Activation of the renin-angiotensin system stimulates biliary hyperplasia during cholestasis induced by extrahepatic bile duct ligation

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Submitted 28 March 2014; accepted in final form 8 January 2015

Afroze SH, Munshi MK, Martínez AK, Uddin M, Gergely M, Szynkarski C, Guerrier M, Nizamutdinov D, Dostal D, Glaser S. Activation of the renin-angiotensin system stimulates biliary hyperplasia during cholestasis induced by extrahepatic bile duct ligation. Am J Physiol Gastrointest Liver Physiol 308: G691–G701, 2015. First published February 12, 2015; doi:10.1152/ajpgi.00116.2014.—Cholangiocyte proliferation is regulated in a coordinated fashion by many neuroendocrine factors through autocrine and paracrine mechanisms. The renin-angiotensin system (RAS) is known to play a role in the activation of hepatic stellate cells and blocking the RAS attenuates hepatic fibrosis. We investigated the role of the RAS during extrahepatic cholestasis induced by bile duct ligation (BDL). In this study, we used normal and BDL rats that were treated with control, angiotensin II (ANG II), or losartan for 2 wk. In vitro studies were performed in a primary rat cholangiocyte cell line (NRIC). The expression of renin, angiotensin-converting enzyme, angiotensinogen, and angiotensin receptor type 1 was evaluated by immunohistochemistry (IHC), real-time PCR, and FACS and found to be increased in BDL compared with normal rat. The levels of ANG II were evaluated by ELISA and found to be increased in serum and conditioned media of cholangiocytes from BDL compared with normal rats. Treatment with ANG II increased biliary mass and proliferation in both normal and BDL rats. Losartan attenuated BDL-induced biliary proliferation. In vitro, ANG II stimulated NRIC proliferation via increased intracellular cAMP levels and activation of the PKA/ERK/CREB intracellular signaling pathway. ANG II stimulated a significant increase in Sirius red staining and IHC for fibronectin that was blocked by angiotensin receptor blockade. In vitro, ANG II stimulated the gene expression of collagen 1A1, fibronectin 1, and IL-6. These results indicate that cholangiocytes express a local RAS and that ANG II plays an important role in regulating biliary proliferation and fibrosis during extrahepatic cholestasis.

angiotensin; bile duct ligation; renin-angiotensin system; cholangiocyte

CHOLANGIOCYTES ARE EPITHELIAL cells that line the intrahepatic and extrahepatic bile ducts and are the target cell type in cholangiopathies such as primary biliary cirrhosis and primary sclerosing cholangitis (4, 61). Cholangiocytes are relatively mitotically dormant unless induced by certain pathological conditions such as bile duct ligation (BDL), partial hepatectomy, and bile acid feeding (23). Cholangiopathies are characterized by an imbalance between proliferation and death of cholangiocytes with proliferation playing a critical role for the maintenance of biliary mass and function during the progression of cholangiopathies (4, 61). Numerous studies have demonstrated that proliferating cholangiocytes develop neuroendocrine phenotypes and secrete and respond to a number of hormones, neuropeptides, and neurotransmitters that modulate proliferation in autocrine and paracrine mechanisms (5, 23, 44).

Angiotensin II (ANG II) is the primary bioactive component of the renin-angiotensin system (RAS) (17, 36). ANG II is produced from angiotensinogen (Ao) in two steps. First, renin cleaves Ao to form angiotensin I (ANG I), which is subsequently cleaved by angiotensin-converting enzyme (ACE) to form ANG II (17, 36). The RAS is well known for the role it plays in the regulation of blood pressure and fluid homeostasis (17, 36). It is not only important as a regulator of the cardiovascular system, but it also plays a critical role in regulating cell physiology such as inflammation, fibrosis, and tissue remodeling in a number of tissue types (7).

The biological actions of ANG II are mediated via two G protein-coupled receptors, angiotensin receptor type 1 (AT1) and angiotensin receptor type 2 (AT2) (17, 36). AT1 and AT2 have distinct downstream targets that counteract the physiological actions of each other (58). For example, activation of AT1 stimulates vasoconstriction and cell proliferation whereas AT2 mediates vasodilatation, inhibits cell growth, and activates apoptosis (58). It has been reported that ANG II induces signal transduction by activating mitogen-activated protein kinases (MAPKs) (ERK1/2, JNK, and p38 MAPK), which play key roles in cellular differentiation, proliferation, migration, and fibrosis (62, 64). ERK1/2-dependent signaling plays a key role in the regulation of cholangiocyte proliferation (23, 44).

