

AT1 receptor role in the hypothalamic and renal function interaction

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Abstract

Angiotensin II (ANG II) is involved in the renal sodium homeostasis in close relation with sympathetic nervous system (SNS) under normal and pathological conditions. Vasopressin (AVP), a hormone that modulates renal sodium and water reabsorption is synthesized and released from supraoptic (SON) and paraventricular nucleus (PVN) under ANG II influence. We hypothesized that brain ANG II AT1 receptors (AT1-R) regulate renal sodium and water reabsorption and excretion through SNS. In this study male Wistar rats with renal denervation/sham and fed with hypersodic (4%) or normal (0.4%) diet were evaluated during 5 days in metabolic cages. On day 5 were injected in lateral ventricle with losartan (AT1-R antagonist) and sacrificed 12 hours later. The urine was daily collected, blood samples and brains were obtained for determinations. The parameters analyzed were: a) c-Fos immunoreactivity in SON, PVN, subfornical (SFO) and organum vasculosum of the lamina terminalis nucleus (OVLT), b) c-Fos-AVP immunoreactivity in SON in sham group, c) sodium and water intake, d) water, sodium and creatinine excretion. Results: c-Fos expression in SFO, OVLT and PVN was differentially affected by hypersodic diet or losartan depending on renal nerve integrity. In sham animals losartan prevented the hypersodic diet effects in water intake, c-Fos and AVP positive neurons in SON. Renal denervation modified the effect of hypersodic diet in water intake, urinary volume and creatinine excretion; losartan prevented these alterations. Food intake was similar in all groups. Our results suggest that brain AT1-R regulate renal sodium and water reabsorption through SNS in close interaction with AVP.

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Abstract

Angiotensin II (ANG II) is involved in the renal sodium homeostasis in close relation with sympathetic nervous system (SNS) under normal and pathological conditions. Vasopressin (AVP), a hormone that modulates renal sodium and water reabsorption is synthesized and released from supraoptic (SON) and paraventricular nucleus (PVN) under ANG II influence. We hypothesized that brain ANG II AT₁ receptors (AT₁-R) regulate renal sodium and water reabsorption and excretion through SNS. In this study male Wistar rats with renal denervation/sham and fed with hypersodic (4%) or normal (0.4%) diet were evaluated during 5 days in metabolic cages. On day 5 were injected in lateral ventricle with losartan (AT₁-R antagonist) and sacrificed 12 hours later. The urine was daily collected, blood samples and brains were obtained for determinations. The parameters analyzed were: a) c-Fos immunoreactivity in SON, PVN, subfornical (SFO) and organum vasculosum of the lamina terminalis nucleus (OVLT), b) c-Fos-AVP immunoreactivity in SON in sham group, c) sodium and water intake, d) water, sodium and creatinine excretion.

Results: c-Fos expression in SFO, OVLT and PVN was differentially affected by hypersodic diet or losartan depending on renal nerve integrity. In sham animals losartan prevented the hypersodic diet effects in water intake, c-Fos and AVP positive neurons in SON. Renal denervation modified the effect of hypersodic diet in water intake, urinary volume and creatinine excretion; losartan prevented these alterations. Food intake was similar in all groups.

Our results suggest that brain AT₁-R regulate renal sodium and water reabsorption through SNS in close interaction with AVP.

Keywords: renal denervation, kidney, vasopressin, Losartan, sodium intake

Introduction

The kidney plays a key role in the hydroelectrolytic equilibrium maintaining homeostasis through water and electrolytes excretion and absorption that is also regulated by the intake. These basic functions have been an osmotic challenge along the evolution in the animals' adaptation to terrestrial life. The kidney can modify the sodium excretion in response to intake changes. It has been found that sodium intake can increase or decrease more than ten times from normal levels, having relatively little changes in extracellular fluid volume. The same phenomenon was observed with water and electrolytes (chlorine, potassium, magnesium, phosphate regulation). Moreover, the kidney exerts a key role in long term blood pressure regulation through sodium and water excretion.

The cardiovascular and hydrosaline homeostasis maintenance requires a fine coordination among neuroendocrine and autonomic systems. The central nervous system (CNS) receives information from multiple sensors (osmolarity, pH, pressure, blood volume and circulating hormones) and integrates the signals generating adaptive responses (Johnson et al., 1992).

The kidney is the target for neurohypophyseal hormones that control water and sodium excretion. Oxytocin and vasopressin (AVP) are released in response to hypovolemia or hyperosmolarity (Mendelsohn et al., 1990, Bourque, 2008). AVP preserves water volume controlling water excretion and the increase in water intake that restores volume fluids. In addition to its neuroendocrine actions AVP induces fast and enduring increase in the SNS activity (Stocker et al., 2006, Shi et al., 2007).

The anatomic and physiological evidences show that sympathetic nerves innervate the juxtaglomerular cells, renal tubules and vasculature (DiBona, 2000). Thus, the renal sympathetic nerve activation (RSNA) frequency mediates the increase in sodium and water excretion regulating tubular renal water and sodium reabsorption in the nephron, promoting changes in renal function, blood flow and glomerular filtration rate. This system also regulates the renal vasculature vasoconstriction and changes in renin angiotensin system through renin release from juxtaglomerular cells (DiBona, 2005, Pontes et al., 2015).

