Hypoxia-induced changes in the expression of rat hepatobiliary transporter genes

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Fouassier L, Beaussier M, Schiffer E, Rey C, Barbu V, Mergey M, Wendum D, Callard P, Scoazec J-Y, Lasnier E, Stieger B, Lienhart A, Housset C. Hypoxia-induced changes in the expression of rat hepatobiliary transporter genes. Am J Physiol Gastrointest Liver Physiol 293: G25–G35, 2007; doi:10.1152/ajpgi.00175.2006.—Cholestatic disorders may arise from liver ischemia (e.g., in liver transplantation) through various mechanisms. We have examined the potential of hypoxia to induce changes in the expression of hepatobiliary transporter genes. In a model of arterial liver ischemia subsequent to complete arterial deprivation of the rat liver, the mRNA levels of VEGF, a hypoxia-inducible gene, were increased fivefold after 24 h. The pattern of VEGF-induced expression and ultrastructural changes, including swelling of the endoplasmic reticulum, indicated that hypoxia affected primarily cholangiocytes, but also hepatocytes, predominantly in the perportal area. Serum and bile analyses demonstrated liver dysfunction of cholestatic type with reduced bile acid biliary excretion. Fluorescence-labeled ursodeoxycholic acid used as a tracer displayed no regurgitation, eliminating bile leakage as a significant mechanism of cholestasis in this model. In liver tissue, a marked reduction in the mRNA levels of Na+/taurocholate-cotransporting polypeptide (Ntcp), bile salt export protein (Bsep), and multidrug resistance-associated protein 2 (Mrp2) and an increase in those of Cfr were detected before bile duct proliferation occurred. In cultured hepatocytes, a nontoxic hypoxic treatment caused a decrease in the mRNA and protein expression of Ntcp, Bsep, and Mrp2 and in the mRNA levels of nuclear factors involved in the transactivation of these genes, i.e., HNF4α, RXRs, and FXR. In bile duct preparations, hypoxic treatment elicited an increase in Cfr transcripts, along with a rise in cAMP, a major regulator of Cfr expression and function. In conclusion, hypoxia triggers a downregulation of hepatocellular transporters, which may contribute to cholestasis, whereas Cfr, which drives secretion in cholangiocytes, is upregulated.

liver ischemia; cholestasis; Na+/taurocholate-cotransporting polypeptide; bile salt export pump; multidrug resistance-associated protein 2; cystic fibrosis transmembrane conductance regulator

Bile formation requires transport activities of both hepatocytes and cholangiocytes (51). The transport of bile salts from blood into bile is the main driving force for bile formation in hepatocytes. This vectorial transport is critically dependent on Na+/taurocholate-cotransporting polypeptide (NTCP, SLC10A1) and the bile salt export pump (BSEP, ABCB11), which mediate the sinusoidal uptake of bile salts and their efflux across the canalicular membrane of hepatocytes, respectively (55). Another important transporter for bile formation in hepatocytes is the multidrug resistance-associated protein MRP2 (ABCC2), which is responsible to a large extent for the generation of bile salt-independent bile flow through the canalicular excretion of glutathione. Cholangiocytes alkaline and dilute canalicular bile through the secretion of a bicarbonate rich fluid. CFTR, a cAMP-regulated chloride channel expressed in cholangiocytes, provides the major driving force for this ductular secretion (28). Both human and experimental studies have provided evidence that any impairment in the expression and/or function of these different hepatobiliary transporters may lead to cholestatic disorders (51).

In different clinical situations, e.g., liver transplantation, liver ischemia may occur and cause or contribute to hepatobiliary dysfunction, which most often is of cholestatic type (9, 21). However, the mechanisms that account for cholestasis in the setting of liver ischemia are generally poorly understood. Work in our laboratory (4) previously showed, in a model of arterial deprivation of the rat liver, that cholestasis was induced by acute arterial liver ischemia, although the mechanisms of cholestasis involved in this model have not been determined. It has been previously demonstrated that hepatobiliary transport systems are subject to extensive regulation, notably at the level of gene transcription (30, 51). It is also known that hypoxia regulates the transcription of multiple genes (54), including genes that encode epithelial transport proteins (7). In the present study, we tested the hypothesis that the expression of hepatobiliary transporters may change in response to hypoxia. In vivo studies were performed in the rat model of arterial liver ischemia that our group previously established (4). In vitro cell preparations were submitted to hypoxia by using a catalytic system (4, 8).

