Ischemia reperfusion of the hepatic artery induces the functional damage of large bile ducts by changes in the expression of angiogenic factors

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Mancinelli R, Glaser S, Francis H, Carpino G, Franchitto A, Vetuschi A, Sferra R, Pannarale L, Venter J, Meng F, Alpini G, Onori P, Gaudio E. Ischemia reperfusion of the hepatic artery induces the functional damage of large bile ducts by changes in the expression of angiogenic factors. *Am J Physiol Gastrointest Liver Physiol* 309: G865–G873, 2015. First published September 21, 2015; doi:10.1152/ajpgi.00015.2015.—Liver transplantation and cholangiocarcinoma induce biliary dysfunction following ischemia reperfusion (IR). The function of the intrahepatic biliary tree is regulated by both autocrine and paracrine factors. The aim of the study was to demonstrate that IR-induced damage of cholangiocytes is associated with altered expression of biliary angiogenic factors. Normal and bile duct ligation rats underwent 24-h sham or hepatic reperfusion after 30 min of transient occlusion of the hepatic artery (HAIR) or portal vein (PVIR) before collecting liver blocks and cholangiocyte RNA or protein. We evaluated liver histology, biliary apoptosis, proliferation and expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in liver sections and isolated small and large cholangiocytes. Normal rat intrahepatic cholangiocyte cultures (NRICC) were maintained under standard conditions in normoxic or under a hypoxic atmosphere for 4 h and then transferred to normal conditions for selected times. Subsequently, we measured changes in biliary proliferation and apoptosis and the expression of VEGF-A/C and VEGFR-2/3. In vivo, HAIR (but not PVIR) induced damage of large bile ducts and decreased proliferation and secretin-stimulated cAMP levels. HAIR-induced damage of large bile ducts was associated with increased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2. In vitro, under hypoxic conditions, there was increased apoptosis and reduced proliferation of NRICC concomitant with enhanced expression of VEGF-A/C and VEGFR-2/3. The functional damage of large bile ducts by HAIR and hypoxia is associated with increased expression of angiogenic factors in small cholangiocytes, presumably due to a compensatory mechanism in response to biliary damage.

angiogenic factors; biliary tree; cholangiocytes; liver; transplantation

CHOLANGIOCYTES LINE THE INTRAHEPATIC biliary epithelium, a complex network of interconnecting ducts of different sizes and functions (5, 35). In humans, cholangiocytes are the target cells in a number of biliary disorders characterized by dysregulation between biliary growth/loss (23). Normally, cholangiocytes have low replicative activity, but proliferate in experimental models that mimic human pathologies, such as ligation

of the extrahepatic bile duct (BDL) and acute carbon tetrachloride administration (CCl$_4$) (3, 32). The human and rodent biliary epithelium is morphologically and functionally heterogeneous (5, 21, 27, 32, 35, 38). In rat liver, isolated small cholangiocytes lining smaller ducts (<15 μm in diameter) are mitotically dormant and exert their functions by activation of the D-*myo*-inositol 1,4,5-trisphosphate (IP$_3$)/Ca^{2+}/calmodulin-dependent protein kinase I signaling pathway (1, 13, 35). On the other hand, isolated large cholangiocytes lining larger ducts (>15 μm in diameter) secrete and proliferate by activation of cAMP-dependent signaling (2, 5, 21, 27, 35).

Several experimental models have shown that ischemia reperfusion (IR) injury plays a role in liver pathophysiology (43). Orthotopic liver transplantation (OLT) has been shown to be a successful treatment choice for patients with end-stage chronic or acute liver failure. However, biliary complications remain a significant source of morbidity (51, 52). Most of these complications consist of anastomotic or nonanastomotic strictures (NAS), biliary necrosis, or leakage (48). Therefore, biliary complications represent major causes of morbidity and graft failure as well as mortality after liver transplantation (26, 34). Hepatic IR elicits hepatic tissue repair, which is characterized by the proliferation of hepatocytes, removal of necrotic tissue, and restoration of the hepatocellular and hepatic microvascular architecture (44). Several mediators, including cytokines and angiogenic factors, regulate the mechanisms underlying hepatocellular regeneration. The function of the intrahepatic biliary epithelium is linked to its vascular supply sustained by the peribiliary arterial plexus (PBP) (15, 47), since alterations of intrahepatic bile duct mass are associated with architectural changes in the PBP (14). The PBP stems from the hepatic artery, nourishes the biliary tree, and sustains a countercurrent of substances reabsorbed from bile toward parenchymal cells (17). After BDL, the increase in intrahepatic bile duct mass is followed by a parallel growth of the PBP, which is fundamental in sustaining the enhanced nutritional and functional demands of the proliferating biliary epithelium (16, 22). Nevertheless, the proliferation of the PBP occurs only after the hyperplasia of the biliary epithelium (17), suggesting a cross-talk mechanism between cholangiocytes and endothelial cells, an interaction that mediates the adaptive changes of these cells during liver damage (14, 45).

VEGF is a family of related growth factors including VEGF-A, -B, -C, -D, and -E and placenta growth factor (11). VEGF is secreted by a number of epithelia including cholan-

giocytes where it modulates functions by autocrine and para-crine mechanisms by interacting with VEGFR-2 and VEGFR-3 (14, 15). Other important angiogenic factors are the Angiopoietins (Ang-1 and Ang-2), which bind to the tyrosine kinase receptor Tie-1/2 (41). Angiopoietins have opposite effects on their receptors: Ang-1 activates Tie-2 by tyrosine phosphorylation, whereas Ang-2 antagonizes the Ang-1/Tie-2 binding (40). In primary biliary cirrhosis the enhanced expression of VEGF-A, Ang-1, Ang-2, and Tie-2 receptor by endothelial cells and periportal hepatocytes is responsible for the angiogenesis occurring in close proximity to the damaged bile ducts (40), which may contribute to recruit the inflammatory cells worsening the pathology. Reduced portal fibrosis and hypertension was observed in cholestatic rats by inhibition of VEGFR-2 (39).

