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INTRACEREBROVENTRICULAR LOSARTAN INFUSION MODULATES ANGIOTENSIN TYPE 1 RECEPTOR EXPRESSION IN THE SUBFORNICAL ORGAN AND DRINKING BEHAVIOUR IN BILE DUCT LIGATED RATS

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Abstract

Bile duct ligation (BDL) causes congestive liver failure that initiates hemodynamic changes including peripheral vasodilation and generalized edema. Peripheral vasodilation is hypothesized to then activate compensatory mechanisms including increased drinking behavior and neurohumoral activation. This study tested the hypothesis that changes in the expression of AT1R mRNA and protein in the lamina terminalis is associated with BDL induced hypoosmolality in the rat. All rats received either BDL or sham ligation surgery. The rats were housed in metabolic chambers for measurement of fluid and food intake and urine output. Angiotensin type 1 receptor (AT1R) expression in the lamina terminalis was assessed by western blot and quantitative real-time PCR (RT-qPCR). Average baseline water intake significantly increased in BDL rats compared to sham and upregulation of AT1R protein and AT1aR mRNA were observed in the subfornical organ (SFO) of BDL rats. Separate groups of BDL and sham ligated rats were instrumented with minipumps filled with either losartan (2.0 µg/µl) or 0.9% saline for chronic intracerebroventricular (ICV) or subcutaneous (SC) chronic infusion. Chronic ICV losartan infusion attenuated the increased drinking behavior and prevented the increased abundance of AT1R protein in the SFO in BDL rats. Chronic SC did not affect water intake or AT1R abundance in the SFO. The data presented here indicate a possible role of increased central AT1R expression in the regulation of drinking behavior during congestive cirrhosis.

Keywords

vasopressin; AT₁R; drinking behaviour; bile duct ligation

INTRODUCTION

Hyponatremia is associated with negative outcomes in many chronic disease states such as congestive heart failure and cirrhosis. In both congestive heart failure and cirrhosis, changes in the osmoregulation of arginine vasopressin (AVP) release from the neurohypophyseal system contribute to water retention and dilutional hyponatremia—thereby increasing morbidity and mortality (Cardenas & Arroyo, 2003; Oren, 2005; Schrier, 2006). Treatment with specific V2 receptor antagonists (V2A) does produce solute-free water excretion and improved plasma osmolality in both heart failure and cirrhosis (Gheorghiade *et al.*, 2003; Gheorghiade *et al.*, 2006; Schrier *et al.*, 2006), however there may be evidence that

increased drinking behaviour from disease and possibly as a side-effect of V2A treatment may affect mortality or re-hospitalization (Rossi *et al.*, 2007; Rozen-Zvi *et al.*, 2010).

Traditionally, increased neurohumoral activation associated with cirrhosis has been attributed to decreased effective circulating volume and hyperdynamic circulatory syndrome (Kim *et al.*, 2010; Schrier, 2010). Increased circulating angiotensin II (Ang II) could contribute to sympatho-excitation, increased AVP release and elevated fluid intake by acting on CNS circumventricular organs (McKinley *et al.*, 2004; Osborn *et al.*, 2007; McKinley *et al.*, 2008).

The lamina terminalis contains two circumventricular organs: the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO). Neurons within these unique forebrain regions lack a functional blood brain barrier (McKinley & Johnson, 2004; Johnson, 2007), which allows these structures to be sensitive to circulating factors such as plasma osmolality and Ang II (Osborn *et al.*, 2007). The OVLT and SFO both project to the median preoptic nucleus (MnPO), which is an important integrative centre for hydromineral and cardiovascular regulation. Angiotensin II type 1 receptor (AT1R) expression is densely localized to these nuclei (Allen *et al.*, 1998; Grob *et al.*, 2004), and the level of AT1R expression in these regions may be important in determining the responsiveness of the brain angiotensin system in regulating hemodynamic physiology. The precise neural and molecular factors that affect the resetting of the thirst and AVP osmotic set-points during heart or liver failure remain undetermined.