Recent studies have demonstrated that the RAS plays a key role in hepatic fibrosis and the activation of hepatic stellate cells (HSC). In a fibrotic liver, the local RAS is induced, which plays a role in the activation of HSC (6–10, 41). Paizis and colleagues (51) demonstrated that ACE and AT1 gene expression was upregulated in response to BDL in total liver and especially in areas of active fibrosis. In liver samples from patients with hepatic fibrosis, the expression of AT1 was downregulated in hepatocytes, whereas expression was increased in HSC, vascular endothelium, and bile duct epithelium (51). Moreover, ANG II induces HSC proliferation via the activation of AT1 (8). Studies have also shown that ACE inhibitors or AT1 receptor blockers noticeably reduce hepatic fibrosis in rat (16, 30, 52, 54, 70, 75). Another recent study demonstrated that ANG II induces tumor progression and fibrosis in intrahepatic cholangiocarcinoma through interaction...
with HSC (49, 50). However, the direct effects of ANG II on cholangiocyte proliferation during extrahepatic cholestasis are not known. Therefore, in the present study, we evaluated the effect of exogenous ANG II on the regulation of cholangiocytes proliferation during BDL. We also explored the underlying signaling mechanism by which RAS regulates cholangiocytes proliferation.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). The real-time PCR primers were purchased from SA Biosciences/Qiagen (Valencia, CA). The primary antibodies for Ao, renin, ACE, AT1, and AT2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse and goat IgG Vectastain ABC kits for immunohistochemistry (IHC) were purchased from Vector Laboratory (Burlingame, CA). The cyclic adenosine monophosphate (cAMP) enzyme immunoassay (EIA) was purchased from Cayman Chemical (Ann Arbor, MI). The angiotensin II (ANG II) EIA kit was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The inositol phosphate-one (IP-One) ELISA kit was purchased from Cisbio US (Bedford, MA).

Animal models. All animal experiments were performed in accordance with protocols approved by the Scott & White and Texas A&M Health Science Center Institutional Animal Care and Use Committee. Male 344 Fischer rats (150–175 g) were purchased from Charles River (Wilmington, MA) and maintained in a temperature-controlled environment (20–22°C) with 12:12-h light-dark cycles. Animals were fed ad libitum standard rat chow and had free access to drinking water. To evaluate the in vivo effect of ANG II on cholangiocyte growth, normal and BDL (immediately after surgery) rats were treated with ANG II (50 ng/kg body wt \(1^{-1}\text{-min}^{-1}\)) (56) or 0.9% NaCl (control) by intraperitoneal implant osmotic minipumps for 2 wk. Normal and BDL (immediately after surgery) rats were also treated with losartan (5 mg/kg body wt intraperitoneal dissolved in saline) (35) for 2 wk. Before each experimental procedure, animals were injected with pentobarbital sodium (50 mg/kg body wt ip) following the regulations of the panel on euthanasia of the American Veterinary Medical Association.

Purification of cholangiocytes and maintenance of biliary cell lines. Cholangiocytes were isolated by immunoaffinity separation by incubation with Molecular Probes secondary antibody (Alexa Fluor 488 or 350), corresponding to the pAb species. The labeled cells were evaluated and the fluorescence-activated cell sorting (FACS) data were analyzed by using C6Flow software (BD Biosciences, San Jose, CA).

RAS component expression in purified cholangiocytes was also confirmed by real-time PCR. Total RNA was extracted by using the RNeasy mini kit (SA Biosciences/Qiagen, Valencia, CA), according to the protocol provided by manufacturer. cDNA was prepared from total RNA by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). SYBR Green PCR Master Mix (SA Biosciences/Qiagen) was utilized in the experimental assay with RT2 PCR rat primers designed specifically for renin (NM_012642) (11), ACE (NM_012544) (28), AT1a (NM_030985) (31, 45), AT1b (NM_031009) (31), and AT2 (NM_012494) (34) as well as for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_017008) (65). The housekeeping gene GAPDH was used to normalize gene expression levels. Real-time PCR was performed with an ABI Prism 7900HT System (Applied Biosystems/Life Technologies, Grand Island, NY). Data were expressed as relative mRNA levels \(\pm SE\) of the selected gene-to-GAPDH ratio.