The hydromineral and osmotic regulation depend on AVP and oxytocin released from SON and PVN and they play a key role in sodium and water excretion. The osmotic sensors are in the subfornical (SFO) and organum vasculosum of the lamina terminalis (OVLT), placed outside the blood brain barrier. Moreover, there are direct and indirect interactions between SNS and CNS through specific innervation or mediated by angiotensin II (ANG II) that regulate the renal sympathetic activity. The evidences show that the increase in the RSNA can be blunted with ACE inhibitors or AT₁-R antagonists. In the same way the effects of ANG II intrarenal administration are decreased after renal denervation. The interactions are modulated by changes in the renin angiotensin system activation that control RSNA and its arterial baroreflex (Kopp et al., 2008). ANG II is generated at renal level and its concentration is higher than in plasma (Braam et al., 1993, Boer et al., 1997). Renal chemoreceptors and mechanoreceptors send information to CNS that is integrated in SFO, PVN, solitary tract nucleus (NTS), noradrenergic cells group A5 or rostral ventrolateral medulla nucleus (RVLM) level (Cao et al., 2015).

In addition to the effects induced by circulating ANG II on circumventricular organs, inside the blood brain barrier the effects are mediated by local ANG II. It has been found that central administration of AT₁-R antagonist is able to modulate changes in brain ANG II activity induced by physiological alterations in sodium intake. This modulation was also observed in a physiopathological model (congestive cardiac insufficiency) where there is an increase in brain ANG II and SNS activity. Intrarenal and extra renal interactions between renin-angiotensin system and RSNA are involved in the neural control of the renal function (DiBona, 2000). The renal sympathetic nerves innervate tubules, vessels and juxtaglomerular granular cells from kidney (Johns et al., 2011). For this reason, changes in RSNA directly influence the functions of these renal components. ANG II through AT₁-R placed in kidney tubules and vessels exert direct actions on sodium, chlorine and water reabsorption as well as in vascular constriction.

Considering the evidence presented above, we aimed to evaluate the brain AT₁-R role in renal function mediated by its interaction with SNS under two independent conditions: sham or renal denervation. The hypersodic diet was used to inhibit peripheral ANG II while it stimulates brain ANG II. This condition is able to unmask the brain AT₁-R influence over SNS under surgical renal denervation (Ye et al., 2002).

Experimental procedures

Animals

Male Wistar rats 250-300g were used. The animals were maintained under controlled environmental conditions (20–24 °C, 12h light/dark cycle with lights on at 7 a.m.) with ad libitum access to food and water and were randomly housed in groups of 5 per cage (34 x 48 x 19). All procedures were approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina (Res. No. 270/18), in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental design

It was performed a 6-day protocol with animals receiving normal sodium (0.4%) or high sodium (4%) diet. Stereotaxic surgery was performed on day 1, Los was administered on day 5 in the right and left lateral ventricles, and 12 hours after Los administration, plasma, kidney, and SNC samples were taken (Figure 1).

Drugs

The selective AT₁-R antagonist, losartan (Sigma Aldrich) was dissolved in artificial cerebrospinal fluid (ACF). The solution was freshly used protected from light and kept at 4°C. The dose (4µg/µl) was selected considering

previous studies (McKinley et al., 1994, Mathai et al., 1997, Mathai et al., 1998).

Surgery for cannulae implantation and denervation

Animals were anesthetized i.p. with ketamine (75 mg/kg, Holliday)/xylazine (5 mg/kg, Köing). In aseptic conditions, rats' skull were exposed and using a stereotaxic device were implanted with bilateral stainless-steel cannula (23 gauge) fixed with dental cement (Subiton, Argentina). Also one stainless-steel screw was anchored to the skull. Cannulae were placed 2 mm above the final place of injection. According to Paxinos & Watson Atlas (Paxinos and Watson, 2009) coordinates for lateral ventricle in respect to bregma were: AP = -0,92 mm, L = +/- 1,5 mm, DV = -6.8 mm. The cannulae were implanted in the right and left side of the lateral ventricle.

In the anesthetized animals the surgical denervation was made under magnifier, firstly the right kidney was exteriorized and the lipid and connective tissue from vessels at renal pelvis level was separated and the nerve was dissected leaving intact the renal artery and vein. To make more accurate the denervation phenol 10% (neurolytic) was applied. The kidney was introduced and the same procedure was performed in the left kidney. Finally the surgery was finished with muscle and skin suture (King et al., 2007, Foss et al., 2013, Hong et al., 2016, Li et al., 2016).

The sham animals were submitted to the same procedure until renal nerve visualization for each kidney,

The renal denervation was verified in kidney slices with hematoxylin-eosin stain (Figure 2). Moreover, renal denervation did not affect the food, sodium and water intake (Figure 3).

Losartan Cerebral microinjection

The animals were placed in metabolic cages from day 1 and were bilaterally administered on day 5 with losartan (4µg/1µl) (Los) or artificial cerebral fluid (ACF) inserting a stainless steel injection cannula (30 gauges) into the guide cannula. The cannula was attached through a polyethylene catheter (P20) to a 25 µl microsyringe (Hamilton). Volumes of 1 µl of ACF or Los solution were gradually injected over 1 min period into left and right sides using an infusion bomb (HARVARD, model 22). The injection cannula was left in place for an additional 20 s to allow complete liquid diffusion (McKinley et al., 1994, Fitts et al., 2005, Llano Lopez et al., 2012).

Urine Collection and blood samples

The daily urine production was collected and registered and 12hs urine production was collected after LOS/ACF injection and centrifuge at 10000 G for 10 min and the supernatant kept at -20 °C until analysis.

At the end of the experiment blood samples were taken from heart left ventricle in anesthetized animals. The samples were centrifuge at 3000 rpm during 5 min and kept at -80 °C until analysis (Figure 1).