MATERIALS AND METHODS

Animal model. Arterial liver ischemia was achieved in rats by complete arterial deprivation of the liver, as previously described (4).
Male Wistar rats (Elevage Janvier, Le Genest St Isle, France) weighing 380–420 g were anesthetized with a subcutaneous injection of chlorpromazine (2 mg/kg) and ketamine (20 mg/kg). In a first step, the liver was prepared as for graft removal during liver transplantation (6). The falciform ligament was divided up to the vena cava. Double ligation and division of the left diaphragmatic vein were performed. After the left lateral lobe was pulled to the right, the left triangular ligament between the left lateral lobe and the posterior abdominal wall was divided and the left accessory hepatic artery running along the esophagus was doubly ligated and divided. Thereafter, the ligament joining the caudate lobe with the stomach and the pars flacida of the lesser omentum was divided. After the right hepatic lobe was retracted to the left and the right triangular ligament was divided, double ligation of the right adrenal vein was performed. The retrohepatic portion of the vena cava was then mobilized. At the completion of this procedure, the liver was isolated from all peripheral vascular supply except for the main hepatic artery, the extrahepatic peribiliary plexus, and the portal vein. In animals defined as sham, no additional vascular intervention was performed. In animals undergoing complete arterial deprivation, double ligation-division of the main hepatic artery and double ligation of the extrahepatic peribiliary vascular plexus were subsequently performed. The common bile duct was cannulated with a polyethylene cuff (~3-mm length, 0.3-mm inside diameter), which was then secured with two circumferential 6-0 silks to interrupt the bile flow. In all animals, investigations were performed 24 h after surgery.

Experiments were performed in accordance with national guidelines for the care and use of laboratory animals. All animals had free access to standard chow and water and were housed in controlled temperature room under 12:12-h light-dark cycles. The protocol was approved by the Paris Department of Veterinary Services.

**Histological and immunohistochemical analyses.** Serial 4-μm-thick sections of formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin-eosin-safran. To determine the number of bile duct sections, we screened high-magnification (×400) fields using an eyepiece equipped with a net micrometer (Carl Zeiss, Jena, Germany). VEGF immunostaining was performed using a rabbit polyclonal antibody (sc-152; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 and an avidin-biotin-peroxidase technique (Vectorstain ABC kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was revealed by 3-amo-no-9-ethylcarbazole (Vector Laboratories). At least 10 portal tracts in 2 liver tissue sections per animal were examined.

**Electron microscopy.** Liver tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 60 min. After postfixation in 1% osmium tetroxide, the tissue was dehydrated and embedded in epoxide resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with a Jeol 100 CX 2 electron microscope (JEOL, Tokyo, Japan).

**Serum and bile analyses.** Blood was withdrawn from the femoral vein of rats under anesthesia. Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γGT), and total bilirubin were measured using standard analytical methods. Bile flow was measured in anesthetized rats with biliary fistulas as previously described (15). Volumes of bile were determined gravimetrically, assuming a density of 1.00 g/ml. Bile was collected every 10 min for 120 min. Approximately 40 min after bile collection was started, steady-state bile flow was achieved. Bicarbonate concentration in bile was measured by potentiometry on a Synchron CX3 Delta analyzer (Beckman Coulter Instruments, Gagny, France). The concentration of bile salts in serum and bile was measured by enzymatic assay using 3α-hydroxysteroid dehydrogenase (Sigma Diagnostics, St. Louis, MO).

