Research article

Participation of the angiotensinergic and vasopressinergic mechanisms in the maintenance of cardiorespiratory parameters in sodium depleted rats

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A B S T R A C T

Changes in blood volume can be caused by different conditions, such as vomiting, diarrhea, alteration of sodium intake, trauma, or the use of diuretics, which can lead to severe health deterioration. Understanding the mechanisms involved in the maintenance of physiological parameters and the hydroelectrolytic balance of the human body during hypovolemia, can help with preventing and handling these high-risk situations. Hence, this study investigated cardiorespiratory [mean arterial pressure (MAP), heart rate (HR), pulmonary ventilation (VE)] and blood parameters, of sodium depleted rats with furosemide and the roles of the central and peripheral renin-angiotensin and the peripheral vasopressinergic systems in controlling blood pressure in these animals. Different groups under the same conditions received subcutaneous (s.c.) injections of furosemide (diuretic/saliuretic) or vehicle, intracerebroventricular (i.c.v.) or intravenous (i.v.) injections of losartan [angiotensin II (ANG II) AT1 receptor antagonist] or saline, and i.v. injections of Manning compound (AVPX, vasopressin V1 receptor antagonist). Sodium depletion increased the VE (708 ± 71, vs. normovolemic: 478 ± 40 mL/min/kg body wt) and did not modify baseline mean arterial pressure (104 ± 4, vs. normovolemic: 105 ± 4 mmHg) and heart rate (334 ± 20, vs. normovolemic: 379 ± 13 bpm). The i.v. losartan (10 mg/kg of body wt) treatment significantly reduced MAP in all groups and elevated HR, with a greater impact in sodium depleted rats before repletion. On the other hand, the i.c.v. losartan (3.3 μg/kg of body wt) and i.v. AVPX (10 μg/kg of body wt) treatments did not alter the MAP and HR in control, sodium depleted, and sodium repleted rats. These results indicate that sodium depletion affects cardiorespiratory control increasing baseline ventilation and peripheral angiotensinergic mechanisms are relevant for maintaining cardiovascular parameters in sodium depleted rats. Besides, this study suggests vasopressin V1 receptors do not participate in the maintenance of MAP and HR in sodium depleted animals with furosemide.

1. Introduction

Animals daily lose water and/or electrolytes through sweat, breathing, and predominantly by urinary excretion. Therefore, regulation of the mechanisms that control the balance between water and electrolyte intake and excretion is essential, especially sodium, the main determinant of extracellular compartment osmolality. Disruption of body fluids regulation is a very common issue encountered in the medical practice. This is mainly due to several illness and conditions that may potentially affect the control of intake and output of water and electrolytes, such as hyponatremia, disrupting this finely balanced mechanism [1].

Hyponatremia occurs when the concentration of sodium in the blood is low, causing a hydroelectrolyte imbalance in the body. This electrolyte

abnormality is commonly encountered in hospitalized patients and it has seen often in the Intensive Care Unit (ICU). In case of deficient or incorrect treatment, hyponatremia may lead to brain edema or demyelination, with life-threatening outcomes [2]. Hyponatremia correlates with increased mortality in several health conditions, for instance, heart failure and acute myocardial infarction in the elderly and in ICU patients. A large cohort study performed between 2000 and 2007 including more than 50,000 hospitalizations, demonstrated the relation between in-hospital mortality and hyponatremia, where the risk of death was increased by 2.3% for each 1 mmol/L decline of serum [Na+] [3].

Integrated neural, cardiovascular, endocrine, renal, and behavioral systems determine the regulation of extracellular fluid volume and osmolality [4]. Reduction in body sodium levels leads to hypovolemia,

which activates the renin-angiotensin system (RAS) and the production of an active peptide named angiotensin II (ANG II). In this circumstance, concentrations of ANG II in the plasma and brain partly induce thirst and/or sodium appetite, which are behavioral responses involved in restore sodium homeostasis. Additionally, the ANG II effects generate an augment in the sympathetic activity, vascular tonus, renal water, and sodium absorption [5, 6, 7, 8].