Biochemical determinations in urine and plasma

The urinary volume was registered and the parameters creatinine, osmolarity, sodium and potassium were determined. The urine samples were diluted in water 1:20 and analyzed for creatinine using Jaffe modified reaction under spectrometry at 510 nm. The osmolarity was measured by an osmometer 3.300 ADVANCE (System) and the ions using a selective electrode

The parameters analyzed in the plasma were: creatinine, osmolarity, sodium potassium and chlorine. Creatinine was measured using a modified Jaffe reaction. Briefly, plasma samples (0.5 ml) were mixed with trichloroacetic acid 5 % in equal volume and centrifuged at 5000 rpm during 10 min, the osmolarity was measured by an osmometer 3.300 ADVANCE (System) and the ions using a selective electrode.

Immunostaining procedure

Twelve hours after Los/ACF administration (Figure 1) the animals were anaesthetized with Urethane 50% (100mg/kg) and transcardially perfused with 200ml of 0.9% saline and heparin (200µl/l), followed by 200ml of 4% paraformaldehyde in 0.1M Phosphate-Buffer (PB, pH 7.4). After removal, the brains were stored at 4°C in

30% sucrose solution. Coronal sections of 40µm were cut using a freezing microtome (Leica CM1510S) and collected in 0.01M Phosphate-Buffer-Saline (PBS, pH 7.4). They were placed in a mixture of 10% H₂O₂ and 10% methanol, washed three times with PBS 0.01 M and then in 10% Normal Goat Serum (NGS; Natocor, Córdoba, Argentina) in 0.1M PBS, for 2h each one. The free-floating slices were incubated overnight, at room temperature in rabbit anti-c-Fos antibody (1:3000; Abcam) in primary antibody solution (2% NGS and 0.3% Triton X-100 (Fluka Analytical) in 0.1M PBS). The following day, the slices were incubated in Biotin-labelled anti-rabbit secondary antibody (1:1000; Vector Laboratories) and Avidin-Biotin-peroxidase Complex (ABC; 1:500; Vector Laboratories) for 2h each one, at room temperature. The peroxidase label was detected with diaminobenzidine hydrochloride (0.5mg/ml, Sigma-Aldrich) and hydrogen peroxide; the solution was intensified with 1% cobalt chloride and 1% nickel ammonium sulphate. This method produces a violet nuclear reaction product (Franchini and Vivas, 1999, Franchini et al., 2002). Also, the series of c-Fos labelled sections containing SON were processed for Arginine vasopressin (AVP) immunohistochemically localization. They were incubated overnight, at room temperature with polyclonal rabbit anti-AVP antibody (1:3000; PS 41 Vasopressin-NP; Rockville, MD, USA) in primary antibody solution. After incubation, the sections were rinsed and incubated with biotin-labelled anti-mouse secondary antibody (1:3000 Jackson Immune Research) and ABC for 2 h each one, at room temperature. Cytoplasmic vasopressin immunoreactivity (AVP-IR) was detected with diaminobenzidine hydrochloride which produces a brown reaction product. Finally, they were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DPX mounting medium (Fluka Analytical).

Cytoarchitectural and quantitative analysis

Images containing Fos-IR nuclei and Fos-AVP-IR were obtained using a computerized system that included a Leica DM 4000B microscope equipped with a DFC Leica digital camera attached to a contrast enhancement device. The brain nuclei evidencing Fos-IR were identified and delimited according to the atlas of Paxinos and Watson (Paxinos and Watson, 2009). The numbers of Fos-IR nuclear profiles in the sections were counted a tone level; the distance from the bregma of the corresponding plates is as follows: for dorsomedial region of PVN= -0.92 mm, SON= -1.30 mm, SFO and OVLT= -1.40 mm. The number of Fos-AVP-IR neurons were counted in SON= -1.3 mm. The image analysis was accomplished by using IMAGE J software from the National Institutes of Health (NIH). Threshold was fixed between intervals of 0–150 in black and white conditions; all higher values were considered background. Fos-IR neurons were identified by dense black nucleus and counted by setting a size range for cellular nuclei (8 to 12 µm of diameter) and AVP-IR neurons were identified by dense brown staining of the cytoplasm. To count of Fos-AVP-IR cells we performed manually. The final value for each brain area is the average of 4 images (bilaterally, in two sections). The counting was made on a 0.37 mm² area (corresponding to 200x magnification). Since the size and section thickness of nuclei did not change between experimental and control groups, any systematic error could be identical for all groups. The counting was made by two operators, on each section analyzed, to ensure that the number of profiles obtained was similar, but only one counting was used. Counting of Fos-IR and Fos-AVP-IR cells was performed blinded to the observer.

Statistical analysis

The experiments were designed to evaluate the brain AT₁-R role in renal function through SNS under two independent conditions: sham or renal denervation. The data were reported as mean ± SEM and analyzed using two-way ANOVA considering the following factors: diet (normosodic/hypersodic) and treatment (losartan/vehicle) followed by post hoc analysis Student-Newman Keuls in each condition. A value of p < 0.05 was considered significant. The analyses were performed by using GraphPad Prism® 6.03 software.

Results

Hypersodic diet altered Fos expression depending on renal nerve integrity. AT₁-R are involved in the neuronal activation in SON

SON : the hypersodic diet increased the number of Fos-IR cells and Los blunted this increase in SHAM animals. Diet factor $F_{(1,9)} = 106.6$ p < 0.0001; treatment factor $F_{(1,9)} = 76.35$ p < 0.0001; interaction $F_{(1,9)}$

= 84.20 $p < 0.0001$. Although, in renal denervated animals (RDN) the diet did not affect Fos expression, however, Los decrease the number of Fos-IR cells. Diet factor $F_{(1,14)} = 0.11$ $p = \text{NS}$; treatment factor $F_{(1,14)} = 4.81$ $p < 0.05$; interaction $F_{(1,14)} = 0.74$ $p = \text{NS}$ (**Figure 4, left panel**).