Chronic bile duct ligation (BDL) in the rat is a commonly used model of hepatic cirrhosis (Better *et al.*, 1980; Brond *et al.*, 2004). As in the human condition, these rats have elevated circulating vasopressin despite significantly decreased plasma osmolality, increased PRA, and hypervolemic ascites formation. Previously, our lab reported that there was a significant positive relationship between PRA and AVP release, and that decreasing PRA by restricting fluid intake to hypertonic saline resulted in a significant decrease in circulating AVP (Carreño *et al.*, 2009).

The purpose of this study was to evaluate the pattern of expression of AT1R mRNA and protein in the lamina terminalis associated with BDL induced hypoosmolality. We further tested the role of central AT1Rs pharmacologically with chronic losartan infusions to functionally test the contribution of central angiotensin signaling in the development of dilutional hyponatremia during BDL cirrhosis.

MATERIALS AND METHODS

Animals

Experiments were conducted on adult male Sprague-Dawley rats (250–350g, Charles River Laboratories, Inc., Wilmington, MA, USA). Rats were individually housed and maintained in a temperature-controlled (23°C) environment under a 12/12h light cycle with light onset at 0700h. Rats had *ad libitum* access to food and water except during the surgical procedures. Rats were provided with food containing 0.32% NaCl. All experimental procedures were conducted in accordance with the guidelines of the Public Health Service and were approved by the University of North Texas Health Science Centre Institutional Animal Care and Use Committee.

Bile Duct ligation model of hepatic cirrhosis

Each animal was anesthetized with 2% isoflurane. After anesthetization, the abdomen was shaved and the area was cleaned with iodine antiseptic. A midline incision was performed on the abdomen and the common bile duct was isolated by blunt dissection and cut between

the two ligatures. Each animal was then returned to the cage and monitored daily until used for the experiment. Visual inspection of ascitic fluid of the peritoneal cavity was performed daily after surgery. Any rat showing morbidity (e.g., failure to thrive, significant decrease in food or water intake) was euthanized with thiobutabarbital (Inactin®, Sigma, St. Louis, MO; 100 mg/kg i.p.) followed by decapitation. Sham ligated controls were subjected to the same surgical procedure with the exception that the bile duct was not ligated or cut. Rats were euthanized 4 weeks (28 days) after BDL or sham surgery for collection of brain and blood samples. Liver fibrosis was observed in successful bile duct ligated animals. Liver/body weight ratio and discoloration of blood serum was used to verify the development of hepatic cirrhosis.

Chronic ICV cannula and osmotic minipump implantation

The rats were allowed to recover for 7 days after BDL or sham surgery. After recovery from surgery a subgroup of BDL and sham rats was instrumented with osmotic mini-pumps (Alzet model 2004, 0.25 μ l h⁻¹; Cupertino, CA, USA) for either intracerebroventricular (ICV) or subcutaneous (SC) infusions to control for peripheral drug effects. The pumps were filled with either losartan (2 μ g/ μ l) dissolved in 0.9% saline vehicle or vehicle alone. The dose of losartan was chosen based on previous in vivo studies (Porter *et al.*, 2004; Zimmerman *et al.*, 2004; Wei *et al.*, 2008; Wei *et al.*, 2009) that showed no change in blood pressure at this dose. The pumps were incubated for at least 48 h in a 37°C water bath and weighed prior to implantation. Surgeries were performed using aseptic technique and the rats were anaesthetized with isoflurane. For ICV infusions, the pumps were connected by polyethylene tubing to a piece of 22 gauge stainless steel tubing (Plastics One, Roanoke, VA); cut 5.0 mm below the pedestal. The free end of the 22 gauge tubing was placed in the left lateral ventricle using stereotaxic technique. A small hole was drilled in the skull at 1.0 mm posterior and 1.5 mm lateral to bregma using the level skull technique (Paxinos & Watson, 1997). The cannula was placed into the hole so that it extended 5 mm from the surface of the skull and then cemented into place with dental acrylic and jeweller's screws. The osmotic pumps were then sutured into a small pocket made under the skin at the base of the neck. For SC infusions, the pumps were prepared and placed under the skin at the base of the neck just as they were implanted for the ICV infusions. All rats were euthanized 28 days after BDL or sham surgery (i.e., 21 days after minipump implantation) and pumps were again weighed to verify drug or vehicle delivery.