In this study, we evaluated the impact of the IR of hepatic artery and portal vein on biliary functions and the role of the angiogenic factors during these conditions.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The selected rat primers were purchased from SABiosciences (Qiagen, Valencia, CA) and designed using sequences with the following NCBI GenBank Accession numbers: PCNA, NM_022381; Bax NM_017059; VEGF-A NM_031836; VEGF-C NM_053653; VEGFR2 NM_013062; VEGFR3 NM_053652; angiopoietin 1 NM_053546; angiopoietin 2 NM_134454; Tie-1 NM_053545; and Tie-2 NM_001105737. The RNeasy Mini Kit to purify total cholangiocyte RNA was purchased from Qiagen. The RIA
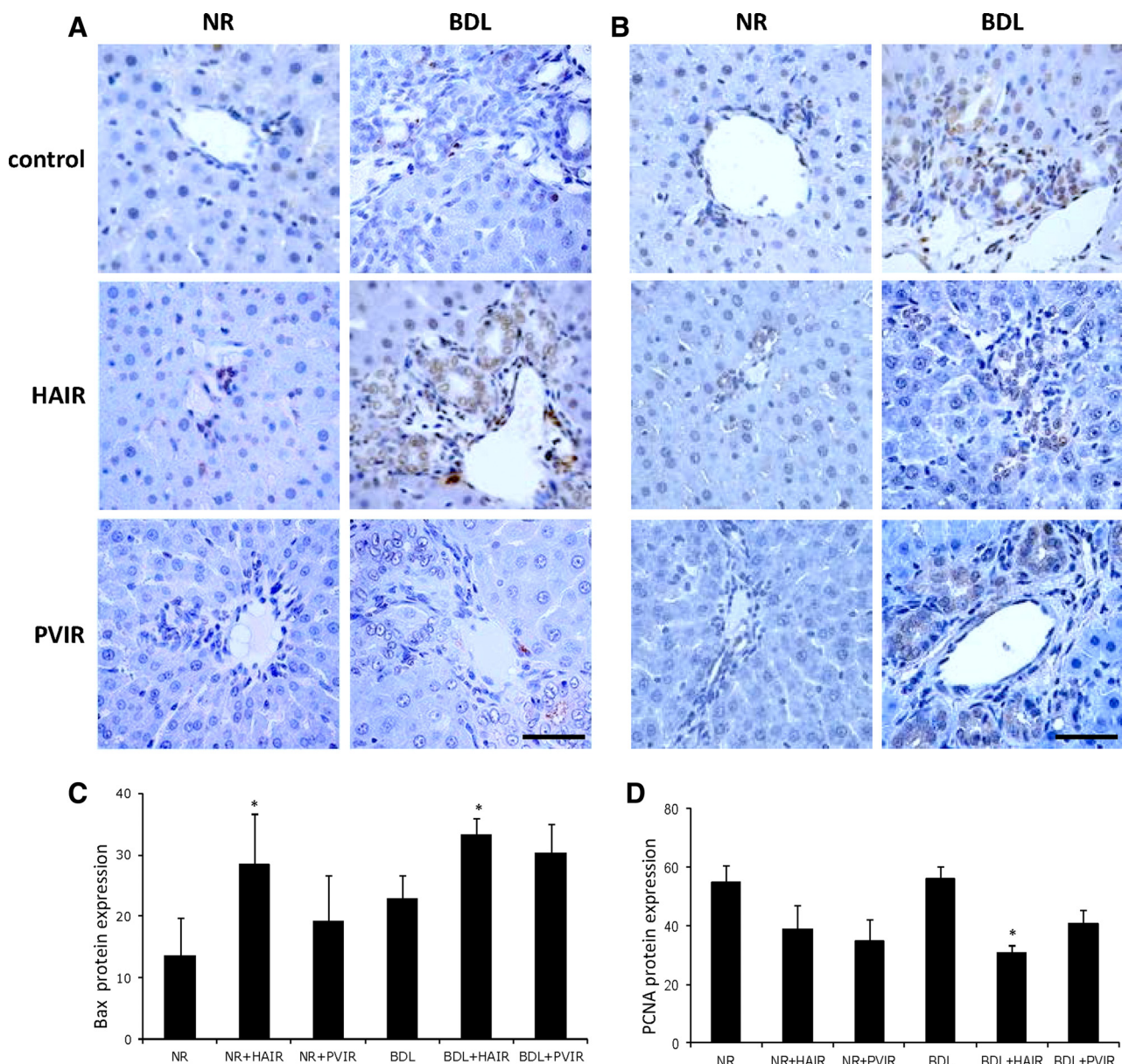

Fig. 1. In bile duct ligation (BDL) rats, hepatic artery ischemia reperfusion (HAIR) increased apoptosis by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) analysis (*A*) and decreased the proliferation by reduced proliferating cell nuclear antigen (PCNA) expression (*B*) of large cholangiocytes in liver sections. Original magnification, ×20; scale bar = 60 μm (for semiquantitative analysis, see Table 1). *C*: there was increased Bax expression (*D*) and reduced PCNA presence in purified cholangiocytes from normal and BDL rats by immunoblots. *P < 0.05 vs. BDL rats.

