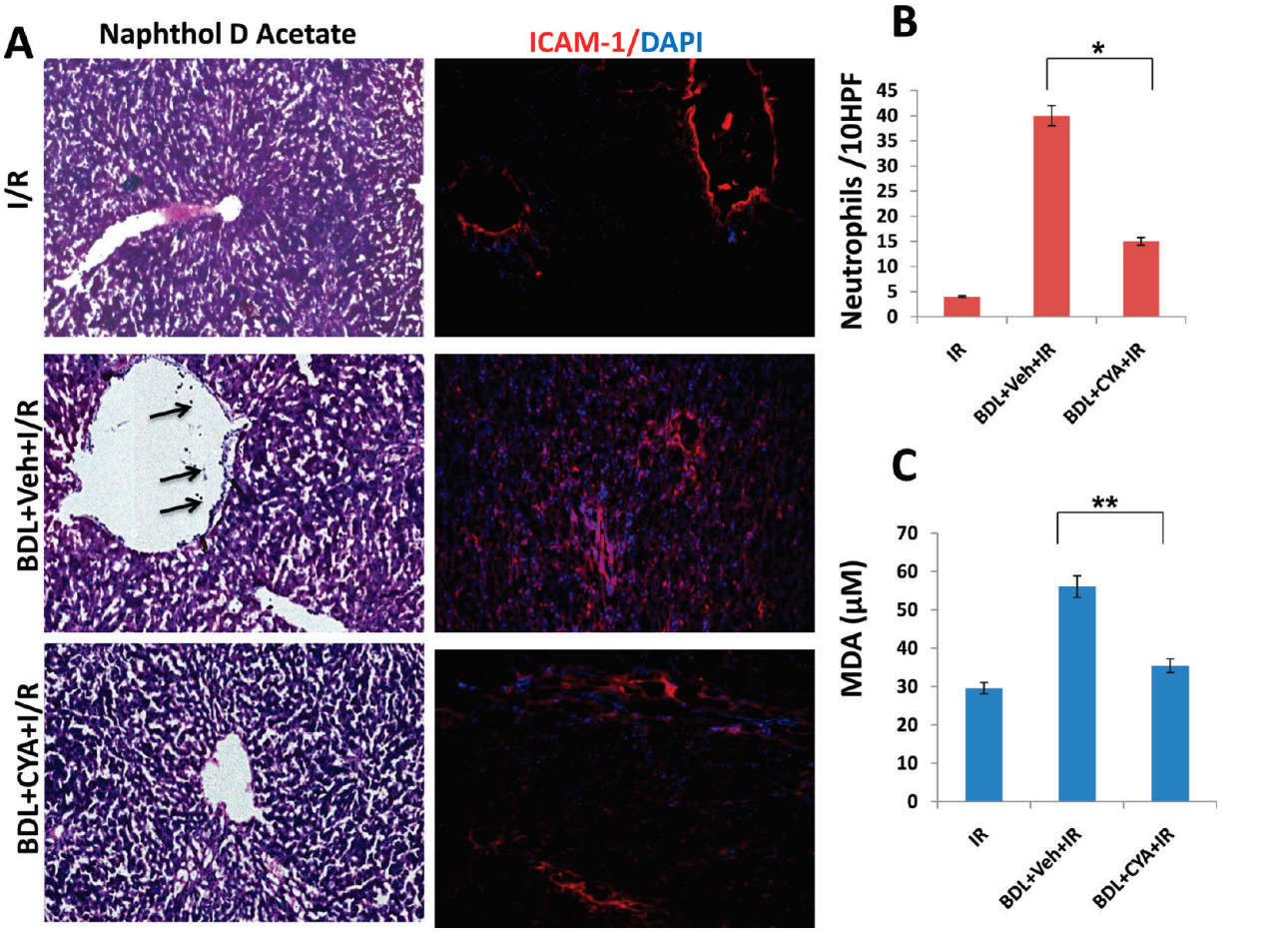

Figure 4. Cyclopamine decreases neutrophilic infiltration, ICAM-1 expression and lipid peroxidation. (A) Naphthol D acetate staining and ICAM-1 expression in liver sections of I/R, vehicle and CYA treated BDL I/R rats. Marked reduction in neutrophils and decreased expression of ICAM-1 was seen in CYA treatment rats. (B) Quantification of neutrophils counted in 10HPF is shown, $^{*}P < 0.01$. (C) CYA treatment reduced MDA levels compared to vehicle treated group, $^{**}P < 0.05$.