The activation of RAS can also generate the release of arginine vasopressin (AVP), a neurohormone directly involved in the hydro-electrolytic balance acting on kidneys water absorption and sodium excretion, as well as on behavioral and cardiovascular responses [9]. Previous studies suggested that AVP has a relevant vasoconstrictor role in sodium depleted rats [10], and the absence of AVP in these animals may lead to hyponatremia [11]. However, Murray *et al.* (1986) observed that sodium depletion with furosemide and a low sodium diet for three days attenuates the pressor response of vasopressin in rats, suggesting that this depletion model may compromise vasopressinergic mechanisms related to blood pressure regulation [12].

Laboratory protocols that elicit disruption of fluids balance, for example, osmotic stimulus and sodium depletion are usually applied to investigate the complex mechanisms that trigger thirst and sodium intake [13]. The acute sodium depletion produced by the subcutaneous injection (s.c.) of the diuretic furosemide accompanied by a sodium deficient diet for 24 h is a model widely used for studies of sodium appetite [14].Additionally, sodium depletion reduces the pressor response to peripheral injection of ANG II and norepinephrine (NOR), but not AVP. The same pressure response attenuation effect was obtained with central injection of ANG II, NOR, and carbachol, suggesting that sodium depletion attenuates mechanisms involved in pressor response mediated by sympathetic activation and ANG II [15]. Moreover, the i.v. administration of furosemide (5 mg kg^{-1}) in anaesthetized rabbits increased the minute ventilation (VE) and consequently decreased end-tidal (ET) CO_2 by furosemide-induced hypovolemia [15].

Previous studies from our laboratory [6] demonstrated that rats with 24 h of sodium depletion by treatment with the diuretic furosemide combined with a sodium deficient diet had no significant changes in blood pressure, despite presenting a reduction in plasma volume [16]. Despite the renin-angiotensin system and vasopressin being crucial in preserving the cardiovascular parameters, the mechanisms involved in maintaining blood pressure in sodium depleted rats with furosemide have not yet been completely elucidated. Thus, the objective of this study was to investigate cardiorespiratory [mean arterial pressure (MAP), heart rate (HR) and pulmonary ventilation (VE)] and blood parameters of sodium depleted rats (treated with furosemide), control rats (treated with vehicle of furosemide), and sodium repleted rats (received water and 0.3 M NaCl for 2 h) and the role of angiotensinergic and vasopressinergic effects in the maintenance of cardiovascular parameters in these animals.

2. Aim

Investigate the cardiorespiratory and blood parameters in sodium depleted rats and whether AT1-type receptor blockade of ANG II with losartan (angiotensin II AT1 receptor antagonist) and receptor blockade of vasopressin with Manning compound (AVPX, vasopressin V1 receptor antagonist) could alter the mean arterial pressure (MAP) and heart rate (HR) in these animals.

3. Materials and methods

3.1. Animals

Male Holtzman rats weighing 280–320 g were housed individually and maintained in a room with controlled temperature (23 $\pm$ 2 °C), humidity (55 $\pm$ 10%), and a 12:12 h light-dark cycle with light onset at 7:30 a.m. Animals had free access to tap water and rodent chow, except when specified in the protocol. All experiments started approximately at

08 a.m, following the Ethics in Animal Use Committee – CEUA of the School of Dentistry of Araraquara – São Paulo State University (protocols n°. 33/2010, 06/2013 and 12/2015) and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize animal discomfort and the number of animals used.

3.2. Brain surgery

The group of rats that received intracerebroventricular (i.c.v.) injections of drugs (central group) were anesthetized with ketamine combined with xylazine and placed in a stereotaxic apparatus (David Kopf Instruments). A stainless-steel cannula (10 × 0.7 mm o.d.) was implanted into the left lateral ventricle (LV) according to the following coordinates: −0.3 mm caudal to bregma, 1.6 mm lateral to the midline, and −3.6 mm deep from dura mater. The cannula was attached to the skull with screws and acrylic resin. After surgery, the animals were housed individually and allowed to recover for 7 days before the beginning of the experiments.

3.3. Peripheral surgery

24 h before the experiment, the central group were anesthetized as described above and a cannula was inserted into the abdominal aorta through the femoral artery for mean arterial pressure (MAP) and heart rate (HR) recordings. Another group of rats also received an abdominal aorta cannula through the femoral artery, following the placement of a cannula in the femoral vein to systemic administration of drugs (peripheral group). After all surgeries, an intramuscular prophylactic dose of benzylpenicillin (80.000 IU) combined with streptomycin and a subcutaneous dose of ketoprofen 1% was injected in all animals.