Table 1. *Measurement of biliary apoptosis by TUNEL assay and proliferation by PCNA expression in small and large bile ducts*

Parameter	Normal		Normal + HAIR		Normal + PVIR		BDL		BDL + HAIR		BDL + PVIR	
	Small	Large	Small	Large	Small	Large	Small	Large	Small	Large	Small	Large
Biliary apoptosis, %	1.89 ± 0.2	2.92 ± 0.3	3.15 ± 0.8	8.79 ± 0.2*	2.97 ± 0.6	6.24 ± 0.4	7.64 ± 0.3	14.42 ± 0.5	7.78 ± 0.2	17.15 ± 0.6*	7.44 ± 0.4	14.94 ± 0.4
PCNA expression	2.06 ± 0.4	9.74 ± 0.2	3.3 ± 0.1	5.64 ± 0.3*	3.13 ± 0.1	7.31 ± 0.4*	4.81 ± 0.6	29.1 ± 0.6	8.64 ± 0.2#	18.2 ± 1.2*	6.82 ± 0.3#	23.1 ± 0.5*

Values are means ± SE. Small bile ducts = <15 µm diameter; large bile ducts = >15 µm diameter. Apoptosis of small and large bile ducts was measured by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) analysis in liver sections. Proliferation of small and large bile ducts was measured by evaluating the expression of PCNA positive cholangiocytes in liver sections. HAIR, hepatic artery ischemia reperfusion; PVIR, portal vein ischemia reperfusion. *P < 0.05 vs. the number of large bile ducts (positive by TUNEL and PCNA) from the corresponding normal and bile duct ligation (BDL) control rats; #P < 0.05 vs. the number of small bile ducts (positive by TUNEL and PCNA) from the corresponding normal and BDL control rats.

Table 2. *Measurement of the percent cholangiocytes positive for VEGF-A/C, VEGFR-2/3, Angio-1/2, and Tie-1/2 in liver sections*

Parameter	Normal		Normal + HAIR		Normal + PVIR		BDL		BDL + HAIR		BDL + PVIR	
	Small	Large	Small	Large	Small	Large	Small	Large	Small	Large	Small	Large
VEGF-A, %	5.5 ± 0.4	10.2 ± 0.8	9.9 ± 0.3*	13 ± 0.5#	8.5 ± 0.2*	10.5 ± 0.2	26.1 ± 0.4	48.9 ± 0.5	38.4 ± 0.3*	44.4 ± 0.3#	31.7 ± 0.1*	45.3 ± 0.3#
VEGF-C, %	5 ± 0.5	8.1 ± 0.6	7.6 ± 0.2*	10.8 ± 0.7	6.9 ± 0.2	10.6 ± 0.2	19 ± 0.3	42.7 ± 0.3	29.4 ± 0.2*	39.3 ± 0.2	22.5 ± 0.3	38.4 ± 0.2
VEGF-R2, %	2.1 ± 0.3	6.5 ± 0.6	5.2 ± 0.2*	7.9 ± 0.3#	3.4 ± 0.3*	5.6 ± 0.2	18.5 ± 0.6	46.1 ± 0.7	29.5 ± 0.2*	42.5 ± 0.2#	23.4 ± 0.3*	41.4 ± 0.4#
VEGF-R3, %	2.3 ± 0.3	4.2 ± 0.6	4.3 ± 0.3*	6.1 ± 0.2#	3.1 ± 0.1*	5.5 ± 0.3	18.6 ± 0.4	38.3 ± 0.5	28.6 ± 0.4*	37.7 ± 0.1	24.2 ± 0.5*	38.6 ± 0.2
Ang-1, %	2.7 ± 0.3	4.2 ± 0.4	4.5 ± 0.2*	5.5 ± 0.2	3.2 ± 0.3*	5.8 ± 0.2#	13.2 ± 0.4	37.7 ± 0.4	27.4 ± 0.2*	31.4 ± 0.9#	20.4 ± 0.1*	34.4 ± 0.2
Ang-2, %	1.3 ± 0.3	6.1 ± 0.2	4.9 ± 0.2*	6.7 ± 0.1	3.3 ± 0.3*	7.7 ± 0.2#	12.1 ± 0.2	40.1 ± 0.6	28.4 ± 0.1*	34.5 ± 0.1#	24.2 ± 0.8*	37.4 ± 0.2
Tie-1, %	1.3 ± 0.4	6.3 ± 0.5	4.8 ± 0.2*	6.4 ± 0.4	3.5 ± 0.3*	7.5 ± 0.2#	13.6 ± 0.2	42 ± 0.3	26.5 ± 0.2*	32.7 ± 0.2	18.5 ± 0.2	38.5 ± 0.3
Tie-2, %	2.1 ± 0.4	5.7 ± 0.3	5.2 ± 0.3*	6.9 ± 0.3#	3.4 ± 0.1*	6.4 ± 0.4	12.4 ± 0.2	29 ± 0.4	21.6 ± 0.3*	26.3 ± 0.4	17.8 ± 0.3*	25.1 ± 0.3#

Values are means ± SE. Semiquantitative analysis of the expression of VEGFA-C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in small and large cholangiocytes in liver sections is shown. *P < 0.05 vs. the number of small bile ducts (positive for VEGFA-C, VEGFR-2/3, Ang-1/2, and Tie-1/2) from the corresponding normal and BDL control rats; #P < 0.05 vs. the number of large bile ducts (positive by TUNEL and PCNA) from the corresponding normal and BDL control rats.

kits for the measurement of intracellular cAMP (cAMP [^{125}I] Biotrak Assay System, RPA509) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

In vivo and in vitro models. Male 344 Fischer rats (150–175 gm) were purchased from Charles River (Wilmington, MA) and kept in a temperature-controlled environment (22°C) with 12-h:12-h light/dark cycles. Animals were fed ad libitum and had free access to drinking water. The studies were performed in normal rats and rats that after