compared to control I/R rats (Figure 5A,B). CYA downregulated mRNA expression of these cytokines. Real time PCR findings were confirmed by Western blot analysis of protein expression in liver homogenates. CYA treated rats showed a striking decline in TNF-α and IL-1β protein expression (Figure 5C).

Cyclopamine Decreases HSC Activation and Bile Duct Proliferation. Next we explored the effect of CYA on early fibrosis. Extensive fibrosis was seen in BDL vehicle treated rats at 2 weeks. Histological evaluation for collagen depositon with Masson trichrome and Sirrus red staining demonstrated expanded areas of portal fibrosis (Figure 6A, upper and middle panels). Treatment with CYA significantly decreased the extracellular matrix deposition in BDL rats. The favorable changes mediated by CYA were also accompanied by marked reduction in expression of α-sma, a marker of activated hepatic stellate cells (Figure 6A, lower panel). These results were confirmed by immunoblotting for protein expression of α-SMA and type I collagen (Figure 6B). Furthermore, there was markedly increased biliary duct proliferation in vehicle treated BDL animals as compared with CYA treatment, the latter showing mild to moderate biliary duct proliferation (Figure 6C). The bile duct proliferation score in vehicle treated and CYA treated BDL rats is shown in Figure 6D, in which it is evidently clear that CYA treatment resulted in reduced biliary proliferation compared to vehicle treated group. Taken together our results indicate that treatment with CYA reduces the activation of hepatic stellate cells and cholangiocyte proliferation after BDL. Concurrent with the above findings we further examined the effect of CYA on hepatocyte proliferation and apoptosis using anti-PCNA antibody and TUNEL respectively. BDL rats treated with saline showed increased proliferation compared to control I/R rats (Figure 6E, upper panel). The number of PCNA positive cells significantly declined in the CYA treated group. Compared to control I/R rats, liver sections from vehicle treated BDL animals showed a large number of TUNEL positive cells with characteristic morphology for apoptosis (Figure 6E, lower panel). Treatment with CYA decreased the number of TUNEL positive cells. The proliferation index and apoptotic index in CYA treated rats was reduced by 53.3% and 40% respectively when compared with the vehicle treated group (Figure 6F).

Cyclopamine Mediates Anti-Inflammatory Effects by Inhibiting the Akt and Mitogen Protein Kinase−ERK1/2 Pathway. We next investigated the association between Hh signaling and known cell survival pathways, i.e. the PI3K/Akt and MAPK signaling. The nonphosphorylated states of Akt and ERK1/2 remain unchanged after CYA treatment (Figure 7A,B). However, cyclopamine treatment induced a decrease in the phosphorylation state of Akt and ERK1/2. Preventing activation of ERK and