PVN : in sham animals no statistical differences were found in Fos expression in diet factor, treatment factor or interaction between both factors. Diet factor $F_{(1,24)} = 0.04$ $p = \text{NS}$; treatment factor $F_{(1,24)} = 0.001$ $p = \text{NS}$; interaction $F_{(1,24)} = 0.30$ $p = \text{NS}$. In RDN group Los decreased the number of Fos-IR cells in animals with hypersodic diet. Diet factor $F_{(1,14)} = 0.71$ $p = \text{NS}$; treatment factor $F_{(1,14)} = 5.63$ $p < 0.05$; interaction $F_{(1,14)} = 1.34$ $p = \text{NS}$ (**Figure 4, right panel**).

SFO : the diet or treatment did not affected Fos expression in sham animals. Diet factor $F_{(1,25)} = 0.33$ $p = \text{NS}$; treatment factor $F_{(1,25)} = 0.19$ $p = \text{NS}$; interaction $F_{(1,25)} = 0.01$ $p = \text{NS}$. Although, in RDN animals the hypersodic diet induced an increase in the number of Fos-IR cells and Los administration did not affected it. Diet factor $F_{(1,14)} = 7.54$ $p < 0.05$; treatment factor $F_{(1,14)} = 0.01$ $p = \text{NS}$; interaction $F_{(1,14)} = 0.12$ $p = \text{NS}$ (**Figure 5, left panel**).

OVLT : in sham animals neither the diet nor treatment affected Fos expression. Diet factor $F_{(1,23)} = 0.44$ $p = \text{NS}$; treatment factor $F_{(1,23)} = 0.001$ $p = \text{NS}$; interaction $F_{(1,23)} = 0.34$ $p = \text{NS}$. However, the hypersodic diet decreased the number of Fos-IR cells and Los did not affect it. Diet factor $F_{(1,15)} = 9.30$ $p < 0.05$; treatment factor $F_{(1,15)} = 0.32$ $p = \text{NS}$; interaction $F_{(1,15)} = 0.41$ $p = \text{NS}$ (**Figure 5, right panel**).

AT₁-R are involved in hypersodic diet-induced increase in vasopressin neurons activation in sham group

SON : hypersodic diet induced an increase in the number of Fos-AVP-IR cells and Los administration prevented it. Diet factor $F_{(1,4)} = 15.37$ $p < 0.05$; treatment factor $F_{(1,4)} = 6.94$ $p = \text{NS}$; interaction $F_{(1,4)} = 23.22$ $p < 0.01$ (**Figure 6**).

Water, food, sodium and potassium intake

As expected the hypersodic diet increased the water intake in sham animals and Los prevented it. Diet factor $F_{(1,27)} = 6.03$ $p < 0.05$; treatment factor $F_{(1,27)} = 0.01$ $p = \text{NS}$; interaction $F_{(1,27)} = 6.08$ $p < 0.05$. In RDN group the hypersodic diet increased the water intake in the group treated with LOS. Diet factor $F_{(1,20)} = 7.1$ $p < 0.05$; treatment factor $F_{(1,20)} = 0.27$ $p = \text{NS}$; interaction $F_{(1,20)} = 2.26$ $p = \text{NS}$ (**Figure 7**).

Food intake expressed as 100g per animal weight was similar in all groups (**Table I**). Sham group: diet factor $F_{(1,27)} = 0.07$ $p = \text{NS}$; treatment factor $F_{(1,27)} = 0.008$ $p = \text{NS}$; interaction $F_{(1,27)} = 3.85$ $p = \text{NS}$ (**Table I**). RDN group: diet factor $F_{(1,20)} = 7.09$ $p = 0.01$; treatment factor $F_{(1,20)} = 0.27$ $p = \text{NS}$; interaction $F_{(1,20)} = 0.14$ $p = \text{NS}$ (**Table I**).

Sodium intake was high in animals exposed to hypersodic diet in sham and RDN groups and Los did not modify it. Sham group: diet factor $F_{(1,27)} = 201.9$ $p < 0.0001$; treatment factor $F_{(1,27)} = 0.20$ $p = \text{NS}$; interaction $F_{(1,27)} = 0.54$ $p = \text{NS}$ (**Table I**). RDN group: diet factor $F_{(1,20)} = 99.77$ $p < 0.0001$; treatment factor $F_{(1,20)} = 0.02$ $p = \text{NS}$; interaction $F_{(1,20)} = 0.08$ $p = \text{NS}$ (**Table I**).

No differences were found in potassium intake induced by diet or treatment either sham or RDN group Sham group: diet factor $F_{(1,27)} = 0.27$ $p = \text{NS}$; treatment factor $F_{(1,27)} = 0.27$ $p = \text{NS}$; interaction $F_{(1,27)} = 1.64$ $p = \text{NS}$ (**Table I**). RDN group: diet factor $F_{(1,20)} = 0.06$ $p = \text{NS}$; treatment factor $F_{(1,20)} = 0.06$ $p = \text{NS}$; interaction $F_{(1,20)} = 0.24$ $p = \text{NS}$ (**Table I**).

Osmolarity, sodium, creatinine, chlorine and potassium plasma levels

No differences were found in these parameters by diet or treatment either sham or RDN group.