**Tracing of aminofluorescein-tagged ursodeoxycholic acid.** To visualize potential bile regurgitation, we used a method based on the imaging of aminofluorescein-tagged ursodeoxycholic acid (UDCA) (22) that was previously reported with modifications (13). Aminofluorescein-tagged UDCA (kindly provided by Dr. Alan Hofmann, University of California, San Diego, La Jolla, CA) was injected in rats 24 h after complete arterial deprivation of the liver, sham operation, or bile duct ligation. Bile duct ligation was performed as previously described (32) and used as a positive control for bile leakage in these experiments. In animals under chlorpromazine-ketamine anesthesia, the liver was perfused through the portal vein with aminofluorescein-

### Table 1. Primers used in real-time PCR

<table>
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<th>Gene</th>
<th>Primers (5′-3′)</th>
<th>GenBank Database Accession Number</th>
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<tr>
<td>Ntcp (Slc10a1)</td>
<td>Forward: CCC TGA TGG CCT TCT CTT G</td>
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<tr>
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<td>Reverse: GAA TCC TGG TTC CAT GCT GAT G</td>
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<td></td>
</tr>
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</tr>
<tr>
<td></td>
<td>Reverse: GCC CAG ACC TGC GTA GGC TA</td>
<td></td>
</tr>
<tr>
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<td>X95927</td>
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</tr>
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<td>NM_012805</td>
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<tr>
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<td></td>
<td>Reverse: TGG GTG TGG ATT CAC ATC TG</td>
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<td>18S</td>
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<td>M10098</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGC ATC GTC TAT GCT CGG AA</td>
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tagged UDCA (0.25 μmol·kg⁻¹·min⁻¹ in 0.9% NaCl, pH 8) for 4 min. Thereafter, the liver was perfused with 0.9% NaCl for 4 min, excised, and immediately snap-frozen in liquid nitrogen. Unfixed cryosections of liver tissue were mounted on slides, air-dried, and immediately examined without mounting with a laser scanning microscope (Leica Microsystems, Eidelberg, Germany).

**Cell preparations and hypoxic treatment.** Hepatocytes were isolated from normal Wistar rats by a method derived from Seglen (48). In brief, after in situ perfusion of the liver with 0.025% collagenase (Boehringer Mannheim, Meglan, France), dispersed hepatocytes were filtered through a 100-μm gauze and then centrifuged twice at 600 rpm. Hepatocytes were 85–90% pure, and cell viability exceeded 90% as tested by erythrosin exclusion. Cells were plated in collagen I-coated dishes. They were allowed to adhere for 4 h and then placed in serum-free William’s medium containing 1 μM hydrocortisone hemisuccinate and 0.25 IU/ml insulin for 24 h before hypoxic treatment.

Intrahepatic bile duct fragments were isolated from normal male Wistar rats using a method described by Mennone et al. (43) with modifications (32). In brief, the liver was perfused in situ, sequentially

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**Fig. 1.** VEGF expression induced by arterial liver ischemia. VEGF expression was assessed using real-time PCR (A) and immunohistochemistry (B) in rat liver 24 h after sham operation or complete arterial deprivation (ischemia). A: data expressed as mRNA levels normalized to 18S are shown relative to mean sham control value. Results of 3 animals are shown as means ± SE. *P < 0.05 vs. sham. B: representative portal tract-centered photomicrographs are shown. In sham image, no VEGF immunolabeling is detectable; in arterial liver ischemia image, VEGF immunoreactivity is visible with equally high intensity in all cholangiocytes (open arrow, bottom inset) and with different levels of intensity from moderate to high (closed arrow, top inset) in periportal hepatocytes.
Fig. 2. Ultrastructural changes in arterial liver ischemia. Compared with cholangiocytes (A) and hepatocytes (B) in normal liver, 24 h after complete arterial deprivation cholangiocytes (C) and hepatocytes (D) displayed typical hypoxic ultrastructural changes. In both cell types, dilatations of the reticulum endoplasmic (er) and the perinuclear space are visible (arrowheads). In addition, in cholangiocytes (C), mitochondria (m) are swollen, and a large hypoxic vesicle (asterisk) is visible in the cytoplasm. Tight junctions (large arrows) are preserved; microvilli (small arrows) located along the apical pole facing the lumen (L) are swollen and distorted. In hepatocytes (D), mitochondria (m) and the bile canaliculus (bc) structure are preserved. Original magnifications: A and C, ×20,000; B and D, ×8,000. N, nucleus.