Metabolic cages

To measure water intake, food intake, and urine output, the animals were housed in metabolic cages (Lab Products Inc., Seaford, DE) starting on the 18th day after BDL or sham surgery. The metabolic cage protocol consisted of 10 days. Days 1–3 were acclimation days in which no measurement was taken. On Days 4–10, food intake, water intake and urine output were recorded at 9:00 am. Food was measured by filling the food containers up to a prespecified weight in grams and subtracting the remaining weight from the prespecified weight 24 hours later. Sodium intake was calculated from sodium content of the food (0.32% by weight, Cat# LM485, Teklad Diets, Madison WI). Water intake was measured using graduated cylinders filled daily to a volume of 100 ml. Urine output was collected in 50 ml Falcon centrifuge tubes and 1 ml from each daily sample was pipetted into a 1.5 ml microcentrifuge tube and centrifuged (20 min; 10,000 $\times$ g). 10 μ l of urine supernatant was then removed for measuring urine sodium concentration using a flame photometer (Jenway PFP7, VWR International, Radnor PA) according to manufacturer's recommendations. All data were normalized to each rat's body weight at sacrifice.

Plasma measurements

A sample of whole blood from each rat was collected into a 1.5 ml microcentrifuge tube. Two heparin-containing capillary tubes were filled with blood from this sample for measuring hematocrit. The rest of the sample was centrifuged (5 min; 10,000×g) and a 200 µl sample of serum was removed for measuring osmolality using a vapour pressure osmometer (Wescor Inc. Logan, UT). Plasma protein was measured by refractometry (national Protometer, National Instruments, Baltimore, MD).

Micropunch dissection of forebrain tissues

Tissue sites containing the SFO, OVLT, and MnPO were micropunched from the forebrain for RT-qPCR and Western blot analysis. Each rat was anesthetized (Inactin; 100 mg/kg, i.p.) and decapitated. The isolated brain was placed in a commercially available brain matrix (Stoelting, Wood Dale, IL). The matrix was used to cut the brain into 1-mm coronal slabs with razor blades. Then, the desired regions from this 1-mm-thick slice were micropunched with 1-ml syringes equipped with blunt 23-gauge needles. The punch samples were expelled into microcentrifuge tubes and snap-frozen on dry ice.

Western Blot

Punches from one set of animals were sonicated in 50 µl of modified radioimmunoprecipitation buffer (RIPA) supplemented with protease and phosphatase inhibitors followed by 30 min incubation on ice. The total homogenates were then centrifuged (14,000 rpm, 30 min at 4°C). Total protein concentration was determined by the Bradford method. Fifty to sixty micrograms of total lysate was loaded onto 4–20% acrylamide SDS gel, electrophoresed in Tris-glycine buffer under denaturing conditions and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) in Tris-glycine buffer with 10–20% methanol. Membranes were blocked for 1 h at room temperature with 5% (wt/vol) non-fat milk in Tris-buffered saline 0.05% (vol/vol) Tween 20 (TBS-Tween; 50 mM Tris base, 200 mM NaCl, 0.05% Tween 20). Membranes were then incubated overnight at 4°C with primary antibody raised against AT₁ receptors (Fitzgerald Industries International, 70R-AC001; 1:500 dilution) and β-actin for normalization (Sigma, A5441; 1:2,000 dilution). Blots were rinsed 3 times 10 min each with TBS 0.05% Tween 20 and then incubated at room temperature for 1 h in a horseradish peroxidase conjugated secondary antibody against the primary antibody host species (1:5,000; Sigma). The proteins were detected by enhanced chemiluminescence (ECL reagents; Amersham, Piscataway, NJ) by acquiring digital gel images from Syngene G-box (Frederick, MD). Densitometry of immunoreactive bands of interest was analysed using Image J software.

RNA extraction and reverse transcription

All procedures were performed using RNase-free conditions. Brain punch samples were homogenized in 200 µl of Trizol (Invitrogen, Carlsbad, CA). Total RNA was isolated from homogenates using a chloroform extraction, isopropanol precipitation, and ethanol wash. Isolated RNA pellets were eluted in TE Buffer and genomic DNA contamination was removed using an RNase-free DNase I digest (DNA Free, Ambion, Austin, TX). RNA concentration and purity was determined using a Nanodrop (Nanodrop, Wilmington, DE). Samples yielding less than 50 ng/µl and/or a 260/280 ratio lower than 1.8 were not used for amplification. Extracted RNA was normalized for concentration and reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and the provided thermal cycling protocol.