1 wk of BDL underwent 30 min of transient occlusion of the hepatic artery (HAIR) or the portal vein (PVIR) followed by 24 h of hepatic reperfusion or sham surgery (28). BDL was performed as described (3). From these groups of animals, we collected liver blocks and cholangiocytes. Before each procedure, animals were injected with euthasol following the regulations of the panel of euthanasia of American Veterinarian Medical Association, and protocols were approved by Baylor Scott & White Institutional
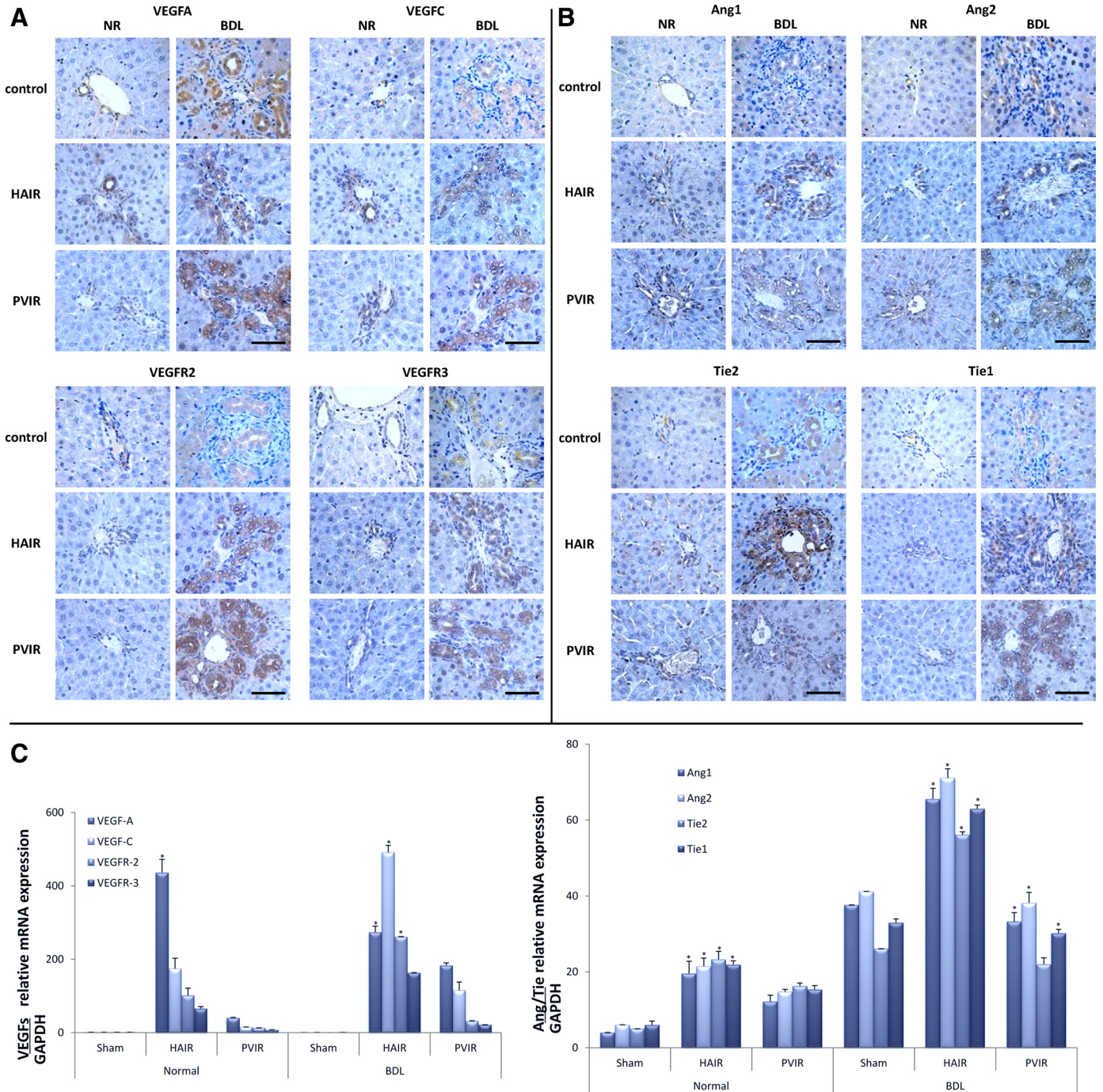

Fig. 2. In BDL rats, there was enhanced expression of VEGF-A/C, VEGFR-2, and R3 (*A*) and Ang-1/2 and Tie-1/2 (*B*) in biliary epithelium compared with normal rats by immunohistochemistry. In BDL + HAIR rats, there was increased expression of VEGF-A/C, VEGFR-2, and R3 (*A*) and Ang-1/2 and Tie-1/2 (*B*) in bile ducts and purified cholangiocytes compared with BDL rats by real time PCR (*C*). The increase in the aforementioned growth factors/receptors was smaller in BDL + PVIR rats compared with BDL + HAIR rats. Original magnification, ×20; scale bar = 60 μm (for semiquantitative analysis, see Table 2). *$P < 0.05$ vs. the corresponding sham value.

Animal Care and Use Committee. The in vitro studies were performed in our normal rat intrahepatic cholangiocyte line (NRICC) that displays morphological and functional phenotypes similar to freshly isolated cholangiocytes (4).

Evaluation of liver histology. We measured necrosis, and the degree of portal inflammation in hematoxylin-eosin (H&E)-stained paraffin-embedded liver sections (4 to 5 μm thick, 3 sections evaluated per group of animals) from the animal groups of Table 1. At least 10 different portal areas (from 3 different sections) were evaluated for each parameter. Liver sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera (49).

Evaluation of cholangiocyte apoptosis. Apoptosis in small and large cholangiocytes was evaluated by quantitative terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) analysis (Apoptag; Chemicon, Billerica, MA) in liver sections and immunoblots (12) for Bax expression in isolated cholangiocytes from the selected groups of animals. Sections were analyzed in a coded manner using BX-51 light microscopy (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic Instrument, Sterling Heights, MI) and processed with an Image Analysis System (Delta Sistemi, Rome, Italy). At least 10 different portal areas (from 3 different sections) were evaluated. Bax expression was performed in protein (10 μg) from whole cell lysates from purified cholangiocytes. Immunoblots were normalized by β-actin. The intensity of the bands was determined by scanning video densitometry using the phospho-imager, Storm 860 (GE Healthcare, Piscataway, NJ) and the ImageQuant TL software version 2003.02 (GE Healthcare) (8).