Fig. 3. Serum biochemical changes in arterial liver ischemia. Serum concentrations of liver enzymes, total bilirubin, and bile acids were measured in rats 24 h after sham operation (open bars) or arterial liver deprivation (filled bars). Results of 4 animals are shown as means ± SE. *P < 0.05 vs. sham.
with 0.019% EDTA and 0.008% collagenase. The portal tract residue was separated from parenchymal tissue, minced, and repeatedly incubated in 0.066% collagenase, 0.006% deoxyribonuclease, and 0.033% pronase or 0.036% hyaluronidase (all enzymes were from Roche Molecular Biochemicals, Mannheim, Germany). Fragments were filtered, and those remaining on a 40-μm filter were collected. More than 90% of the cells in bile duct preparations were cholangiocytes, as ascertained by cytokeratin 19 and γ-GT labeling (32). Bile duct preparations were suspended in DMEM-Ham’s F-12 supplemented with 2% Ultroser G (Biosepra, Villeneuve-la-Garenne, France) and plated in collagen I-coated dishes. They were allowed to adhere for 12 h before hypoxic treatment.

Hypoxia was achieved as reported previously (8) using the AnaeroGen system (Oxoid, Dardilly, France), which catalytically reduces oxygen concentration to <1% within 30 min. Both hepatocytes and bile duct preparations were exposed to hypoxia at 37°C for 4 h, which did not affect cell viability, as previously assessed by the release of lactate dehydrogenase (4, 8). Controls included parallel cultures in which cells were maintained under normoxia.

Reverse transcription and real-time PCR. Total RNA was extracted from tissue and cultured cells by RNA Plus lysis solution (Quantum, Montreuil-sous-Bois, France), according to the method of Chomczynski and Sacchi. Complementary DNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase. Real-time PCR was performed with the TaqMan system (Applied Biosystems, Foster City, CA) and the SYBR green Master Mix (Applied Biosystems). The primers were designed according to published rat cDNA sequences in the GenBank database using Primer Express software (version 1.5; PE Applied Biosystems) (Table 1). 18S RNA was used as internal control. One-step RT-PCR was performed for both target gene and endogenous controls. Duplicate critical threshold (CT) values were analyzed in Microsoft Excel using the comparative CT ($ΔΔ$CT) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-ΔΔC_T}$) was obtained as normalized to 18S.

cAMP assay. To determine intracellular cAMP content, we immediately permeabilized cells by incubation at 4°C for 10 min in 500 μl of 2 × 10⁻⁵ M digitonin in 20 mM Tris·HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF, and 10⁻⁴ M IBMX. After incubation, the medium from each well was collected and stored at −70°C until determination of cAMP content. cAMP was measured by radioimmunoassay using a commercial RIA (NEN Life Science Products, Paris, France) as previously described (14). The protein content of cell samples was determined by bicinchoninic acid protein assay (Pierce, Bezons, France).

Membrane fractionation and immunoblotting. To examine the expression of transporter proteins, we analyzed preparations of hepatocyte membranes by immunoblotting. Hypoxic and control hepatocytes were washed twice in ice-cold PBS, scraped, and collected in ice-cold Tris-sucrose buffer [10 mM Tris·HCl, pH 7.5, 0.25 M sucrose, 0.2 mM CaCl₂, and protease inhibitor (Complete tablet; Roche)]. Cell lysates were incubated (10 min at 4°C) and sonicated, and EDTA was added to a final concentration of 1 mM. Nuclear and unbroken cells were discarded by centrifugation (1,000 g, 10 min, 4°C). The resulting supernatant was centrifuged (100,000 g, 60 min, 4°C). The pellet containing crude membranes was resuspended in ice-cold Tris-sucrose buffer (20 mM Tris·HCl, pH 7.5, 0.25 M

![Fig. 4. Bile secretory changes in arterial liver ischemia. Bile flow (A) and biliary output of bile salts (B) and bicarbonate (C) were measured in rats 24 h after sham operation (open bars) or arterial liver deprivation (filled bars). Results of measurements performed over 10 min after 1 h in 4 animals are shown as means ± SE. *P < 0.05 vs. sham.](image1)