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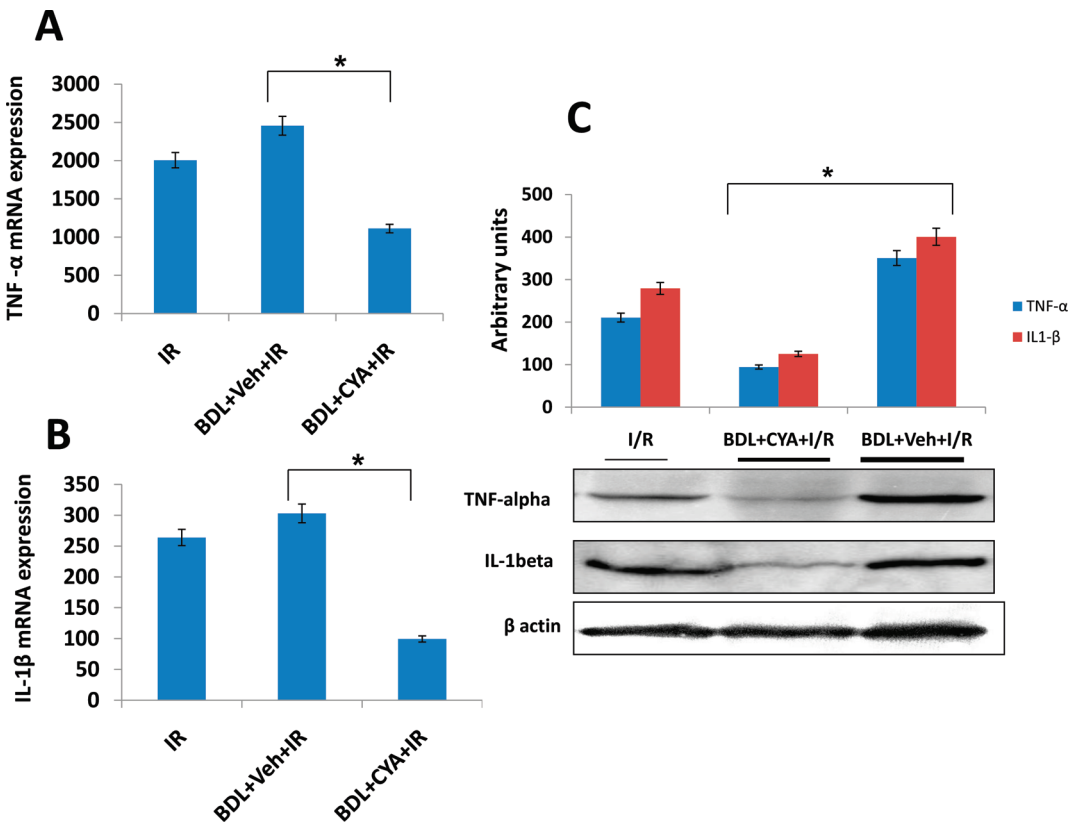

Figure 5. Cyclopamine induces downregulation of proinflammatory cytokines. (A, B) Total RNA was isolated from I/R, vehicle and CYA treated BDL I/R rats, and expression of TNF-α and IL-1β was assessed by real time RT-PCR, *P < 0.01. (C) Total protein from liver was extracted and subjected to Western blotting for detection of TNF-α and IL-1β, *P < 0.01.

Akt indicates pivotal role of these signaling pathways in HSC activation and propagating inflammatory injury.

Discussion. Liver transplantation is now considered as a cure for end stage liver disease. However, as the number of patients on the liver transplant waiting list continues to grow, the demand for donor organs increases. Although expansion of donor criteria to include marginal livers such as those which are steatotic or cholestatic has led to their becoming a significant source of transplantable organs, their use is hampered by the higher risk of postoperative complications. Ischemia reperfusion is pivotal in causing hepatocellular damage in these marginal livers. We recently reported that the Hh pathway is constitutively upregulated in healthy liver and renders it susceptible to I/R injury.[15] Based on these observations we extended our hypothesis that Hh signaling may mediate inflammatory changes in cholestatic liver I/R injury. The principal findings of this study were as follows: (1) the Hh pathway is upregulated in cholestatic liver; (2) The severity of ischemia reperfusion injury is more marked in cholestatic liver and parallels Hh expression; (3) Hh inhibition with CYA is hepatoprotective by reducing neutrophil infiltration, lipid peroxidation, and release of proinflammatory cytokines; (4) CYA reduces early fibrosis by downregulating activation of hepatic stellate cells and profibrogenic genes; and (5) CYA reduces the proliferation of biliary epithelium. Taken together our results provide new evidence for the role of endogenous Hh signaling in cholestatic liver subjected to warm IR injury.

Consistent with previously published reports, the present study demonstrates increased Hh expression in BDL rats by RT-PCR, immunoflorescence and Western blot analysis.[24−27] Hh pathway inhibiting experiments were performed by preconditioning BDL animals with CYA. Vehicle treated BDL rats subjected to 30 min of ischemia and 4 h of reperfusion showed marked increase in serum AST, ALT and bilirubin compared to CYA treated rats. Morphometric analysis of liver sections from vehicle treated BDL rats showed marked increase in inflammatory injury compared to CYA rats. Taken together, it is evidently clear that I/R elicited responses were greater when ischemia was superimposed upon cholestatic liver. These findings provide new evidence that endogenous Hh signaling promotes hepatocellular injury in cholestatic liver, which can be attenuated significantly by inhibiting this pathway.