Evaluation of cholangiocyte proliferation. Cholangiocyte proliferation was studied in liver sections by PCNA immunohistochemical expression. Immunohistochemistry was performed in 3 to 4 μm thick sections. Sections were deparaffinized and endogenous peroxidase activity was blocked by a 30-min incubation in methanolic hydrogen peroxide (2.5%). Later, the endogenous biotin was blocked by a biotin blocking system (code X0590; Dako, Copenhagen, Denmark) according to the instructions supplied by the vendor. Sections were then hydrated in graded alcohol and rinsed in 1$\times$ PBS (pH 7.4) before applying the selected primary antibody. Sections were incubated overnight at 4°C with PCNA polyclonal antibodies (Santa Cruz Biotechnology, Milan, Italy). The following day, samples were rinsed with PBS for 5 min, incubated for 20 min at room temperature with secondary biotinylated antibody (LSAB Plus system; Dako, Milan, Italy) and then with Dako ABC (LSAB Plus system; Dako), and finally developed with 3,3'-diaminobenzidine. To confirm the specificity of immunoreaction, negative controls were performed for all immunoreactions. We measured the percentage of PCNA-positive small and large cholangiocytes (36). At least 10 different portal areas (from 3 different sections) were evaluated. Intrahepatic bile duct mass (BDM) was evaluated by determining the area fraction of liver parenchyma occupied by bile ducts using BX-51 light microscopy (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic Instrument, Sterling Heights, MI) and processed with an Image Analysis System (Delta Sistemi, Rome, Italy) (20). BDM was expressed as percentage of area occupied by bile ducts with respect to the total liver parenchyma. Proliferation was also evaluated by measurement of PCNA protein expression by Western blots using specific antibody and normalized by β-actin as seen previously.

Expression of angiogenic factors in cholangiocytes. The immunohistochemical expression of VEGF-A, VEGF-C, VEGF-R2, VEGF-R3, Ang-1, Ang-2, Tie-1, and Tie-2 (Santa Cruz Biotechnology, Milan, Italy) in small and large cholangiocytes was evaluated in liver sections. Immunohistochemistry for these proteins was performed as described above for PCNA staining. We measured the percentage of cholangiocytes expressing the selected angiogenic factors (36). At least 10 different portal areas (from 3 different sections) were evaluated for each parameter. Real time PCR analysis was performed using specific primers designed against rat VEGF-A, VEGF-C, VEGFR-2,

VEGFR-3, Ang-1, Ang-2, Tie-1, and Tie-2 genes. A delta delta of the threshold cycle ($\Delta\Delta$CT) analysis was performed using normal cholangiocytes as the control sample; as housekeeping, we used GAPDH (8).

Measurement of cAMP levels in purified cholangiocytes. We measured basal and secretin-stimulated cAMP levels, a functional marker of cholangiocyte proliferation (24, 31), in purified cholangiocytes from the selected groups of animals. Following incubation for 1 h at 37°C (5, 29), cholangiocytes (1 $\times$ 10^5 cells) were stimulated at room temperature for 5 min with 0.2% BSA (basal) or secretin (100 nmol/l in 0.2% BSA). Intracellular cAMP levels were assessed with commercially available kits (31, 32).

In vitro effect of normoxia and hypoxia on apoptosis, proliferation, and expression of VEGF-A/-C and VEGFR-2/-3 in NRICC. The in vitro experiments were performed in our NRICC (4). NRICC were maintained in culture with DMEM-F-12, MEM nonessential amino acids solution, Insulin-Transferrin-Selenium-X, chemically defined lipid concentrate, MEM vitamin solution, L-glutamine, Pen/Strep, Gentamicin, Bovine pituitary extract, Dexamethasone, 3,3',5-triiodo-L-thryonine, EGF, FBS, and forskolin. NRICC were maintained under standard conditions in normoxic atmosphere of 21% O_2, 74% N_2, and 5% CO_2 to reflect physiologic conditions or under a hypoxic atmosphere of 5% O_2, 90% N_2, and 5% CO_2 for 4 h and transferred to normal conditions for different times (1–3 h) with or without pre-incubation with an antibody anti-VEGF-A or anti-Angiopoietin-1. Cells were plated at 80% confluence 1 day before the incubation under hypoxic or normoxic conditions. The cells were seeded in Petri dishes and maintained in their medium. Cells were then placed in the incubator at 37°C and exposed to hypoxia in a modular incubator chamber (C-Chamber Hypoxia chamber) with continuous monitoring and automated adjustments to maintain the several parameters stable (ProOx controller; Biospherix, Redfield, NY). Afterward, we extracted from NRICC proteins and total RNA to measure changes in *1*) proliferation and apoptosis by immunoblotting and real-time PCR for PCNA and Bax, respectively; and *2*) mRNA expression of VEGF-A/C and VEGFR-2/3 by real-time PCR (12). A $\Delta\Delta$CT analysis was performed using NRICC maintained at normal levels of oxygen for 7 h as the control sample.