![Fig. 5. Distribution of aminofluorescein-tagged ursodeoxycholic acid (UDCA) tracer in arterial liver ischemia. Aminofluorescein-tagged UDCA was visualized in rats 24 h after sham operation (A), arterial liver deprivation (B), or bile duct ligation (C). Photomicrographs show fluorescent labeling of bile ducts in sham (A) and arterial liver ischemia (B) with leakage of fluorescent UDCA (arrowheads) into the portal tract after bile duct ligation (C). Representative of 3 animals in each group. L, lumen.](image2)
sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM 2-mercaptoethanol, and protease inhibitors), sonicated, and centrifuged (100,000 g, 60 min, 4°C). The supernatant was collected as a solubilized membrane fraction. Membrane proteins (10–50 μg) were then subjected to electrophoresis through a 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Immunoblotting was performed using previously reported polyclonal antibodies against rat Ntcp (1:5,000), Mrp2 (1:2,000), and Bsep (1:1,000) (5) and a monoclonal antibody against Na⁺,K⁺-ATPase α₁-subunit (1:2,500) from Abcam (Cambridge, UK). The protein level of Na⁺,K⁺-ATPase, unaffected by hypoxia (52), was used as an internal standard for membrane proteins. Immunoreactivity was revealed by ECL Plus (Amersham Biosciences). The detected bands were quantified with ImageJ (National Institute of Mental Health, Research Services Branch, Bethesda, MD).

Statistical analysis. Quantitative data are shown as means ± SE. Comparisons between pairs were made using the Mann-Whitney U-test. Comparisons between multiple groups were made using two-way analysis of variance with repeated measures (Statview, Abacus Concept, CA) followed by pairwise comparison. Statistical significance was set at P < 0.05.

RESULTS

Hypoxic changes induced by arterial liver ischemia. Because hypoxia is a major stimulus of VEGF expression in different tissues, including the liver (8, 12, 45), we examined VEGF expression as a marker of hypoxia in the liver of rats with arterial liver ischemia.Twenty-four hours after arterial deprivation, the mRNA levels of VEGF were increased approximately fivefold in rats with liver ischemia compared with controls (Fig. 1A). The localization of VEGF-induced expression was determined by immunohistochemistry. In liver tissue sections from sham animals, there was no VEGF immunoreactivity in cholangiocytes (Fig. 1B, top). Twenty-four hours after complete arterial deprivation of the liver, VEGF staining appeared in all cholangiocytes with an equal intensity (Fig. 1B, bottom). In sham animals, VEGF staining in hepatocytes was restricted to the first layer of perivenular hepatocytes (data not shown). Twenty-four hours after complete arterial deprivation of the liver, VEGF staining was detected in all the hepatocytes, although at higher levels in periportal areas (Fig. 1B, bottom). Consistent with the presence of hypoxia in both cell types, ultrastructural changes that are typical of hypoxia were detected in the vast majority of both cholangiocytes and hepatocytes at electron microscopy. Compared with the aspect of cholangiocytes and hepatocytes in normal liver (Fig. 2, A and B), vesicular dilatation of the endoplasmic reticulum and the nuclear membrane were detected in both cell types in liver ischemia (Fig. 2, C and D). These dilatations sometimes formed large vacuoles (Fig. 2C), which were more frequent in cholangiocytes. Swelling of mitochondria and microvilli were visible only in cholangiocytes (Fig. 2C). No alteration of mitochondria or bile canaliculi was observed in hepatocytes (Fig. 2D). In both cell types, tight junctions appeared intact, as shown Fig. 2C in cholangiocytes.

Hepatic dysfunction of cholestatic type in arterial liver ischemia. By the time hypoxic changes had occurred in the livers of rats 24 h after arterial deprivation, the animals had developed liver dysfunction. Biochemical serum analyses were typical of cholestatic injury as shown by a significant rise in the concentrations of liver enzymes (AST, ALT, γ GT), bilirubin, and bile acids (Fig. 3). Cholestasis was confirmed by the demonstration that bile flow was significantly decreased in these animals compared with sham animals (Fig. 4A). The biliary output of bile salts and bicarbonate, two major determinants of bile secretion, were both significantly reduced in these animals (Fig. 4, B and C).