The expression of renin, ACE, AT1, and AT2 was evaluated by IHC in frozen liver sections from normal and BDL rats as described (5 μm thick; n = 3 per each group of animals). Immunofluorescence was performed for renin, ACE, AT1, and AT2 expression was performed in NRIC as described (21). Images were taken in a blinded fashion on an Olympus IX71 fluorescence microscope (Tokyo, Japan) with a DP70 digital camera.

Evaluation of proliferation of biliary and NRIC. We evaluated the effects of in vivo administration of ANG II and losartan to normal and BDL rats on cholangiocyte proliferation by PCNA (29) and CK-19 (13) IHC as previously described (29). Proliferation assays were also performed in NRIC following in vitro treatments with ANG II (10^{-7} M), L-162,313 (AT1 agonist, 10^{-7} M) (68), CGP-42112A (AT2 agonist, 10^{-7} M) (15), losartan [angiotensin receptor blocker (ARB), 10^{-5} M] (18), H89 [cAMP-dependent protein kinase A (PKA) inhibitor, 30 μM] (22), or PD98059 (ERK inhibitor, 10 nM) (22). Cell proliferation was measured at 48 h as described (14). NRIC proliferation was also evaluated in the presence of benzazepin (ACE inhibitor, 1 to 40 μM) at 48 h. NRICs were seeded (7,000 cells/well) onto 96-well plates in complete medium and allowed to adhere overnight at 37°C. Cells were then serum-starved in medium containing 0.5% FBS for 24 h, washed twice with 1 × phosphate buffered saline (PBS); 8 replicates were subsequently treated with ANG II (10^{-7} M) and DMSO (vehicle) or different agents. Cells were incubated for 48 h with the respective treatments, at which time 10 μl of Cell Titer 96 was added to each well. Absorbance at 490 nm was determined with a microplate reader (Spectra Max 3400, Molecular Devices, Sunnyvale, CA). Absorbance is directly correlated to the number of viable cells (43).

Evaluation of ANG II levels and evaluation of intracellular signaling mechanisms. The concentration of ANG II in cell supernatants and plasma were measured by an ANG II EIA (Phoenix Pharmaceuticals, Burlingame, CA). NRIC (1 × 10^{6} cells per well) were cultured in a six-well plate overnight. Then, NRICs were stimulated with vehicle (0.2% BSA) and ANG II (10^{-7} M) for 10 min at room temperature. cAMP levels were determined by a competitive EIA kit (Cayman) (40). Next, the effects of ANG II (10^{-7} M at 37°C for 90 min) on the activity of PKA were evaluated in NRIC with the PepTag assay for
Mean Fluorescence Intensity

Gene expression (fold-change)

Gene expression (fold-change)

Gene expression (fold-change)

A

B

C

D

AT_1

AT_2

Renin

ACE

Positive

Negative

Normal

BDL

AT_1

AT_2

Renin

ACE

Negative
nonradioactive detection of PKA according to the manufacturer’s protocol (Promega, Madison, WI) (6). The effect of ANG II on ERK1/2 phosphorylation was evaluated by the CASE ELISA kit (SuperArray, Frederick, MD) (67). The kit includes a complete antibody-based detection system for colorimetric quantification of the relative amount of phosphorylated protein and total ERK1/2. For the assay, NRIC were seeded into 96-well plates and were stimulated with ANG II (10^{-7} M), for 5, 10, 30, 60, and 180 min. In another experiment NRIC were treated with ANG II (10^{-7} M), ARB (10^{-7} M) + ANG II (10^{-7} M), H89 (30 μM) + ANG II (10^{-7} M), or ARB (10^{-7} M) for 10 min to evaluate the effect of inhibitors on ANG II-induced ERK1/2 phosphorylation. Detection of total and phosphorylated protein expression was determined according to the manufacturer’s protocol. Finally, we measured the effect of ANG II (10^{-7} M at 37°C for 90 min) on the activity and phosphorylation of CREB in NRIC in the absence or presence of pretreatment with H-89 (PKA inhibitor, 10 μM). Activation and phosphorylation of CREB were evaluated by a transcription factor assay kit (Active Motif, Carlsbad, CA), which detects binding activity of phosphorylated CREB (Ser-133) (41).