Quantitative real-time PCR

Taqman gene expression assays were purchased from Applied Biosystems containing FAM and VIC probe-labelled primers (AT1aR—Assay ID: Rn01435427_m1, Gene ID: 24180; GAPDH—Assay ID: Hs99999905_m1). Using a 96 well PCR optical plate, duplexed primers, and at least 2 µl of sample cDNA were added in triplicate and assayed with Taqman gene expression master mix (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed by a Bio-Rad CFX96 Real-time PCR detection system. The CFX96 initially warms the sample to 50°C for 2 minutes then heat activates the DNA-Polymerase for 10 minutes at 95°C. Then, 40 cycles of PCR are performed as follows: (1) 15 seconds at 95°C, (2) 1 minute at 60°C. Fluorescence data are captured by the optical camera module at the end of each cycle using CFX Manager software v1.6.541.1028 (Bio-Rad, 2008).

Quantification and analysis of PCR results

Data were plotted across cycles for detected fluorescence above the background signal. The x coordinate at the threshold of amplification was designated the Ct and used as a measure of mRNA quantitation. To validate sample viability, the slope of tangential lines to the threshold point for each sample was compared and samples with slope values deviating more than 5% from the mean were discarded. Standard dilution efficiency curves were generated using previously determined primers. Standard curves for both genes had correlation coefficients greater than 0.990. Interaction tests during multi-plex between the primers were negative. The relative mRNA expression of GAPDH mRNA was used for normalization. The data were analysed by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

Experimental protocol

There were eight separate groups used for metabolism cage studies: drug/vehicle naïve sham ligated (n = 9), drug/vehicle naïve BDL (n = 11), vehicle ICV infused sham ligated (n = 9), losartan ICV infused sham ligated (n = 8), vehicle ICV infused BDL (n = 10), losartan ICV infused BDL (n = 10), SC losartan sham ligated (n = 10), SC losartan BDL (n = 10). These animals were used for punch collection and Western blot analysis of SFO AT1aR abundance. Some samples were not included in the analysis due to poor protein or mRNA recovery. The duration of the experiment for all animals was 28 days from sham ligation or BDL surgery. A subset received either ICV cannula or SC minipump implantation 7 days after sham ligation or BDL surgery as described above. At the end of the experiment period, rats were anesthetized with thiobutabarbital (Inactin®, Sigma, St. Louis, MO; 100 mg/kg ip) and decapitated. Samples were collected for analysis as described above.

Statistical Analysis

All results are presented as means ± SEM. Daily water, urine, sodium, and food measurements were analysed using two-way repeated measures ANOVA. Post-hoc analysis of significant main effects was carried out using Student Newman–Keuls t-test (Sigmaplot, San Jose, CA). Other data were analysed by one-way analysis of variance. Significance was set at $P < 0.05$.

RESULTS

EFFECTS of BDL on fluid balance and AT1 receptors

Plasma osmolality, hematocrit and plasma protein values from naïve sham ligated and BDL rats are shown in Table 1. BDL rats exhibited significantly lower plasma osmolality and hematocrit volume fraction levels ($p < 0.05$) compared to sham ligated animals (Table 1). BDL rats that completed the experiment displayed jaundice with elevated serum bilirubin,

hepatosplenomegaly, and developed a cirrhotic liver as evaluated by gross visual examination. Liver-weight-to-body-weight ratios at time of sacrifice were significantly higher in all BDL rats compared to sham ($p<0.001$). Therefore, experimental BDL led to liver pathology associated with extracellular volume expansion as measured by hematocrit. We also observed a trend in decreased weight gain in BDL animals (Table 1, $p=0.07$ sham vs. BDL). As a result, all metabolic data were normalized to body weight.

BDL animals drank significantly greater amounts of water compared to sham ligated controls (Figure 1A, $p<0.05$) but there were no differences in urine volume (Figure 1A). Minipump naïve BDL animals exhibited decreased renal sodium excretion compared to sham animals (Figure 2B, $p<0.001$).