One important feature of liver damage after I/R injury is inflammation.[28,29] The inflammatory response is more intense and exponentially amplified in cholestatic livers experiencing I/R injury.[30] Most prominent among the inflammatory cells are neutrophils, which are recruited within hours of reperfusion.[31] Neutrophil recruitment contributes to cytotoxic inflammatory injury by increased ICAM-1, and facilitating upregulation of proinflammatory cytokines such as TNF-α and interleukins.[32−34] Neutrophils can further aggravate I/R injury in cholestatic liver.[35] Cumulatively, our data indicated extensive liver injury manifesting as markedly elevated liver enzymes, intense neutrophilic infiltration and ICAM-1 expression in vehicle treated BDL rats (Figure 4). This aggravated liver injury was substantially reduced by CYA. Recent studies have confirmed the role of proinflammatory cytokines like TNF-α and IL-1β in initiating and propagating inflammatory responses after I/R.[36−39] These cytokines recruit neutrophils by upregulating vascular cell adhesion molecules and

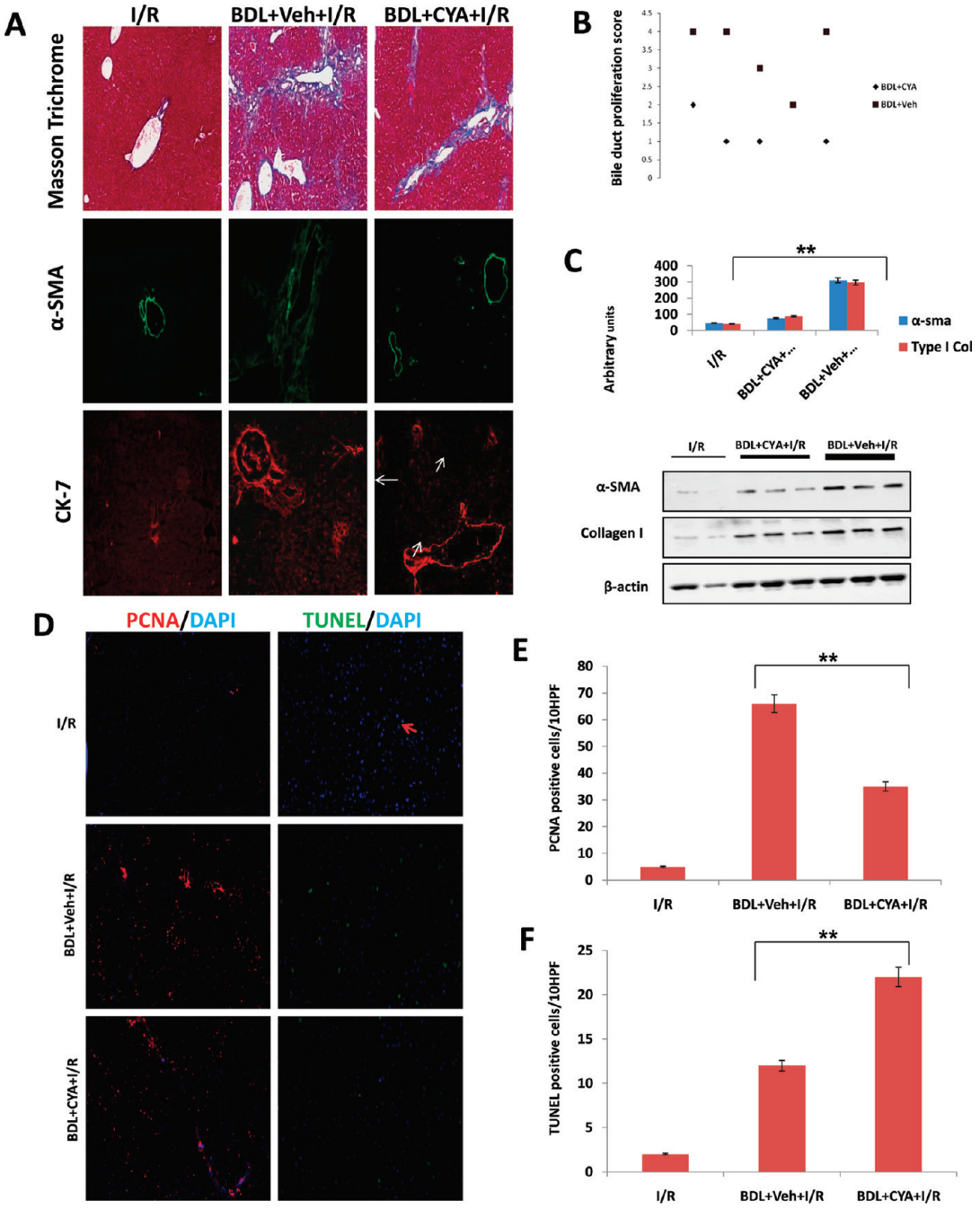

Figure 6. Cyclopamine reduces liver fibrosis and bile duct proliferation. (A) Preconditioning with cyclopamine reduced collagen deposition (Masson trichrome and Sirrus red staining) and α-smooth muscle actin expression. Original magnification ×40 (Zeiss AX10 microscope). (B) Total protein from liver was extracted and subjected to Western blotting for detection of α-sma and type 1 collagen, $^{**}P < 0.05$. (C) H&E and CK-7 expression in proliferating