Plasma osmolarity; Sham group: diet factor $F_{(1,27)} = 0.73$ $p = \text{NS}$; treatment factor $F_{(1,27)} = 0.54$ $p = \text{NS}$; interaction $F_{(1,27)} = 1.14$ $p = \text{NS}$ (**Table II**). RDN group: diet factor $F_{(1,20)} = 0.00012$ $p = \text{NS}$; treatment factor $F_{(1,20)} = 0.10$ $p = \text{NS}$; interaction $F_{(1,20)} = 0.49$ $p = \text{NS}$ (**Table II**).

Plasma sodium, Sham group: diet factor $F_{(1,27)} = 1,07$ p=NS; treatment factor $F_{(1,27)} = 0,76$ p= NS; interaction $F_{(1,27)} = 0,22$ p = NS (**Table II**). RDN group: diet factor $F_{(1,20)} = 0,17$ p=NS; treatment factor $F_{(1,20)} = 1,63$ p = NS; interaction $F_{(1,20)} = 2,45$ p = NS (**Table II**).

Plasma creatinine; Sham group: diet factor $F_{(1,27)} = 0,028$ p=NS; treatment factor $F_{(1,27)} = 1,62$ p= NS; interaction $F_{(1,27)} = 0,11$ p = NS (**Table II**). RDN group: diet factor $F_{(1,20)} = 0,41$ p=NS; treatment factor $F_{(1,20)} = 0,001$ p = NS; interaction $F_{(1,20)} = 2,10$ p = NS (**Table II**).

Plasma chlorine, Sham group: diet factor $F_{(1,27)} = 2,16$ p=NS; treatment factor $F_{(1,27)} = 0,50$ p= NS; interaction $F_{(1,27)} = 0,01$ p = NS (**Table II**). RDN group: diet factor $F_{(1,20)} = 0,0009$ p=NS; treatment factor $F_{(1,20)} = 0,58$ p = NS; interaction $F_{(1,20)} = 0,44$ p = NS (**Table II**).

Plasma potassium, Sham group: diet factor $F_{(1,27)} = 0,76$ p=NS; treatment factor $F_{(1,27)} = 2,98$ p= NS; interaction $F_{(1,27)} = 6,41$ p = NS (**Table II**). RDN group: diet factor $F_{(1,20)} = 0,0002$ p=NS; treatment factor $F_{(1,20)} = 0,75$ p = NS; interaction $F_{(1,20)} = 0,83$ p = NS (**Table II**).

Urinary volume and sodium excretion

Regarding urinary volume, the increase induced by the hypersodic diet was blunted with Los in sham group. Diet factor $F_{(1,27)} = 36,83$ p < 0,0001; treatment factor $F_{(1,27)} = 24,16$ p < 0,0001; interaction $F_{(1,27)} = 2,00$ p = NS. In RDN group the hypersodic diet increased the urinary volume in animals administered with LOS. Diet factor $F_{(1,16)} = 7,23$ p < 0,05; treatment factor $F_{(1,16)} = 2,66$ p = NS; interaction $F_{(1,16)} = 3,97$ p = NS (**Figure 7**).

In sham group with hypersodic diet the sodium excretion was significantly high and Los did not affect it. Diet factor $F_{(1,27)} = 35,42$, p < 0,0001; treatment factor $F_{(1,27)} = 0,34$ p = NS; interaction $F_{(1,27)} = 0,55$ p = NS. The same phenomenon was observed in RDN group. Diet factor $F_{(1,16)} = 15,31$ p < 0,001; treatment factor $F_{(1,16)} = 1,35$ p = NS; interaction $F_{(1,16)} = 1,39$ p = NS (**Figure 7**).

In sham group the hypersodic diet induced an increase in sodium fractional excretion and Los did not affect it. Diet factor $F_{(1,27)} = 36,83$ p < 0,0001; treatment factor $F_{(1,27)} = 0,24$ p = NS; interaction $F_{(1,27)} = 0,99$ p = NS. In RDN group the hypersodic diet increased sodium fractional excretion in animals administered with LOS. Diet factor $F_{(1,20)} = 19,34$ p < 0,005; treatment factor $F_{(1,20)} = 2,23$ p = NS; interaction $F_{(1,20)} = 3,93$ p = NS (**Figure 7**).

Urinary osmolarity and creatinine excretion

In sham group the hypersodic diet induce an increase in osmolarity and Los administration prevented this response. Diet factor $F_{(1,27)} = 10,10$ p < 0,005; treatment factor $F_{(1,27)} = 1,65$ p = NS; interaction $F_{(1,27)} = 0,49$ p = NS. In RDN group no differences were found by diet or treatment. Diet factor $F_{(1,20)} = 2,55$ p = NS; treatment factor $F_{(1,20)} = 0,25$ p = NS; interaction $F_{(1,20)} = 2,19$ p = NS (**Table III**).

Regarding creatinine excretion the hypersodic diet induce a decrease that was prevented by Los administration, in sham group. Diet factor $F_{(1,27)} = 15,77$ p < 0,001; treatment factor $F_{(1,27)} = 0,67$ p = NS; interaction $F_{(1,27)} = 0,77$ p = NS (**Table III**). However, in RDN group the hypersodic diet decreased creatinine excretion only in the animals administered with Los. Diet factor $F_{(1,20)} = 4,32$ p < 0,05; treatment factor $F_{(1,20)} = 0,01$ p = NS; interaction $F_{(1,20)} = 3,76$ p = NS (**Table III**).

Potassium excretion

Regarding potassium excretion in sham group the differences did not reach statistical significance. Diet factor $F_{(1,27)} = 0,15$ p = NS; treatment factor $F_{(1,27)} = 0,74$ p = NS; interaction $F_{(1,27)} = 2,85$ p = NS (**Table III**). In RDN group no differences were found by diet or treatment (**Table III**).