Distribution of aminofluorescein-tagged UDCA in arterial liver ischemia. Hypoxia was previously shown to decrease epithelial barrier function through tight junctional disruption in different tissues (e.g., lung, intestine) (29). We anticipated that if such alterations occurred in liver epithelia, they would cause bile regurgitation and thereby contribute to cholestatic manifestations. To examine potential leakage of bile in rats with
arterial liver ischemia, we used aminofluorescein-tagged UDCA as a tracer in these rats and in sham and bile duct-ligated rats used as controls. In sham-operated rats, as previously reported in normal mice (13), fluorescent labeling suggested that aminofluorescein-tagged UDCA underwent rapid secretion in bile and cholangiocyte uptake (Fig. 5A). The labeling pattern of fluorescence was identical in rats 24 h after arterial deprivation compared with sham rats (Fig. 5B). By contrast, in bile duct-ligated rats at 24 h, the periductal area of portal fields was frequently labeled with fluorescence (Fig. 5C), attesting to leakage of UDCA out of bile ducts in these animals. Such periductal labeling was never found in rats with arterial liver ischemia, a finding consistent with the ultrastructural appearance of tight junction integrity between adjacent cholangiocytes. Together, these findings suggested that altered cell junction permeability was not a mechanism that significantly contributed to cholestatic injury in this model.

Gene expression of hepatobiliary transporters in arterial liver ischemia. Next, we examined the possibility that arterial liver ischemia may affect the expression of hepatobiliary transporter genes that are of crucial importance for bile formation in hepatocytes (Ntcp, Bsep, Mrp2) or in cholangiocytes (Cftr). Using real-time PCR, we measured expression of these genes within the liver of rats 24 h after arterial deprivation compared with sham controls. The mRNA levels of the hepatocyte transporters Ntcp, Bsep, and Mrp2 were significantly decreased (~80%, 70%, and 76%, respectively) in rats with liver ischemia compared with controls (Fig. 6, A–C). By contrast, the mRNA levels of Cftr, a major transporter in cholangiocytes, were significantly increased (~4-fold) in rats with liver ischemia compared with controls (Fig. 7A). This increase was not explained by bile duct proliferation. In agreement with previous data indicating that ductular reaction is detected later in this model (after 48 h) (4), we found that the count of bile duct sections was identical in rats at 24 h after arterial liver ischemia and in controls (Fig. 7B).

Hypoxia-induced changes in the expression of hepatobiliary transporters in cultured cells. To determine whether hypoxia by itself was able to trigger changes in the expression of hepatobiliary transporters, we examined their expression in rat liver cell preparations exposed to hypoxia in vitro. Hepatocytes and cholangiocytes preparations were submitted to a hypoxic treatment that was noncytotoxic and induced VEGF expression in both cell types (4, 8). In hepatocytes, hypoxia induced significant changes in the mRNA levels of Ntcp, Bsep, and Mrp2, which were reduced ~75, 80, and 70%, respectively (Fig. 8A). These changes were accompanied by a decrease in the mRNA levels of transcription factors acting on the promoters of these transporter genes, i.e., HNF4α, RXRα, and FXR, which were decreased 80, 70, and 70%, respectively (Fig. 8B). Also, as soon as 4 h after the onset of hypoxia, the amounts of Ntcp, Bsep, and Mrp2 proteins detected in the plasma membrane of hepatocytes were decreased by 60–65% (Fig. 8C). In bile duct preparations, hypoxia induced significant changes in the mRNA levels of Cftr, which were increased ~2.5-fold (Fig. 9A). The upregulation of Cftr expression in bile duct preparations was accompanied by a dramatic increase in intracellular cAMP, a signaling molecule of critical importance in the regulation of Cftr expression and function (Fig. 9B).