bile ducts in I/R, vehicle and CYA treated BDL I/R rats. CK-7 staining in vehicle treated BDL I/R group showed florid and abundant biliary duct proliferation compared with CYA treated rats. (D) The degree of biliary proliferation was scored as in material and methods. (E) CYA treatment resulted in reduced number of PCNA and TUNEL positive cells. Original magnification ×40 (Zeiss AX10 microscope). (F) Proliferation index and apoptosis index in each group was calculated by counting PCNA and TUNEL positive cells in 10 random high power fields (40×), $^{**}P < 0.05$.

inducing neutrophil CXC cytokines.[40] Genetic or pharmacological inhibition of TNF-α is an emerging strategy to reduce the burden of multiorgan I/R injury.[37,41] Our data in vehicle treated BDL rats showed increased expression of TNF-α and IL-1 β. CYA treatment was hepatoprotective by reduction in the expression of these proinflammatory cytokines (Figure 5). Lipid peroxidation was also reduced by CYA possibly secondary to overall decreased activation of neutrophils and inhibition of proinflammatory cytokines. There has been a recent report concluding that cholestasis protects mouse liver from I/R injury.[42] On the contrary the results of our study and others indicate that cholestatic liver is more susceptible to I/R injury.[43] Genetic differences in the animal models or other molecular mechanisms could affect the hepatocellular inflammatory responses to I/R injury in cholestatic livers.[44]

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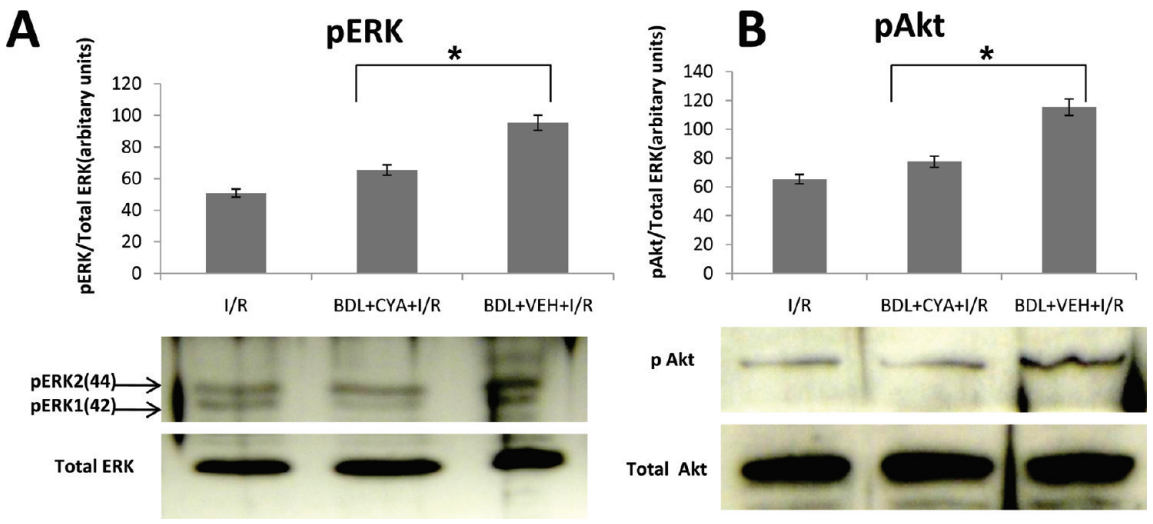