Urinary creatinine, osmolar and free water clearance

No differences were found in creatinine clearance by diet or treatment either sham or RDN group (**Table III**). Sham group: diet factor $F_{(1,27)} = 0,13$ p=NS; treatment factor $F_{(1,27)} = 0,29$ p= NS; interaction $F_{(1,27)}$

= 0,06 p = NS (**Table III**). RDN group: diet factor $F_{(1,20)} = 0,10$ p=NS; treatment factor $F_{(1,20)} = 0,09$ p = NS; interaction $F_{(1,20)} = 2,14$ p = NS (**Table III**).

Regarding osmolar clearance in sham group, the hypersodic diet induced an increase that was blunted by Los administration. Diet factor $F_{(1,27)} = 11,52$ p < 0,005; treatment factor $F_{(1,27)} = 0,05$ p = NS; interaction $F_{(1,27)} = 1,01$ p = NS (**Table III**). In RDN group no differences were found in creatinine clearance by diet or Los administration (**Table III**). Diet factor $F_{(1,20)} = 3,42$ p = NS; treatment factor $F_{(1,20)} = 0,04$ p = NS; interaction $F_{(1,20)} = 0,17$ p = NS (**Table III**).

In sham group free water clearance reached no statistical differences in response to diet or Los administration. Diet factor $F_{(1,27)} = 2,83$ p = NS; treatment factor $F_{(1,27)} = 0,07$ p = NS; interaction $F_{(1,27)} = 0,19$ p = NS (**Table III**). Similar results were observed in RDN group. Diet factor $F_{(1,20)} = 0,16$ p = NS; treatment factor $F_{(1,20)} = 1,48$ p = NS; interaction $F_{(1,20)} = 4,12$ p = NS (**Table III**).

Discussion

The main finding of this study is the ANG II central action through AT₁-R conforming a complex interaction system between CNS and SNS in the renal function control. There is considerable evidence supporting the fact that RAS components are present in the tissue and plasma, and they can act independently from each other or linked through regulatory pathways (Mendelsohn et al., 1984, Saavedra, 1992, Phillips and Summers, 1998, Zhuo et al., 1998).

The SNS involvement in the renal sodium equilibrium maintenance under normal and pathological conditions is modulated by ANG II actions over AVP. Moreover, it has been shown that Los reduces RAS and SNS activity. Renal denervation is a delicate and specific surgical approach, very useful since it allows recreating similar conditions to renal transplanted individuals.

In the present study we perform the experiments six days after renal denervation, based on the fact that in this time period the anatomical-physiological renal nerve interruption persist, allowing the renal function evaluation without sympathetic influence (King et al., 2007, Foss et al., 2013, Hong et al., 2016, Li et al., 2016). The applied experimental protocol using renal denervation and central Los administration evidenced the ANG II role at brain level, independently from its functions through SNS modulation.

Hypersodic diet induced-neuronal activation

The available evidence shows that brain ANG II -through AT₁-R- is involved in blood pressure control, SNS stimulation, AVP release and water and sodium intake (Saavedra, 1992). It has been described the presence of AT₁-R in PVN, SON, OVLT and SFO brain areas involved in arterial blood pressure control and hidroelectrolytic homeostasis (Saavedra, 1992, Vivas et al., 2014).

The marked increase in the number of Fos-IR neurons in the SON from intact animals was blunted by Los administration, suggesting that AT₁-R mediates the neuronal activation induced by a moderated hypersodic diet (McKinley et al., 2001). The renal denervation was able to avoid the described increase in neuronal activation induced by the hypersodic diet, evidencing the close interaction between SON vasopressinergic neurons and SNS activity. These constitute the essential circuit loop SON-SNS-kidney, being the principal components involved in the sodium overload neuroregulation in this experimental paradigm (Unger et al., 1989, Sakai et al., 2007).

The PVN physiological relevance appears to be lower than SON since the neuronal activity was not affected by the hypersodic diet. It is important to highlight the great cell heterogeneity in PVN in contrast to SON. In this sense, PVN is a highly complex nucleus where CRH, GNRH, AVP and oxytocin synthesis take place and receives inputs from several brain regions (Sofroniew, 1983, Stern, 2015). Meanwhile, SON presents less complexity where the neurons that synthesize oxytocin and AVP are bigger and easily localized and identified (Brown et al., 2013).

The SNS activity interruption induced a marked decrease in SFO neuronal activity, indicating a basal stimulatory tone in this nucleus that is not observed in OVLT. Nevertheless, in both nuclei, the sodium

overload alters the neuronal activation under renal denervation conditions, evidencing a differential regulatory role from SNS on the neural components of these nucleuses (Sakai et al., 2007). SFO and OVLT exert key functions in the extracellular sodium concentrations, since they are related to sodium appetite and are sensitive to ANG II (Vivas et al., 1990, Franchini and Vivas, 1999, Antunes-Rodrigues et al., 2004, Sakai et al., 2007).

Renal effects: Water and sodium balance

The variations in sodium excretion affect the extracellular volume activating complex response mechanisms that reestablish the hydrosaline homeostasis.

It is known that AVP and oxytocin play a key role in the osmoregulation through natriuresis and diuresis (Unger et al., 1989). The anatomo-physiological evidences show that the sympathetic nerves regulate changes in the urinary sodium and water excretion through the tubular reabsorption at the nephron level. The collector tubules are AVP target and its release is under brain ANG II regulation through AT₁-R (Pontes et al., 2015). In this sense, the renal denervation allows to unmask the central actions of these receptors and their interaction with SNS in the renal sodium regulation.