AT1R protein abundance was significantly increased in the SFO of BDL rats compared to sham (Figure 2A; $p<0.05$). AT1aR mRNA abundance also increased in BDL rats compared to sham (Figure 2B; $p<0.05$). Western Blot analysis of OVLT AT1R abundance showed a slight increased expression in BDL rats (Figure 3A $p<0.05$). However, there was no corresponding increase in AT1aR mRNA in the OVLT (Figure 3B). There was no significant difference in AT1R protein or AT1aR mRNA abundance in the MnPO between treatment groups (Figure 3C and 3D respectively).

Effects of chronic losartan infusions on fluid balance and AT1 receptor expression in BDL rats

Similar to minipump naïve rats, plasma osmolality and hematocrit were lower in BDL animals that received saline or losartan infusion either ICV or subcutaneously (Table 2). Notably, ICV losartan treatment did not affect plasma osmolality in BDL rats (Table 2). This observation indicates that ICV losartan treatment at this concentration may not affect vasopressin release or the resulting plasma hypoosmolality. Furthermore, sham ligated animals implanted with a subcutaneous losartan minipump had significantly higher body weight at the end of the experiments as compared to all other treatment groups (Table 2, $p<0.05$). As a result, all metabolic data were normalized to body weight.

When treated with ICV losartan the increased drinking behaviour exhibited by BDL animals was prevented (Figure 4; $p<0.05$). BDL rats that received subcutaneous minipumps containing the same concentration of losartan as a control for peripheral actions of ICV infused losartan showed no inhibition in drinking behaviour or change in renal sodium handling. This observation indicates the site of action of losartan is in the CNS. We also did not see any evidence that subcutaneous losartan modified the disease process. Interestingly, BDL animals infused intracerebroventricularly with 0.9% saline vehicle showed greater urine output compared to sham animals (Figure 4) possibly due to the increased water intake and partial vasopressin escape.

We did not observe any difference in renal sodium excretion in BDL animals in the ICV or subcutaneous minipump treatment groups (Figure 5). There was no difference in food intake between all the treatment groups (data not shown).

Losartan infused chronically into the lateral ventricles prevented the increased AT1R protein expression that we observed in drug naïve and vehicle infused rats (Figure 6). Western blot analysis of AT1R expression in the OVLT (Figure 7A) and MnPO (Figure 7B) of BDL animals treated with losartan ICV did now show a significant difference among the treatment groups.

DISCUSSION

The main findings of this study are that 1) water intake increased in BDL rats, 2) BDL is associated with an upregulation in the abundance of SFO AT1R protein and AT1aR mRNA, and 3) infusion of the AT1R antagonist losartan into the ventricles blocked the increased water intake and increased abundance of AT1R protein in the SFO. A corollary finding from this study is that plasma osmolality was not affected by ICV losartan infusion; which suggest that central angiotensin signalling may not play a role in BDL associated inappropriate vasopressin release. Taken together, these results indicate that central angiotensin signalling may play a role in increased SFO AT1aR expression and the regulation of drinking behaviour but not the activation of SON neurosecretory cells in the context of BDL cirrhosis.

In a previous experiment, our lab provided a group of BDL rats with 2% saline to drink to decrease PRA by increasing sodium delivery to the distal tubule of the kidney (Carreño *et al.*, 2009). This manipulation was associated with a significant decrease in PRA and circulating vasopressin compared to BDL rats provided only with water (Carreño *et al.*, 2009). These finding provided correlational evidence that the renin-angiotensin system may contribute to increased plasma vasopressin concentrations in BDL rats. Earlier in vivo studies have shown that neurons in the lamina terminalis contain a functional renin-angiotensin system that extends into the SON (Chen & Toney, 2001; Y. Ozaki, 2004; Cato & Toney, 2005; Oz *et al.*, 2005), and in vitro electrophysiological studies have also shown that Ang II can act post-synaptically to activate non-specific cationic conductance to depolarize SON neurons and pre-synaptically to facilitate glutamate release (Y. Ozaki, 2004; Oz *et al.*, 2005). Losartan can cross the blood-brain barrier at sufficiently high concentrations (Huang *et al.*, 2009), but we did not test receptor binding after losartan administration. Therefore we cannot provide direct information about the anatomical specificity of the observed effects. Circulating Ang II may play a role in the activation of circumventricular organ afferents to the SON; however, the present data indicate that central angiotensin signaling is not necessary for decreased plasma osmolality during BDL cirrhosis. Two previous studies showed that losartan can alter the BDL disease process by affecting liver fibrosis, portal hypertension and hyperdynamic circulation (Croquet *et al.*, 2002; Yang *et al.*, 2002). However, the present study used a concentration of losartan that was much smaller than the minimum dose that is required to modify the progression of cirrhosis or hyperdynamic circulation.