**Evaluation of proliferation of biliary fibrosis and profibrotic gene expression.** Liver fibrosis was evaluated by Sirius red staining for identifying interstitial collagen with red color in paraffin-embedded liver sections (4–5 μm thick, 10 different fields analyzed from each sample obtained from three different animals). Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used for semiquantitative analysis of the staining. Briefly, a series of digital images was captured from the Sirius red-stained sections. The percentage of the fibrotic area per total liver area was calculated based on the percentage area of red color in each individual slide from each group. Fibrosis was also evaluated by IHC for fibronectin (1:100; Abcam, Cambridge, MA) as described above. Fibrotic marker expression in NRIC was also confirmed by real-time PCR as described above. RT2 PCR rat MA) as described above. Fibrotic marker expression in NRIC was evaluated by a transcription factor assay kit (Active Motif, Carlsbad, CA), which detects binding activity of phosphorylated CREB (Ser-133) (41).

**Evaluation of proliferation of biliary fibrosis and profibrotic gene expression.** Liver fibrosis was evaluated by Sirius red staining for identifying interstitial collagen with red color in paraffin-embedded liver sections (4–5 μm thick, 10 different fields analyzed from each sample obtained from three different animals). Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used for semiquantitative analysis of the staining. Briefly, a series of digital images was captured from the Sirius red-stained sections. The percentage of the fibrotic area per total liver area was calculated based on the percentage area of red color in each individual slide from each group. Fibrosis was also evaluated by IHC for fibronectin (1:100; Abcam, Cambridge, MA) as described above. Fibrotic marker expression in NRIC was also confirmed by real-time PCR as described above. RT2 PCR rat primers designed specifically for collagen Iα1 (NM_055304) (37), fibronectin 1 (FN1; NM_019143) (63), and IL-6 (NM_012589) (46) as well as for GAPDH were utilized (65). The housekeeping gene GAPDH was used to normalize gene expression levels. Real-time PCR was performed with an ABI Prism 7900HT System (Applied Biosystems/Life Technologies). Data were expressed as relative mRNA levels ± SE of the selected gene-to-GAPDH ratio.

**Statistical analysis.** All data are expressed as means ± SE. Differences between two groups were analyzed by the Student unpaired t-test when two groups were analyzed by Mann-Whitney U-test and Kruskal-Wallis H-test when more than two groups were analyzed, followed by an appropriate post hoc test. A value of P < 0.05 was considered significant.

**RESULTS**

**Cholangiocytes express the RAS.** Cholangiocytes isolated from normal and BDL rats as well as NRIC expressed RAS components including Ao, renin, ACE, AT_1a, and AT_1b receptors (Fig. 1). There was a significant increase in the gene expression levels of Ao, renin, ACE, AT_1a, and AT_1b in cholangiocytes isolated from BDL compared with normal rats (Fig. 1A). Consistent with gene expression levels, there was a significant increase in the protein expression levels of Ao, renin, ACE, and AT_1 in cholangiocytes isolated from BDL rats compared with normal rats as determined by FACS (Fig. 1B). No change in AT_2 gene expression was observed during BDL (Fig. 1A). A small increase in AT_2 protein expression was observed in BDL cholangiocytes as evaluated by FACS (Fig. 1B). The RAS components were also expressed in NRIC as evaluated by immunofluorescence (Fig. 1C). There was higher expression of RAS components in the bile ducts of BDL rat liver sections compared with normal rat as evaluated by IHC (Fig. 1D). Hepatocytes are also positive for the expression of RAS components as expected (76). As shown in Table 1, serum from BDL rats showed higher levels of ANG II compared with normal rats. Also, primary cultures of cholangiocytes from BDL rats secrete higher amounts of ANG II in the medium compared with normal cholangiocytes (Table 1). In addition, the normal cholangiocyte cell line, NRIC, secretes ANG II in the culture media (Table 1).

**ANG II stimulates biliary proliferation.** Liver sections from the BDL rats, infused with ANG II, showed higher expression of CK-19-positive cholangiocytes per portal area compared with normal control rats (Fig. 2, A and B). Similar to the increase in the number of CK-19-positive cholangiocytes per portal area (Fig. 2B), there was also a significant increase in the number of PCNA-positive cholangiocytes per portal area (Fig. 2C). The number of both CK-19- and PCNA-positive cholangiocytes per portal area was significantly reduced in BDL rats treated with the ARB (losartan) compared with BDL + ANG II-treated rats (Fig. 2, B and C).