3.4. MAP and HR measurements

The cannulas were tunneled subcutaneously to the back of the rat and, at the beginning of the experiment, the arterial cannula was connected using polyethylene tubing (PE-10 connected to a PE- 50) to a pressure transducer (Stathan P 23 Db) coupled to a multichannel recorder (Power Lab, ADInstruments). MAP and HR were recorded in conscious and unrestrained rats.

3.5. Determination of pulmonary ventilation

The arterial cannula was connected to a pressure transducer (MLT844, ADInstruments, Sydney, NSW, Australia) coupled to a pre-amplifier (Bridge Amp, ML221, ADInstruments, Sydney, NSW, Australia) connected to a Powerlab computer data acquisition system (PowerLab 16/30, ML880, ADInstruments). The respiratory frequency (fR, breaths/min) and the tidal volume (VT, mL/kg body wt) in conscious, unrestrained rats were measured by whole-body plethysmography as described in detail previously [17, 18]. Briefly, the animals were kept in a plexiglass recording chamber (5 L) that was ventilated with room air. The calibration volume was performed during each ventilation measurement throughout the course of the experiments by injecting a known air volume (1 mL) inside the chamber. VT was calculated using the formula described by Malan (1973) [17] and used in previous studies [19, 20, 21]. Minute ventilation (VE) was calculated as the product of VT and fR and it is presented at ambient barometric pressure and at body temperature, saturated with water vapor at this temperature (BTPS). Rectal body temperature was measured using a thermometer.

3.6. Blood collection

Blood was collected from femoral artery for determination of plasma concentration of protein, sodium, potassium and osmolality via the implanted arterial cannula. Samples were collected in tubes containing

sodium EDTA (2 mg/ml of blood) to obtain plasma. All samples were centrifuged for 15 min. Plasma osmolality was measured in a Model 3250 Osmometer (Advanced Instruments, Norwood, MA, U.S.A) by freezing point depression. The plasma concentration of sodium and potassium were measured by ion-specific electrode (Nova) and the plasma protein concentration was measured in a refractometer (ATAGO).

3.7. Determination of pH and blood gases

Arterial blood samples were also obtained via the implanted arterial cannula. The measurements were made using an I-STAT portable clinical analyzer and EG7+ cartridges (Heska, Waukesha, WI). Ninety-five (95) microliters of arterial blood were sampled for immediate analysis of arterial pH (pHa), arterial carbon dioxide partial pressure (PCO_2), arterial oxygen partial pressure (PO_2), plasma bicarbonate (HCO_3^-) and hematocrit (Hct).

3.8. Drugs injected

- Losartan (angiotensin II AT1 receptor antagonist) or vehicle (sterile saline) were prepared at concentrations of 3.3 µg/kg of body wt per injection (i.c.v.) and 10 mg/kg of body wt per injection (i.v.).
- Angiotensin II (ANG II) was prepared at a concentration of 50 ng/µL (i.c.v.) and 50 ng/0,1 mL/rats (i.v.).
- Solution of furosemide (20 mg/kg of body wt, diuretic/saliuretic) was prepared from a stock solution dissolved in isotonic saline with pH close to 9,0, adjusted with sodium hydroxide (NaOH) solution and administered s.c., and its vehicle (saline pH 9,0 adjusted with NaOH) s.c.
- Manning compound (AVPX [Mercapto-Cyclopentamethylenepropionyl1, O-Me-Tyr2, Arg8]), (vasopressin V1 receptor antagonist), 10 µg/kg of body wt per injection (i.v.), dose used by Blanch et al., 2007 [22].
- Arginine vasopressin (AVP) was prepared at a concentration of 12.5 ng/0.1 mL/per rat (i.v.).

3.9. Protocol of acute sodium depletion for 24 h

The sodium depletion was performed with a single dose of 1 mL/rat of diuretic furosemide s.c. followed by a sodium deficient diet (powdered com meal, 0.001% sodium and 0.33% potassium) and water ad libitum for 24 h. This sodium depletion (induced by treatment with s.c. furosemide + sodium deficient diet) induces 1.5 to 2.0 mEq loss of sodium and consistent intake of hypertonic sodium solutions [23, 24, 25, 26]. The control group received the treatment with the vehicle of furosemide and was kept for 24 h with a standard diet (rodent chow pellets, 0.5% sodium) and water ad libitum.