Our results show that the increase in water intake induced by hypersodic diet is mediated by brain AT₁-R, since this increase was prevented by Los administration. Moreover, this regulation involves the SNS considering that renal denervation was able to avoid the increase in water intake induced by the hypersodic diet and Los administration reestablished this response (Cao et al., 2015). A similar phenomenon was observed in the urinary volume increase induced by hypersodic diet in intact animals. This increase was abolished by renal denervation and reestablished by AT₁-R blockade. These results not only confirm the existence but also the critical regulatory function of the SNS-brain ANG II circuit loop.

Regarding daily food intake we did not observed differences between diets, meaning that sodium did not modified palatability. In the same way renal denervation or Los administration did not affect food intake. Although, water intake was increased by hypersodic diet as expected (Franchini et al., 2002, Daniels et al., 2009, Cao et al., 2015). Our experimental design allowed independent evaluating of water and sodium intake, representing an advantage over protocols where the sodium is administered through drinking water.

In accordance with other authors, we found that 6 days of hypersodic diet (4%) did not affect plasmatic sodium, creatinine and osmolarity (Jacob et al., 2003, Nishi et al., 2015). Moreover, it has been described that this experimental protocol did not increase blood pressure (Jacob et al., 2003, Foss et al., 2013, Gasparini et al., 2019). Although, renal denervation diminished sodium excretion modifying the sodium balance, while water balance remained unaffected. In relation to potassium, differences in excretion and balance were not observed.

The decreased urinary osmolarity and creatinine excretion concomitant with increased urinary volume induced by hypersodic diet revealed the AVP role over renal function. This last is also supported by the effects induced by renal denervation. Moreover, the ANG II role over AVP become evident since the AT₁-R antagonist restores the AVP actions at renal level. Considering altogether, it is possible to suggest that the hypersodic diet could promote AVP release as a consequence of ANG II acting on AT₁-R.

Declarations

Author statement

Celia Ruberto: Methodology, Investigation, Writing

Victoria B Occhieppo: Methodology, Investigation.

Claudia Bregonzio: Writing - Review & Editing Funding acquisition

Gustavo Baiardi: Supervision, Formal analysis, Project administration, Funding acquisition, Writing - Review & Editing

Declarations of interest

None

Compliance with Ethical Standards

All procedures were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and the EU (Eighth Edition, 2011) and approved by the Animal Care and Use Committee, School of Chemical Sciences (Res HCD n° 46/15 and 270/18), National University of Cordoba. Experiments were made to minimize the number of animals used and their suffering.

Data Accessibility Statement

The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included in the paper.

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Table I. Intake parameters	Table I. Intake parameters	Table I. Intake parameters	Table I. Intake parameters	Table I. Intake parameters
	SHAM	SHAM	SHAM	SHAM
	NSD-VEH	HSD-VEH	NSD-Los	NSD-Los
Body Weight (g, bw)	264.3±9.2	264.3±9.2	260.0±6.7	260.0±6.7
Food Intake (g/100gbw)	2.43±0.28	2.94±0.26	2.90±0.20	2.90±0.20
Na ⁺ (mEq)	0.38±0.03	4.09±0.45*	0.45±0.04	3.81±0.45*
K ⁺ (mEq)	2.29±0.18	2.46±0.27	2.71±0.25	2.71±0.25

Table II. Plasma parameters	Table II. Plasma parameters	Table II. Plasma parameters	Table II. Plasma parameters
	SHAM	SHAM	SHAM
	NSD-VEH	HSD-VEH	NSD-Los
Osmolarity (mOsm/Kg)	370.1±18.4	333.1± 16.6	335.5±22.0
Na ⁺ (mmol/L)	136.7±3.3	135.3±1.7	135.7±2.2
Creatinine (mg/dL)	0.21±0.02	0.24±0.04	0.29±0.06
Cl ⁻ (mmol/L)	95.1±2.3	92.0±1.4	93.5±2.0
K ⁺ (mmol/L)	7.2±0.7	5.7±0.2	6.9±0.3

Table III. *Urinary parameters*

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	SHAM NSD-VEH	SHAM HSD-VEH	SHAM NSD-Los	SHAM HSD-Los	RDN NSD-VEH	RDN HSD-VEH
Osmolarity (mOsm/Kg)	1867.7±243.1	1014.0±181.0*	1429.7±236.3	886.6±153.2	1496.3±199.1	1467.7±199.1
Na ⁺ (mmol/L)	0.6±0.1	5.2±1.2*	0.7±0.1	4.3±1.0*	0.7±0.2	2.5±0.7*
Creatinine (mg/dL)	50.8±7.0	18.1±5.6*	39.3±7.9	18.5±2.7	54.2±9.5	52.1±12.1
Cl ⁻ (mmol/L)	0.9±0.2	5.8±1.3	1.0±0.2	4.7±1.2	0.9±0.2	3.5±1.1
K ⁺ (mmol/L)	1.0±0.2	1.9±0.4	1.4±0.3	1.3±0.1	1.1±0.2	1.2±0.3
Creatinine clearance	0.50±0.09	0.57±0.07	0.60±0.14	0.61±0.09	0.47±0.11	0.77±0.21
Osmolar clearance	0.03±0.00	0.08±0.02*	0.04±0.01	0.06±0.01*	0.02±0.00	0.04±0.00
Free water clearance	-0.02±0.00	-0.05±0.02	-0.03±0.01	-0.04±0.01	-0.02±0.00	-0.03±0.00

Table legends

Table I: Intake the food, sodium and potassium, parameters following intracerebroventricular injection (ICV) of Losartan (Los) or vehicle (VEH; saline, 0.9% NaCl) in adult male rats that underwent renal denervation (RDN), or renal nerve intact (Sham), and kept in a normal sodium (NSD) or high-sodium (HSD) diet during 5 days. Body weight (g), Food Intake (g/100g bw), Sodium intake (mEq), Potassium intake (mEq). Groups: NSD-VEH, HSD-VEH, NSD-Los and HSD-Los. Animals with RDN=7 and Sham=6 in each group. Values are expressed as means ± SEM. * p < 0.05 with respect to the normal sodium diet.