As shown in Fig. 3A, the AT_1 agonist induced a similar increase in NRIC proliferation compared with ANG II treatment at 48 h. However, the AT_2 agonist had no effect on NRIC proliferation (Fig. 3A). ANG II-induced proliferation was inhibited by ARB (losartan) (Fig. 3A). Since cholangiocytes express RAS and secrete ANG II, we evaluated the role of ANG II in the regulation of basal biliary proliferation in NRIC treated with benazepril (ACE inhibitor). Benazepril induced a significant dose-dependent reduction in NRIC proliferation at 48 and 72 h (Fig. 3B). This was also confirmed in NRIC treated with ARB, in which a significant reduction in basal proliferation was observed at 48 h.

**ANG II stimulates cAMP-, PKA-, ERK1/2-, and CREB-dependent intracellular signaling mechanisms and proliferation.** In NRIC, cAMP and inositol 1,4,5-trisphosphate (IP_3) levels were evaluated to begin to elucidate ANG II mediated intracellular signaling mechanisms. ANG II caused an increase in intracellular cAMP but not IP_3 levels (not shown), compared with basal values (Fig. 4A). Since ANG II stimulated intracellular cAMP levels, we measured cAMP-dependent PKA activity. There was enhanced PKA activity in NRIC treated with ANG II that was inhibited by ARB (Fig. 4B). ERK1/2 has been shown to play a key role in proproliferative signaling mechanisms in cholangiocytes (20, 22, 52). A significant activation of ERK1/2 was observed in the ANG II-stimulated NRIC in a time-dependent manner (Fig. 5A).

### Table 1. Measurement of Ang II concentration in plasma of NR and BDL rats and Ang II secretion in the supernatant of NR and BDL cholangiocytes as well as in the supernatant of NRICs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ang II, pg/ml</th>
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<tr>
<td>Normal serum</td>
<td>110.8 ± 11.4</td>
</tr>
<tr>
<td>BDL serum</td>
<td>142.8 ± 6.5*</td>
</tr>
<tr>
<td>Normal cholangiocyte supernatant</td>
<td>128.4 ± 9.5</td>
</tr>
<tr>
<td>BDL cholangiocyte supernatant</td>
<td>157.4 ± 6.2*</td>
</tr>
<tr>
<td>NRIC supernatant</td>
<td>0.6 ± 0.01</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. normal rat (NR) plasma (n = 6); †P < 0.05 vs. normal cholangiocyte supernatant (n = 6). Ang II, angiotensin; BDL, bile duct ligation; NRICs, normal rat intrahepatic cholangiocyte cell line.
There was higher phosphorylation of ERK1/2 in NRIC within 5–10 min of ANG II treatment and the phosphorylation levels of ERK1/2 remained unchanged up to 180 min (Fig. 5A). ANG II-induced ERK phosphorylation was attenuated both by ARB (losartan) and H89 (PKA inhibitor) (Fig. 5B). This data suggests that PKA is the upstream modulator of ERK1/2, which is supported by the inhibition of ANG II-dependent proliferation by H89 and PD98059 (ERK 1/2 pathway inhibitor) (Fig. 3A). 

Finally, we evaluated the effects of ANG II on downstream CREB activity, which has been shown to play an important role in modulating cholangiocyte proliferation (19, 23). Stimulation with ANG II and AT1 agonist increased pCREB DNA binding activity in NRIC, which was attenuated by ARB (losartan) (Fig. 5C). Stimulation with ARB alone also resulted in decreased pCREB DNA binding activity (Fig. 5C).

**ANG II stimulates biliary fibrosis and expression of profibrotic genes.** Liver sections from the BDL rats, infused with ANG II, showed significantly higher levels of Sirius red staining (i.e., fibrosis) in the portal areas compared with normal control rats (Fig. 6A). Sirius red staining was significantly reduced in BDL rats treated with the ARB (losartan) compared with BDL + ANG II-treated rats (Fig. 6A). Similar findings were observed for fibronectin staining (Fig. 6B). In vitro, ANG II stimulated increased collagen 1A1 (Col1a1), fibronectin 1 (FN1), and IL-6 gene expression in NRIC, which was blocked by ARB (Fig. 6C).