Studies of drinking behaviour in BDL rats have reached mixed conclusions. Our finding that BDL rats consume more water is consistent with a previous study of rats that were not housed in metabolic cages (Carreño *et al.*, 2009). However, another study showed no difference between sham ligated and BDL rat drinking behaviours (Croquet *et al.*, 2002). In this latter study, the rats were housed in metabolic cages for 6 days without a period of acclimation during which time the rats were given oral doses of water by gastric gavage as a control for losartan drug administration. The use of gastric gavage may have influenced baseline water intake due to stress or the volume of fluid delivered directly to the gastrointestinal tract.

Other studies have shown that BDL rats have increased sodium appetite, but did not find evidence of increased water intake (Lane *et al.*, 1998; Fitts *et al.*, 1999). It has also been reported that the increase in sodium intake is independent of the renin-angiotensin system (Lane *et al.*, 1998; Fitts *et al.*, 1999). The differences between these observations may be due to the different strain of animal used in the previous work (Lane *et al.*, 1998; Fitts *et al.*, 1999), which has been shown to have different dipsogenic responsiveness to osmotic or blood pressure challenges (Fregly *et al.*, 1990). There was a significant increase in body

weight at the end of the experiment among sham ligated animals that did not receive the ICV cannula (Table 2), and therefore we normalized all metabolic data to body weight. We did not find any significant differences in food intake, and we did not measure animal activity levels.

While animals normally regulate plasma osmolality during acute osmotic challenges through differential vasopressin release to alter renal free water clearance, drinking behaviour is another way that animals can regulate plasma osmolality. In the physiological context of progressively decreasing plasma osmolality associated with BDL cirrhosis, increased water intake, along with inappropriate vasopressin, could contribute to hyposmolality. Two circumventricular organs, the SFO and OVLT are involved in the central control of fluid intake related to changes in plasma Ang II (McKinley *et al.*, 2006; Johnson, 2007) and may participate in elevated water intake in BDL rats. However, the effect of Losartan on drinking behaviour may not be specific to SFO AT1Rs and therefore the precise regions and molecular mechanisms responsible for increased water intake in BDL remain to be determined.

From our analysis of AT1R expression in the lamina terminalis, we concluded that BDL is associated with an increased abundance of AT1aR mRNA and AT1R protein in the SFO of BDL rats compared to sham controls. These data are similar to other studies that showed increased abundance of AT1R in the SFO of animal models that had elevated peripheral renin-angiotensin system activation (Wei *et al.*, 2008; Wei *et al.*, 2009). Normally receptors down-regulate when presented with an abundance of ligand. However, in response to elevated circulating Ang II, AT1Rs have paradoxically increased in abundance. This phenomenon was first observed in studies of rats deprived of water (Hwang *et al.*, 1986; Nazarali *et al.*, 1987; Sanvitto *et al.*, 1997; Barth & Gerstberger, 1999) and spontaneous hypertension rats (Hwang *et al.*, 1986; Nazarali *et al.*, 1987; Sanvitto *et al.*, 1997; Barth & Gerstberger, 1999), but has since been confirmed in a rat model of congestive heart failure (Wei *et al.*, 2008; Wei *et al.*, 2009).

ICV administration of Ang II has been shown to potently stimulate drinking in rats, and the MnPO appears to be both necessary and sufficient for dipsogenic effect (Buggy & Johnson, 1978; Lind *et al.*, 1984; O'Neill & Brody, 1987). Other studies utilizing transgenic mice have shown that the increased production of SFO Ang II increased water intake (Sakai *et al.*, 2007), and that chronic brain-restricted overexpression of AT1R results in higher baseline water intake and an increased water intake response to exogenous ICV Ang II (Lazartigues *et al.*, 2008). Studies of spontaneously hypertension rats (Gutkind *et al.*, 1988; Pérez-Delgado *et al.*, 2000), rats deprived of water (Sanvitto *et al.*, 1997; Barth & Gerstberger, 1999), and rats with congestive heart failure (Wei *et al.*, 2008; Wei *et al.*, 2009) have also shown increased expression of AT1R in the SFO, which is associated with increased water intake where measured. Taken together, these data suggest an important role for central Ang II and especially AT1R in the regulation of water intake by SFO neurons projecting to the MnPO. Although the role of SFO angiotensin signalling in stimulating drinking behaviour has been well described, changes that occur in this system during cirrhosis are not well understood.