Table II: Plasma parameters after an intracerebroventricular injection (ICV) of Losartan (Los) or vehicle (VEH; saline, 0.9% NaCl) in adult male rats that underwent renal denervation (RDN) or renal nerve intact (Sham), and that were kept in a normal sodium (NSD) or high-sodium (HSD) diet during 5 days. Osmolarity (mOsm/Kg). Sodium (Na⁺), Creatinine (mg/dL), Chlorine (Cl⁻), Potassium (K⁺). Groups: NSD-VEH, HSD-VEH, NSD-Los, and HSD-Los. Animals with RDN=7 and Sham=6 in each group. Values are expressed as means ± SEM.

Table III: Urinary and plasma parameters following an intracerebroventricular injection (ICV) of Losartan (Los) or vehicle (VEH; saline, 0.9% NaCl) in adult male rats that underwent renal denervation (RDN) or renal nerve intact (Sham), and kept in a normal sodium (NSD) or high-sodium (HSD) diet during 5 days. Urine volume (mL), Osmolarity (mOsm/Kg). Sodium (Na⁺), Creatinine (mg/dL), Chlorine (Cl⁻), Potassium (K⁺). Groups: NSD-VEH, HSD-VEH, NSD-Los and HSD-Los. Animals with RDN=7 and Sham=6 in each group. Values are expressed as means ± SEM. * p < 0.05 with respect to the normal sodium diet.

Figure legends

Figure 1: Experimental design: 6-day protocol with normosodic and hypersodic diet. The stereotaxic surgery was performed at the beginning of the protocol and vehicle/losartan was administered on day five. Twelve hours later the animals were sacrificed.

Figure 2: Representative microphotograph of histological sections of the kidney A) SHAM, renal nerve intact, B) RDN, renal nerve lesioned. Renal nerve lesion verification in hematoxylin-eosin stained 100x sections.

Figure 3: Food, sodium and water intake in rats treated with a 0.4% normosodic diet (white) and with a

4% hipersodic diet (black) for 5 days prior to intracerebroventricular administration with vehicle/losartan (Left Panel SHAM) and (Right Panel RDN). A) Food intake (g/100g/bw), B) Sodium intake (mEq) and C) Water intake (ml/100g/bw)

Figure 4: Immunostaining anti-c-Fos in the supraoptic nucleus (SON), left panel, and supraoptic nucleus (PVN), right panel. A) Number of Fos-IR cells in rats treated with a normal sodium diet (0.4%) and with a hypersodium diet (4%) and injected intracerebroventricularly with vehicle or losartan (4 μ g / μ l). *p <0.05 compared with the normal sodium diet. #p <0.05 compared with the animals injected with vehicle, in SHAM control animals (n = 7) and RDN denervated animals (n = 6). Mean \pm S.E.M. B) Representative microphotograph of coronal sections c-Fos labelled in the SON and PVN (200 x magnification). The bar represents 100 μ m. Veh: vehicle; Los: losartan; NSD: normosodic diet; HSD: hypersodic diet.

Figure 5: Immunostaining anti-c-Fos in the subfornical nucleus (SFO), left panel, and organum vascular lamina terminalis (OVLT), right panel. A) Number of Fos-IR cells in rats treated with a normal sodium diet (0.4%) and with a hypersodium diet (4%) and injected intracerebroventricularly with vehicle or losartan (4 μ g / μ l). * p <0.05 compared with the normal sodium diet, in SHAM control animals (n = 7) and RDN denervated animals (n = 6). Mean \pm S.E.M. B) Representative microphotograph of coronal sections c-Fos labelled in the SFO and OLVT (200 x magnification). The bar represents 100 μ m. Veh: vehicle; Los: losartan; NSD: normosodic diet; HSD: hypersodic diet.

Figure 6 : Double immunostaining anti-c-Fos and AVP in the SON of control animals (SHAM). A) Number of Fos-AVP-IR cells in rats treated with normal sodium diet (0.4%) and with a hypersodium diet (4%) and injected intracerebroventricularly with vehicle or losartan (4 μ g / μ l). * p <0.05 compared with the normal sodium diet. #p <0.05 compared with those animals injected with vehicle (n = 6). Mean \pm S.E.M. B) Representative microphotograph of coronal sections c-Fos and AVP labelled (200 x magnification). The bar represents 100 μ m. Veh: vehicle; Los: losartan; NSD: normosodic diet; HSD: hypersodic diet.

Figure 7 : Water intake, urinary volume, and fractional sodium excretion. A) Water intake (ml) per 100 g of body weight. B) Urinary volume (ml) and fractional excretion of sodium EFNa (%) in rats treated with a normosodic diet (0.4%) and with a hypersodium diet (4%) and injected with vehicle or losartan (4 μ g / μ l). *p <0.05 compared with the normosodic diet. #p <0.05 compared with the animals injected with vehicle, in SHAM control animals (n = 7) and RDN denervated animals (n = 6). Mean \pm S.E.M.

Graphical abstract legend

Angiotensin II, through its AT₁ receptors, has a critical role in the brain and kidney crosstalk mediating the interactions between the central nervous system, the sympathetic nervous system and the renal function.

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