**DISCUSSION**

The major findings in the study relate to the expression of a local RAS by cholangiocytes and the regulation of biliary proliferation by ANG II during extrahepatic cholestasis. We found that 1) cholangiocytes express the components of the RAS and produce ANG II; 2) RAS components and ANG II levels are elevated during extrahepatic cholestasis induced by BDL; 3) administration of exogenous ANG II to normal and BDL rats increased intrahepatic bile duct mass; 4) administration of the ARB (losartan) during BDL significantly reduces intrahepatic bile duct mass; and 5) ANG II stimulates cholangiocyte proliferation via the activation of AT1 triggering cAMP-, PKA-, ERK1/2-, and pCREB-dependent signaling mechanisms. Upregulation of the RAS may play an important role in the regulation of biliary mass during extrahepatic cholestasis via autocrine and paracrine signaling mechanisms.
Previous studies have shown that RAS plays an important role in advanced liver diseases (17, 36). Several groups have evaluated the expression of RAS components in animal models of hepatic fibrosis and human tissue samples. Paizis et al. (51) demonstrated that the gene expression for ACE and AT1 were upregulated by BDL in total liver and were highly expressed in areas of active fibrosis. Similar to our findings, the biliary epithelium in tissue samples from patients with advanced liver cirrhosis expressed AT1 by IHC (59). In this study, the expression of AT1 was downregulated in hepatocytes, whereas the expression was increased in HSC, vascular endothelium, and bile duct epithelium (59).

The endocrine (or classical) RAS is known for its role in the regulation of blood pressure, electrolyte balance, and fluid homeostasis (36). Recently, studies have demonstrated the expression of all of the “classical” RAS components required for a functioning local RAS in the pancreas (38, 53). Our findings that cholangiocyte express the components necessary for the formation of ANG II, such as renin and ACE, indicate that cholangiocytes have a functional RAS. This is similar to findings observed in activated hepatic stellate cells, which have a local RAS and produce ANG II that stimulates fibrosis via activation of NADPH oxidase (77). This concept is supported by the observation in our study that cholangiocytes express the message and protein for Ao, which is the precursor for ANG II. Ao expression has also been shown in liver cells such as Kupffer cells, hepatocytes, and HSCs (42). In addition to expressing angiotensinogen, cholangiocytes also produce ANG II, which is similar to findings in activated HSCs (77). Both in vitro and in vivo, HSCs have higher expression levels of active renin and ACE and synthesis ANG II (10). Cholangiocyte production of ANG II is significantly increased during BDL in both serum and cholangiocyte supernatants. In patients with cirrhosis or chronic liver disease, there were also higher expression levels of angiotensin and plasma renin activity (69). RAS plays a key role in the alterations of portal pressure during decompensated liver cirrhosis (1). Finally, the involvement of RAS during cholestasis is supported by our finding that renin and ACE expression levels are increased in cholangiocytes during BDL. The expression of RAS in numerous liver cell types indicates that cholangiocyte proliferation may be modulated in paracrine mechanisms in addition to autocrine mechanisms. The activation of RAS in cholangiocytes may play a role in the activation of other cell types throughout the liver contributing to ANG II-induced fibrosis.

Fig. 3. Evaluation of the effects of angiotensin II (ANG II) on the proliferation of NRICs. A: ANG II and the AT1 agonist stimulate the proliferation of NRIC at 48 h. ANG II-stimulated proliferation is blocked by ARB, H89 (PKA inhibitor), and PD98059 (ERK1/2 pathway inhibitor). ARB (losartan) decreased basal proliferation. *P < 0.05 vs. basal (control). #P < 0.05 vs. basal. Data are presented as means ± SE, n = 6. B: benazepril (ACE inhibitor) dose dependently inhibits NRIC proliferation at 48 and 72 h. *P < 0.05 vs. basal. Data are presented as means ± SE, n = 6.

Fig. 4. Effect of ANG II on intracellular cAMP levels and PKA activity in NRICs. A: ANG II stimulated intracellular cAMP levels in NRICs. *P < 0.05 vs. normal. Data are presented as means ± SE, n = 6. B: ANG II stimulated an increase in PKA activity compared with basal, which is prevented by ARB (losartan). *P < 0.05 vs. basal (control). #P < 0.05 vs. basal. Data are presented as means ± SE, n = 6.
We next evaluated the effects of ANG II on biliary proliferation in both normal and cholestatic BDL rats. Chronic administration of ANG II stimulated a significant increase in biliary mass and proliferation in both normal and BDL rats, which was blocked by ARB (losartan) (AT\(_1\) inhibitor). Blockage of the RAS signaling either through ARB or ACE inhibition has been shown to attenuate fibrosis and to suppress activated HSCs and hepatic TGF-\(\beta_1\) expression in animal models of chronic liver injury (including CCl\(_4\) and BDL) (30, 48, 66, 72, 73, 75). In vitro, in NRIC we found that ANG II and an AT\(_1\) agonist stimulate a significant increase in proliferation, which was blocked by ARB. Interestingly, treatment of NRIC with ARB resulted in a significant reduction in basal proliferation, which supports the concept of the presence of a local RAS in cholangiocytes. We also found a significant reduction in proliferation of NRIC when treated with the ACE inhibitor benazepril. This is similar to findings reported by Li and Wanchun (39) that benazepril reduces intimal proliferation.