RESULTS

Effect of HAIR and PVIR on liver histology and biliary apoptosis and proliferation. There were no significant histological differences between the experimental groups of normal rats and normal rats plus HAIR or PVIR (not shown). On the
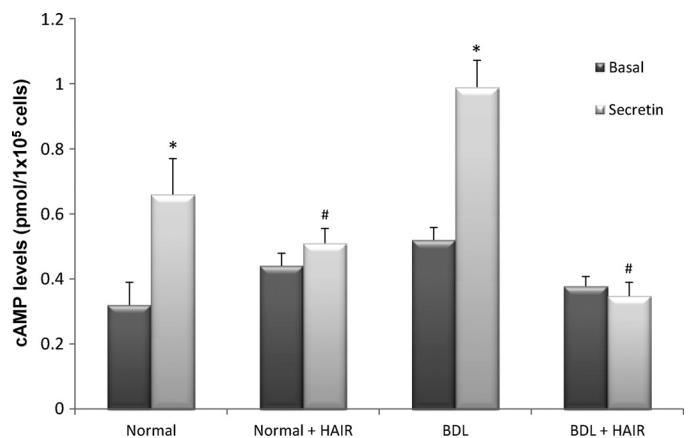

Fig. 3. Basal cAMP levels of cholangiocytes from BDL rats were higher than cAMP levels of normal cholangiocytes. In BDL (but not BDL + HAIR) rats, secretin increased cAMP levels in purified cholangiocytes. Data are means $\pm$ SE of 6 experiments. *$P < 0.05$ vs. the corresponding basal value; #ns, not significant vs. the corresponding basal value.

contrary, both HAIR and PVIR induced lobular damage and areas of focal necrosis and portal inflammation in normal compared with BDL rats. In both normal and BDL rats, HAIR (and at lower degree PVIR) induced apoptosis of large bile ducts as evidenced by TUNEL in liver sections (Fig. 1*A* and Table 1) and decreased proliferation of large cholangiocytes (by reduced PCNA expression in liver sections) (Fig. 1*B* and Table 1). There were increased Bax and reduced PCNA expression in purified cholangiocytes from the selected groups of animals (Fig. 1, *A* and *B*, lower graphs).

Expression of angiogenic factors in cholangiocytes. In BDL rats, there were *1*) enhanced expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in small bile ducts (in liver sections) (see Table 2) and purified pooled cholangiocytes (that include both small and large cholangiocytes) (5) compared with normal rats; and *2*) decreased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in large bile ducts (Fig. 2, *A* and *B*, and Table 2). In BDL rats, HAIR (and

at lower degree PVIR) determined a further increased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in small bile and decreased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in large bile ducts compared with BDL sham rats (Fig. 2, *A* and *B*, and Table 2); there was low expression of these factors in hepatocytes (Fig. 2, *A* and *B*). The increase in the aforementioned growth factors/receptors was lower in BDL + PVIR rats compared with BDL + HAIR rats (Fig. 2, *A* and *B*, and Table 2). The same results were obtained by real time PCR in purified pooled cholangiocytes, where angiogenic factors and receptors were present at higher levels after HAIR both in normal and BDL rats compared with control animals (Fig. 2*C*).

Measurement of basal and secretin-stimulated cAMP levels. Consistent with previous findings (19), basal cAMP levels of cholangiocytes from BDL rats were higher than cAMP levels of normal cholangiocytes (Fig. 3). In BDL (but not BDL + HAIR)
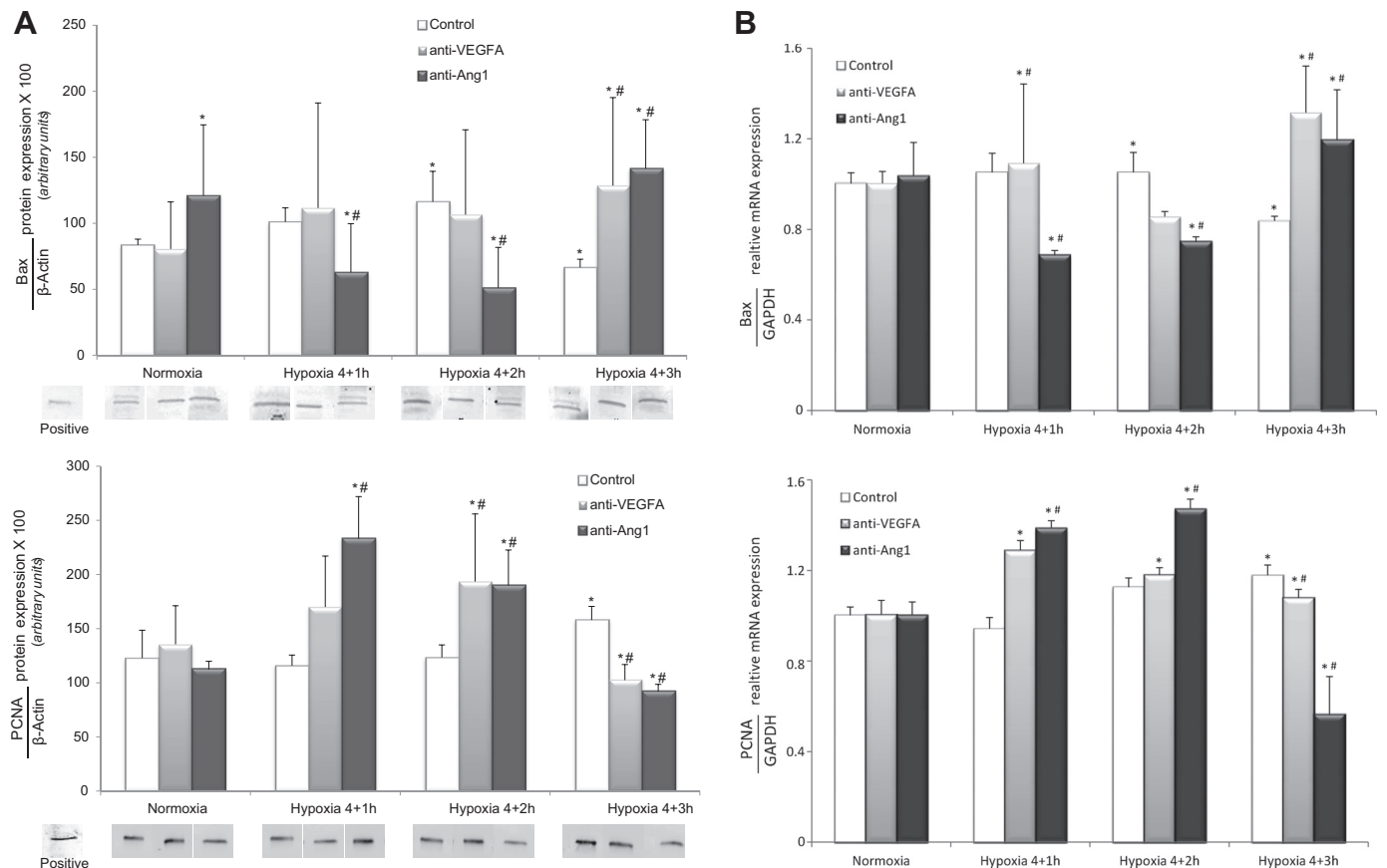