DISCUSSION

Decreased blood flow resulting in liver tissue hypoxia is common in a number of clinical situations (e.g., liver trans-
plantation, shock syndromes) (9, 21, 40). Tissue hypoxia is also a contributory factor in chronic liver disease states (8, 35, 53). In the present study, we have shown that hypoxia induces changes in the expression of hepatobiliary transporter genes. Because in hepatocytes these changes consist of the downregulation of transporters with key functions in bile formation, we infer that they could contribute, at least partly, to the cholestatic manifestations that occur in the setting of liver ischemia.

The liver has the specificity to be vascularized by both the hepatic artery and the portal vein. Hepatic arterial damage or thrombosis may occur and cause arterial liver ischemia during hepatobiliary surgery, liver transplantation, or intra-arterial chemotherapy and/or embolization (23, 49). Although hepatic parenchyma receives dual arterial and venous blood supply, bile ducts depend mostly on arterial blood flow (17, 46). In keeping with these lines, ischemic cholangitis is recognized as a typical complication of arterial liver ischemia (3, 23). However, in ischemia-related cholestatic disorders, features of cholangitis are inconstant, and other causes of liver injury including reperfusion, immune-mediated, or drug-induced toxicity are often superimposed. To better define the pathophysiological consequences of selective arterial liver ischemia, our group previously designed a model of complete arterial deprivation of the rat liver (4). The expression of VEGF that was used as an indicator of reduced oxygen concentration suggests that, in this model, hypoxia is induced primarily in bile ducts but also in hepatocytes, with a clear predominance in the periportal region in keeping with previous work (23). Accord-

Fig. 8. Hypoxia-induced changes in the expression of transporters and transcription factors in cultured hepatocytes. The mRNA levels of Ntcp, Bsep, and Mrp2 (A) and of hepatic nuclear factor (HNF-4α), retinoid X receptor (RXRs), and farnesoid X receptor (FXR) (B) were measured using real-time PCR in rat hepatocytes in primary culture, maintained under normoxia, or exposed to 4 h of hypoxia. Data expressed as mRNA levels normalized to 18S are shown relative to mean control value and represent means ± SE of results obtained from 3 preparations. *P < 0.05 vs. normoxia. C: Western blots of Ntcp, Bsep, Mrp2, and Na⁺-K⁺-ATPase in plasma membrane preparations of hepatocytes maintained under normoxia or exposed to 4 h of hypoxia, from 3 experiments. In quantitative analyses, the protein levels were normalized to Na⁺-K⁺-ATPase and are shown relative to normoxia as means ± SE. *P < 0.05 vs. normoxia.
shown relative to mean control value and represent means using real-time PCR. Data expressed as mRNA levels normalized to 18S are normoxia or exposed to hypoxia, Cftr mRNA levels were measured for cAMP content in rat cholangiocyte preparations. A: in rat bile duct preparations maintained under normoxia or exposed to 4 h of hypoxia, Cftr mRNA levels were measured using real-time PCR. Data expressed as mRNA levels normalized to 18S are shown relative to mean control value and represent means ± SE of 3 preparations. *P < 0.05 vs. normoxia. B: intracellular cAMP was measured using RIA and was reported as cell protein content. Data represent the means of results obtained by duplicate analyses from 2 preparations.

A major functional consequence of arterial liver deprivation was a decrease in bile secretion. The possibility that the biliary cuff placed to ligate the peribiliary vascular plexus caused obstruction was previously excluded by the absence of cholestasis in control animals that underwent the same procedure (4). In the present study, neither the ultrastructural appearance of tight junctions nor the labeling pattern of aminofluorescein-tagged UDCA provided evidence that abnormal paracellular permeability, which may be hypoxia induced (29), contributed to cholestatic features in rats with arterial liver ischemia. Likewise, it was previously shown that tight junctional integrity and transepithelial resistance are relatively resistant to ischemia in bile ducts (11).