Western blot and RT-qPCR analysis of OVLT and MnPO AT1R protein and mRNA abundance failed to show consistent differences between sham and BDL groups indicating that increased expression of AT1R in BDL rats may be limited to the SFO. The primary antibody used for the Western blot analysis does not discriminate between AT1aR and the Angiotensin type 1b (AT1b) receptor subtype. Therefore, increases in AT1bR abundance may have contributed to our results. However, AT1aR mRNA in the SFO was increased. Therefore, it is likely that the increased AT1R protein abundance in the SFO reflects

increased expression of AT1aR. Both AT1a and AT1b receptor subtypes exhibit unique regulatory functions (Iwai *et al.*, 1992; Burson *et al.*, 1994; Llorens-Cortes *et al.*, 1994); however they also share 95% homology in mRNA and amino acid sequences, and have similar Ang II binding profiles (Chiu *et al.*, 1993). It is possible that the increased drinking behaviour we observed may have been due to increased activation of AT1b receptors. Davisson *et al.* (2000) showed that brain AT1b receptors are important mediators of drinking behaviour due to central administration of Ang II. However, it was also shown that central AT1aR overexpression increased both baseline and Ang II-induced drinking behaviour (Lazartigues *et al.*, 2008). Both of these transgenic studies were performed in mice, and it is possible that the drinking behaviour of rats may respond differently to AT1aR knockdown or silencing. Although the difference in AT1aR and AT1bR may be specific to rodents, future studies that selectively target AT1a and/or AT1b receptor subtypes in the SFO during BDL, such as with viral-mediated shRNA knockdown microinjected into the SFO (Hommel *et al.*, 2003; Chen *et al.*, 2006; Xue *et al.*, 2011) may be very beneficial in further defining its role in cardiovascular and hydromineral homeostasis in this model.

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1. What is the central question of this study?

Dilutional hyponatremia increases morbidity and mortality associated with heart or liver failure. Vasopressin release and increased fluid intake contribute to hypoosmolality but the underlying mechanisms are poorly understood. Our study tests the role of the central renin-angiotensin system in hypoosmolality in a rat model of liver failure.

2. What is the main finding and its importance?

Hepatic cirrhosis produced hypoosmolality with increased water intake and angiotensin receptors in the subforincal organ, a forebrain circumventricular organ. Central infusions of the angiotensin receptor blocker losartan normalized both water intake and the increase in angiotensin receptors. However, the rats remained hypoosmotic suggesting a selective role for the central renin-angiotensin system.

PERSPECTIVES

We tested the contribution of central angiotensin receptors to the changes in body fluid regulation produced by BDL. The results demonstrate that increased water intake associated with BDL was attenuated by chronic central infusions of losartan. Increased AT1R expression in the SFO was also blocked by ICV losartan. In contrast, ICV losartan did not produce an increase in urine output or raise plasma osmolality and hematocrit as would be expected if AVP release were significantly decreased. This suggests that the inhibition of central angiotensin receptors is not sufficient to reverse or delay the development of hypoosmolality produced by BDL. Nonetheless, these findings do help identify a potential role for SFO AT1Rs in excess drinking during liver failure although due to the route of losartan administration other regions may also contribute to this effect. Better understanding of how water intake and vasopressin release are regulated both osmotically and non-osmotically may influence treatment protocols for dilutional hyponatremia.

Figure 1.

Average daily water intake and urine output (A) and average daily sodium intake and renal sodium excretion (B) from Sham and BDL rats. Values are normalized to body weight (Kg) and represented as means $\pm$ SEM. *Statistically significant, $P < 0.014$ vs. Sham. Parentheses indicate the number of animals.