Fig. 4. A, *top*: evaluation of cholangiocyte apoptosis (by immunoblotting analysis for Bax) in normal rat intrahepatic cholangiocyte cultures (NRICC) maintained in condition of normoxia or hypoxia for 4 h and transferred to normal conditions for different times (1–3 h) with or without pre-incubation with an antibody anti-VEGF-A or anti-Ang-1. Bax expression was increased when NRICC were incubated under hypoxia; this effect was higher after 4 h of hypoxia followed by 2 h of normoxia, but it decreases after 4 h of hypoxia followed by 3 h of normoxia. These effects were reversed administering an antibody anti-VEGF-A or anti-Ang-1, where the levels of Bax expression were enhanced after 4 h of hypoxia + 3 h of normoxia. A, *bottom*: measurement of cholangiocyte proliferation (by immunoblotting analysis for PCNA) in the same samples. Without the pre-incubation with the antibodies anti-VEGF-A and anti-Ang-1, PCNA expression was increased only after 3 h of normoxic conditions. Conversely, administration of the antibodies increased proliferation in the first hours of normoxia but inhibited it after 3 h. Data are means ± SE of 6 blots. *$P < 0.05$ vs. its corresponding control value; #$P < 0.05$ vs. normoxia control value. B, *top*: cholangiocyte apoptosis was also assessed by real time PCR for Bax in the several treatments of NRICC. The apoptotic degree was lower in the last sample, where the cells returned to the normoxic conditions for 3 h after 4 h of hypoxia. Instead, pre-incubation with an anti-VEGF-A or an anti-Ang-1 enhanced the mRNA expression of Bax. B, *bottom*: cholangiocyte growth was investigated by real time PCR for PCNA in the same in vitro samples. PCNA mRNA expression was significantly increased after 3 h of reoxygenation, whereas it was worsened with the administration of the antibodies, in particular after treatment with an anti-Ang-1. Data are means ± SE of 6 blots. *$P < 0.05$ vs. its corresponding control value; #$P < 0.05$ vs. normoxia control value.

rats, secretin increased cAMP levels of purified pooled cholangiocytes (Fig. 3).

In vitro effect of normoxia and hypoxia on apoptosis, proliferation, and expression of VEGF-A/C and VEGFR-2/3 in NRICC. To verify if the in vivo findings were specific to cholangiocytes, we performed in vitro studies in NRICC during hypoxic (4 h) conditions using antibodies against VEGF-A and Ang-1 or controls (nonimmune serum). Consistent with previous studies showing increased apoptosis during hypoxic conditions (46, 53), there was increased Bax expression when NRICC were incubated under hypoxia; this effect was higher after 4 h of hypoxia and 2 h of normoxia, but it was increased after 3 h of reoxygenation if we pre-incubated cells with the antibodies for anti-angiogenic factors (Fig. 4, *A* and *B*). NRICC also displayed a lower proliferative rate (by PCNA expression) following hypoxic conditions that was enhanced using an anti-VEGF-A or an anti-Ang-1 antibody (Fig. 4A-B). Recovery of the normoxic conditions led to restoration of cholangiocyte proliferation and prevention of biliary apoptosis in a time-dependent manner (Fig. 4, *A* and *B*). During hypoxic conditions, there was a significant increase in mRNA expression of VEGF-A, VEGF-C, VEGFR-2, and VEGFR-3 (measured as ratio to GAPDH mRNA) (Fig. 5). These increased values returned to normal levels following restoration of normoxic conditions for 1, 2 or 3 h (Fig. 5).

DISCUSSION

Our study has shown that in normal and BDL rats, HAIR (and to lesser extent PVIR) induced a functional damage of bile ducts as demonstrated by increased apoptosis of large bile ducts in liver sections and Bax expression in purified pooled cholangiocytes. In BDL rats, HAIR decreased proliferation of large cholangiocytes in liver sections, and PCNA expression and secretin-stimulated cAMP levels of pooled cholangiocytes compared with BDL sham rats. In BDL rats, HAIR and PVIR induced lobular damage and focal areas of necrosis compared
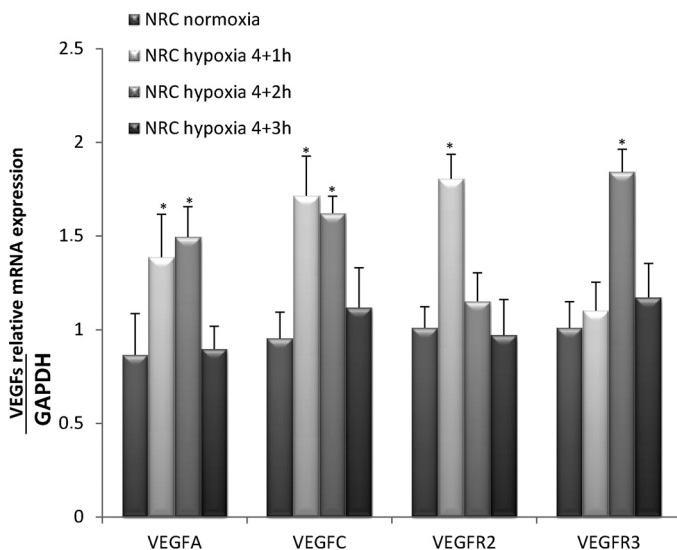