Next, we evaluated the intracellular signaling mechanisms regulating ANG II-induced proliferation. We found that ANG II stimulated intracellular cAMP levels but not IP\(_3\), which is in contrast to many studies (2, 12, 25). The majority of studies have show that activation of AT\(_1\) produces second messengers such as IP\(_3\), diacylglycerol, and reactive oxygen species (26). However, our data are supported by the findings that ANG II stimulates increased intracellular cAMP in glomerular epithelial cells (60). Another study has shown that ANG II can activate cAMP formation in renal mesangial cells (55). The cAMP/PKA/ERK/CREB intracellular signaling pathway has been shown to play an important role in the regulation of biliary proliferation both in vitro and in vivo (20, 44). Therefore, we explored the downstream PKA/ERK/CREB signaling pathway related to ANG II. Similar to our previous studies, ANG II stimulated PKA activity, ERK1/2 phosphorylation, and pCREB DNA binding activity. Supporting the concept that the PKA/ERK/CREB signaling pathway plays an important role in ANG II-stimulated biliary proliferation, inhibition of PKA or ERK1/2 signaling attenuated proliferation. The concept of the presence of a local RAS in cholangiocytes is also supported by the findings that treatment with ARB alone reduced ERK1/2 phosphorylation and pCREB DNA binding activity below basal levels.

Finally, we also evaluated the effects of chronic ANG II administration on biliary fibrosis and the expression of proproliferative/profibrotic genes. We found, similar to other studies, that ANG II stimulates a significant increase in fibrosis surrounding the portal area as well as an increase in portal fibronectin expression, which were both blocked by ARB (33, 47, 74). In a similar fashion, we found that ANG II stimulates the gene expression of collagen 1A1 and fibronectin 1 in NRIC, which was blocked by pretreatment with ARB. In vitro, we also found that ANG II stimulates a significant increase in IL-6 gene expression in cholangiocytes, which is a proproliferative cytokine that plays a key role in regulating biliary hyperplasia (71). ANG II has been shown to increase IL-6 expression levels in several cell types (24, 32). Further studies will be necessary to fully elucidate the relationship between ANG II and IL-6 on biliary proliferation and fibrosis.

The present study demonstrates that the local RAS plays an important role in the regulation of normal and cholestatic cholangiocyte proliferation and the activation of biliary fibrosis. Modulation of the RAS and the ANG II/AT\(_1\) signaling pathway in cholangiocytes may represent a novel therapeutic approach for cholangiopathies.
Fig. 6. Evaluation of Sirius red staining, fibronectin expression in liver sections, and ANG II-induced IL-6 expression in NRIC. A: there is increased levels of fibrosis in the portal areas of the ANG II treated Normal + ANG II and BDL + ANG II compared with the NR rat liver. Losartan (ARB) treatment significantly downregulates fibrosis. Original magnification ×40. B: there is increased fibronectin expression in the portal areas of ANG II treated Normal + ANG II and BDL + ANG II compared with the NR rat liver. Losartan (ARB) treatment downregulates fibronectin expression. Original magnification ×40. C: in vitro, ANG II stimulated the gene expression levels of Col1a1, FN1, and IL-6, which were blocked by ARB. *P < 0.05 vs. normal. **P < 0.05 vs. BDL. #P < 0.05 vs. BDL. Data are presented as means ± SE, n = 6.
ACKNOWLEDGMENTS

This material is the result of work supported with resources and the use of facilities at the Central Texas Veterans Health Care System, Temple, Texas. The views presented are those of the authors and do not necessarily represent the views of the Department of Veterans Affairs. The authors thank Dr. Gianfranco Alpini for reading the manuscript and for providing the NRIC line. We also thank Anna Web for assistance with confocal imaging.

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GRANTS

This work was supported by an NIH R01 grant (DK-081442), VA CDA-2, and VA Merit Award to S. Glaser.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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