These findings further argue against the possibility that obstruction, which would have affected cell junction permeability as shown in bile duct-ligated rats, occurred in this model. Bile formation is critically dependent on canalicular transporters, of which Bsep (constituting the rate-limiting step in the overall transport of bile salts from the sinusoidal blood into the canaliculus) and Mrp2 contribute quantitatively to a great extent (51, 55). However, the uptake of bile salts, which in rodents is largely mediated by Ntcp (44), may have an influence on the regulation of canalicular bile salt secretion, because it has been demonstrated that bile salts can regulate Bsep levels in the canalicular membrane (16). We may thus anticipate that the marked downregulation in the expression of these three transporters accounted at least partly for cholestasis in the model of arterial liver ischemia. On the basis of previous studies showing that hypoxia causes ATP depletion and cytoskeletal changes (11, 20, 47), it is likely that additional mechanisms were involved, including possible defects in the activity and the localization of transporters at plasma membranes.

The profound decrease within 24 h in the mRNA levels of the three transporters, Ntcp, Bsep, and Mrp2, is unique in the present model compared with other cholestatic models, i.e., endotoxin or ethinylestradiol administration and common bile duct ligation (34, 36). In particular, little or no significant change in the mRNA levels of Bsep (34, 36) and Mrp2 (36) were previously reported in the bile duct ligation model. This suggested that the changes in transporter expressions were not just secondary events but resulted at least partly from hypoxia. It was previously reported that bile salt transport is by far more active in the periportal zone compared with other zones with lower oxygenation (19, 25). This contention 1) further supports the possibility that bile salt transporter genes are oxygen regulated and 2) implies that in the model of arterial liver ischemia, hypoxia is more severe in regions of predominant transporter expression (i.e., periportal) and may thus be sufficient to explain at least partly the marked reduction of mRNA levels in whole tissue extract.

Direct evidence that the expression of these transporters in hepatocytes and that of Cftr in cholangiocytes may be regulated by hypoxia was provided by in vitro studies. In these studies, hepatocytes and bile duct preparations were submitted to hypoxic conditions, which were nontoxic, induced VEGF expression, and caused variations in transporter mRNA levels to an extent similar to that in vivo. We found in hypoxic hepatocytes a marked decrease in the expression of hepatic nuclear factor (HNF4α) and retinoid X receptor (RXRα) genes, which have been shown to be repressed by hypoxia in previous studies (24, 42), and also of farnesoid X receptor (FXR). Whereas HNF4α transactivates the rat Ntcp promoter (27), RXRα forms heterodimers with FXR that transactivate BSEP/Bsep and Mrp2 genes (1, 18, 31), with pregnane X receptor (PXR) and constitutive androstane receptor (CAR) that transactivate Mrp2 (31), and with retinoid acid receptor (RARα) that transactivate rat Ntcp and Mrp2 genes (10, 27). Hypoxia was previously shown to deactivate another nuclear factor, peroxisome proliferator-activated receptor-α (PPAR-α), by reducing the availability of its obligate partner, RXRα (24). We thus inferred from the present findings that the downregulation of Ntcp, Bsep, and Mrp2 genes by hypoxia could be mediated by downregulation and/or deactivation of
their transcription factors. Although it has been reported that gene repression by hypoxia is mediated by hypoxia-inducible factor-1-dependent (37,41) or -independent mechanisms (24), we found no evidence of hypoxia responsive element in any of these nuclear factor or transporter gene promoters. Another response found in the hypoxic liver was the upregulation of Cftr, which preceded bile duct proliferation in this model. In vitro studies demonstrated that Cftr expression was triggered by hypoxia in bile ducts. Because cAMP response element binding protein-CRE is a key element of both the transactivation of many genes in response to hypoxia (33,50) and the upregulation of Cftr in cholangiocytes, the upregulation of Cftr in cholangiocytes secretion is driven by Cftr, the upregulation of bile salt and organic anion transporters in hepatocytes may contribute to cholestasis in hypoxic liver tissue.

In conclusion, this study indicates that hypoxia regulates the expression of hepatobiliary transporters. Contrasting with an upregulation of Cftr in cholangiocytes, the downregulation of bile salt and organic anion transporters in hepatocytes may contribute to cholestasis in hypoxic liver tissue.

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REFERENCES

HEPATOBLIARY TRANSPORTER EXPRESSION IN HYPOXIA