Figure 7. Cyclopamine reduces activation of PI-3K and MAPkinase signaling. (A, B) Total protein was extracted from liver tissue of I/R, vehicle and CYA treated BDL I/R rats, and phosphorylation of ERK1/2 and Akt was performed by Western blotting. Membranes were stripped and reprobed with total ERK and Akt antibodies to verify protein levels, $^*P < 0.05$.

Cholestasis and I/R injury lead to significant alterations in both portal and arterial hemodynamics resulting in HSC activation and cholangiocyte proliferation. The activated HSCs release proinflammatory cytokines and cell adhesion molecules thereby aggravating the existing inflammatory injury.[45−47] HSCs and cholangiocytes express target Hh genes and proliferate under Hh signaling resulting in increased ECM deposition and intrahepatic ductal mass.[13] In our study, *in vivo* administration of CYA to BDL rats decreased expression of α-sma and type 1 collagen resulting in decreased fibrosis. It also decreased the number of proliferating bile ducts. We also evaluated the effect of CYA on hepatocyte proliferation and apoptosis. In normal rat liver, the hepatocytes are mitotically quiescent with rare apoptosis.[48,49] However, increased hepatocyte proliferation was noted under pathological conditions such as cholestasis. CYA treatment was associated with reduced hepatocyte proliferation and apoptosis. In support of our findings, studies have shown antiproliferative effects of CYA in other cell types.[50,51] The exact mechanism by which CYA exerts its antiproliferative effects is presently not clearly understood. However there is growing evidence showing regulation of proliferation and survival of HSCs and expression of profibrogenic genes by both MAPkinase and PI3K-Akt signaling.[52−54] In the present work we show that CYA treatment in BDL animals inhibited the activation of these signaling components suggesting a putative role of these well-characterized signaling molecules. However, more studies need to be carried out to completely delineate this signaling in our experimental system. Ductal proliferation seen after BDL is fundamentally crucial to sustain the enhanced functional and nutritional needs in addition to compensating for ongoing loss of ductal mass due to increased biliary pressure. One could argue that the inhibition of ductal proliferation by CYA would negatively affect liver regeneration and remodeling. Future studies on the effect of CYA at different time points should determine whether CYA is detrimental in remodeling of I/R cholestatic liver.

In summary, the results of this study provide convincing evidence of hepatoprotection following warm ischemia reperfusion injury by Hh antagonist cyclopamine in a rat model of cholestasis.

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ABBREVIATIONS USED

ALT, alanine transaminase; AST, serum aspartate transaminase; BDL, bile duct ligation; CK-7, cytokeratin-7; CYA, cyclopamine; DAPI, 4′,6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; EMT, epithelial mesenchymal transformation; ERK, extracellular signal regulated kinase; Gli-1, Glioblastoma-1; HBCD, 2-hydroxypropyl-β-cyclodextrin; Hh, Hedgehog; HSC, hepatic stellate cells; ICAM-1, intercellular adhesion molecule; IL-1β, interleukin-1β; I/R, ischemia reperfusion; MDA, malondialdehyde; NBF, neutral buffered formalin; Ptch-1, Patched-1; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene fluoride; α-SMA, alpha- smooth muscle actin; Shh, Sonic Hedgehog; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

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