Fig. 5. Treatment in hypoxic conditions induced a significant increase in the message expression of VEGF-A, VEGF-C, VEGFR-2, and VEGFR-3 (measured as ratio to GAPDH mRNA). This increase returned close to normal values proportionally following restoring of normoxia for 3 h. Data are means ± SE of 6 experiments. *$P < 0.05$ vs. the corresponding basal value.

with normal and BDL rats without HAIR or PVIR. HAIR-induced damage of large bile ducts was coupled with increased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in small bile ducts and decreased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2, and Tie-1/2 in large bile ducts compared with BDL sham rats. The increased expression of angiogenic factors in small cholangiocytes is likely due to a compensatory mechanism due to the damage of large BDL cholangiocytes by HAIR. In vitro, during hypoxia/normoxia conditions there was increased apoptosis and reduced proliferation of NRICC concomitant with enhanced expression of VEGF-A/C and VEGFR-2/3.

The liver is susceptible to I/R injury that often occurs after conditions such as shock, trauma, surgical hepatectomy, and transplantation. Hepatic I/R leads to an acute inflammatory response, causing significant hepatocellular damage and liver dysfunction. The mechanisms of hepatic I/R injury are complex and involve multiple mechanisms (50). For example, I/R-induced apoptosis of bile ducts may contribute to the pathogenesis of biliary complications after liver transplantation (9). To begin to understand the mechanisms of I/R-induced biliary disorders, we used, in vivo, an experimental model of BDL rats that underwent 24 h of hepatic reperfusion immediately following sham or 30 min of transient occlusion of HAIR or PVIR. The in vitro model consisted of NRICC that were exposed to normoxic or hypoxic conditions for 4 h and then to normoxia for 1 to 4 h.

Previous studies have shown that 45 min of ischemia in cholestatic rats resulted in extensive damage during 24 h of reperfusion, leading to the premature death of most of these animals (30). Therefore, for our experiments, ischemia time was reduced to 30 min to achieve a nonlethal I/R injury model. In our model, the reperfusion time was extended to 24 h, resulting in hepatic necrosis and an inflammatory response. Another study in mice has demonstrated that cholestasis protected against I/R injury (18). The study suggested that hyperbilirubinemia induces decreased inflammatory response and subsequent reduced injury (42). As an alternative protective mechanism, the study suggested that cholestatic mice failed to activate NF-κB and TNF-α synthesis, two mediators of postischemic liver inflammation (18). In our study a marked inflammatory response persisted in the presence of cholestasis, possibly indicating a species difference in the inflammatory response during cholestasis. In addition, we also evaluated during I/R damage the role of selected angiogenic such as VEGF, which upon multiple stimuli is produced by endothelial cells, macrophages, activated T cells, and cholangiocytes (22, 36). VEGF plays important roles in both physiologic as well as pathologic vasculogenesis and angiogenesis (11, 37). Because ischemia is a potent stimulus for VEGF synthesis, it has been suggested to play a role during I/R-induced liver injury (33). Also, a recent study has shown that endogenous VEGF in the liver is expressed and released after transplantation and that the administration of neutralizing VEGF antibody during reperfusion attenuated liver damage in a cold I/R injury model (6). Among other angiogenic factors, Ang-1 is a strong vascular protective agonist of the Tie-2 receptor responsible for suppressing vascular leakage, maintaining endothelial cell survival, and inhibiting vascular inflammation. Ang-2 promotes in a dose-dependent manner destabilization, vessel leakage, and inflammation (7, 10). In the kidney, I/R induces a dysregula-

tion in Ang-2/Ang-1, which is accompanied by a loss of endothelial cells, proliferation of pericytes, and development of fibrosis.

With regard to the expression/role of vascular factors on the biliary epithelium after I/R injury, we propose that small cholangiocytes express higher levels of VEGF after HAIR most likely due to a compensatory mechanism to maintain biliary homeostasis following the injury of large cholangiocytes following I/R. In fact, the in vitro study with NRICC treated in hypoxic conditions displayed a lower proliferative rate (by PCNA expression) following the first 2 h of normoxic conditions, but proliferation began to increase after 3 h of normoxia. An effect that was blocked by the pre-incubation with antibodies against VEGF-A and Ang-1. In fact, cholangiocyte growth is inhibited to confirm the key role of these angiogenic factors in the recovery after hypoxic damage. In fact, it has been shown that *1*) angiogenic factors modulate cell growth by autocrine and paracrine mechanisms in liver; and *2*) overexpression in small cholangiocytes, together with the inhibitory effects of the antibodies in cholangiocyte proliferation, show that under hypoxic conditions, VEGFs and angiopoietins may contribute to hepatic repair. On the other hand, we also found that the biliary apoptosis is controlled by angiogenic factors as protective factors, since the pre-incubation with an anti-VEGF-A or an anti-Ang-1 induces an increase in cholangiocyte apoptosis after the first hours of reoxygenation. Supporting this finding, a number of studies support the concept that small cholangiocytes are more resistant than large cholangiocytes to hepatic injury (32, 35). The higher resistance of small cholangiocytes to damage may be due to their undifferentiated nature, whereas large cholangiocytes are more differentiated and more susceptible to injury (25). The locally produced VEGF may be an early mediator promoting hepatic I/R injury. Therefore, the blockade of this endogenous VEGF may have a cytoprotective effect. On the other hand, exogenous VEGF may also exert cytoprotection during I/R injury. Additional studies need to be performed to clarify the underlying mechanisms, but the manipulation of the small cholangiocyte compartment may be the key in the regulation of the expression/secretion of angiogenic factors and for the management of I/R induced biliary injury.

GRANTS

DISCLAIMERS

DISCLOSURES

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