Figure 2. Western Blot analysis of AT₁R abundance (A) and RT-qPCR analysis of AT₁aR mRNA (B) from brain punches containing the SFO of Sham and BDL rats taken 28 days after BDL surgery. The data are presented as mean ± SEM relative protein and mRNA abundance. * Statistically significant, P<0.05. Parentheses indicate number of animals.

Figure 3. Western Blot analysis of AT₁R abundance (top) and RT-qPCR analysis of AT₁R mRNA (bottom) from brain punches containing the OVLT (A & B) and MnPO (C & D) of sham ligated and BDL rats taken 28 days after BDL surgery. The data are presented as mean ± SEM relative protein and mRNA abundance. *Statistically significant, P<0.05. Parentheses indicate number of animals.

Figure 4.

Average daily water intake and urine output normalized to body weight (Kg) from Sham and BDL rats treated with either vehicle (Veh), losartan (Los), or sub-cutaneous losartan (SCLos). Values are means $\pm$ SEM. *Statistically significant, $P < 0.01$ vs. all Sham groups. †Statistically significant, $P < 0.02$ vs. all BDL groups. Animal numbers are 8–10 for each group.

Figure 5. Average daily sodium intake and renal sodium excretion from Sham and BDL rats treated with either vehicle (Veh), losartan (Los), or sub-cutaneous losartan (SCLos). Values are means $\pm$ SEM. Animal numbers are 8–10 for each group.

Figure 6.

Western Blot analysis of AT1R abundance from brain punches containing the SFO of Sham and BDL rats from each treatment group taken 28 days after BDL surgery. * is statistically significant, $P < 0.05$ vs. all other ICV groups, # is statistically significant vs. sham ICV groups $P < 0.05$. Numbers within the bars indicate the number of animals.

Figure 7. Western Blot analysis of AT₁R expression in brain punches of the A) OVLT, and B) MnPO of Sham and BDL rats treated with either vehicle or losartan ICV taken 28 days after BDL surgery. Numbers within the bars indicate the number of animals.

Measurements of plasma osmolality, hematocrit, plasma protein, liver weight to body weight ratio, and final body weight from sham (Sham) and bile duct ligated (BDL) rats.

Table 1

	n	Osmolality (mOsm/kg)	Hematocrit (%)	Plasma Protein (g/dl)	Liver Wt/Body Wt	Final Body Weight (g)
Sham	11	302 ± 2	48 ± 1	7.3 ± 0.1	0.033 ± 0.003	470 ± 9
BDL	15	295 ± 1 *	44 ± 1 *	7.4 ± 0.1	0.071 ± 0.002 *	441 ± 11

Data are mean ± SEM.

* p<0.05 compared to sham ligation.

Table 2

Measurements of plasma osmolality, hematocrit, plasma protein, liver weight to body weight ratio and final body weight from sham (Sham) and bile duct ligated (BDL) rats that were treated with either vehicle ICV (Veh), losartan ICV (Los), or losartan subcutaneously (SCLos).

	n	Osmolality (mOsm/kg)	Hematocrit (%)	Plasma Protein (g/dl)	Liver Wt/Body Wt	Final Body Weight (g)
Sham Veh	10	303 ± 3	46 ± 0.8	7.4±0.17	0.029 ± 0.004	438.6 ± 10 [‡]
Sham Los	9	300 ± 2	47 ± 0.7	7.4±0.18	0.032 ± 0.004	425.3 ± 12 [‡]
Sham SCLos	10	304 ± 1	49 ± 0.5	7.7±0.17	0.032 ± 0.004	482.3 ± 8
BDL Veh	10	293 ± 2 [*]	41 ± 1.8 [*]	7.6±0.17	0.066 ± 0.004 [*]	407.9 ± 14 [‡]
BDL Los	9	292 ± 3.3 [*]	42 ± 0.9 [*]	7.7±0.18	0.068 ± 0.004 [*]	410.9 ± 8 [‡]
BDL SCLos	10	297 ± 1.0 [‡]	46 ± 1.0 [‡]	7.5±0.17	0.075 ± 0.004 [*]	426.3 ± 6 [‡]

Data are mean ± SEM.

* p<0.05 compared to all sham ligation;

[‡] p<0.05 compared to Sham SC Los.