3.10. Protocol of sodium repletion

For fluid intake, during 2 h water and 0.3 M NaCl were presented in 0.1 mL graduated glass burettes fitted with metal drinking spouts. During the sodium repletion, animals were not fed.

3.11. Histology

At the end of protocols rats were deeply anesthetized with an intraperitoneal (i.p.) dose of sodium thiopental (80 mg/kg of body wt, Cristália, Brazil) and then received an injection of Evan's blue dye (100 nl of a 2% solution) into the LV. Rats were perfused transcardially with saline followed by 10% buffered formalin. The brain was removed, stored in buffered formalin, and cut in 50 µm coronal sections with a microtome. Slices were stained by the Giemsa method and analyzed by light microscopy. Only rats with a clear spread of dye in the LV were used for data analysis. A representative photomicrograph of a brain slice from one rat is presented in Fig. S2 in the Supporting Information (SI).

3.12. Statistical analysis

The mean and standard error of means (S.E.M.) were represented graphically. Two-way ANOVA and post-test of Student-Newman-Keuls were used for comparisons between different treatments and groups. Differences were considered significant at $p < 0.05$. The charts with the p values for significant differences between the groups are presented in Figs. S3 to S10 in the SI.

4. Experimental protocol

A first group of animals were individually placed in Plexiglass chambers (5 L) and allowed to move freely while the chamber was flushed with room air during approximately 30 min. The rats were exposed to three different conditions: normovolemic (free access to food, water and 0.3 M NaCl), sodium depleted (24 h after s.c. injection of furosemide) or vehicle treatment (24 h after s.c. injection of vehicle) and sodium repleted (2 h after free access to 0.3 M NaCl and water). On the first day of experiments, cardiorespiratory parameters and blood parameters were measured in normovolemic condition. Thereafter, the rats received an injection of 1 mL of furosemide (sodium depleted rats) or 1 mL vehicle (control rats). After 24 h, cardiorespiratory parameters and blood parameters were measured again. After that, the rats returned to their cages and 0.3 M NaCl and water were offered to the animals in 0.1 mL graduated glass burettes for 2 h to restore their sodium balance. Cumulative 0.3 M NaCl and water intakes were measured at 30, 60, 90 and 120 min. Cardiorespiratory parameters and blood parameters were measured again in sodium repleted rats.

Central and peripheral groups of animals also randomly received an injection of 1 mL of furosemide or 1 mL of vehicle. Then, animals treated with furosemide were kept in individual cages with low-sodium food, and animals treated with vehicle were kept with a standard diet; both groups received water ad libitum for the next 24 h. Following this period, these animals were connected to the pressure transducer while maintaining their free movement. During a period ranging from 15 to 60 min, the animals could explore their boxes until they calmed and the cardiovascular parameters stabilized. Following this stage, MAP and HR were measured. After a period ranging from 10 to 30 min, half of the central group treated with furosemide received a central injection of losartan (3.3 µg/kg body wt, i.c.v.) or saline, and half of the peripheral group received a peripheral injection of losartan (10 mg/kg, i.v.) or saline. To confirm that the ANG II AT1 receptors were blocked effectively, after 10 min, all animals received a central administration of ANG II (50 ng/µL, i.c.v.) or a peripheral administration of ANG II (50 ng/0,1 mL/rats, i.v.), according to the former via of administration of losartan or saline. This way, there were eight different groups analyzed, divided in i.c.v. and i.v. injections, namely: vehicle + saline; vehicle + losartan; furosemide + saline and furosemide + losartan.

A second peripheral group of animals also randomly received an injection of 1 mL of furosemide or 1 mL of vehicle. Then, these two subgroups were kept in individual cages and treated as previously described. After 24 h, all animals were also connected to the pressure transducer, they were free to explore their boxes for 15–60 min until they calmed and their cardiovascular parameters stabilized. Then, MAP and HR were measured. After a period ranging from 10 to 30 min, half of the rats treated with furosemide received a peripheral injection of AVPX (10 µg/kg/body wt, i.v.) and the other half a peripheral injection of saline. To confirm that the V1 receptors were blocked effectively, all animals received a peripheral administration of AVP (12.5 ng/0.1 mL/per rat, i.v.), 10 min after the injections of AVPX or saline. Thus, four different groups were analyzed with i.v. injections: vehicle + saline; vehicle + AVPX; furosemide + saline, and furosemide + AVPX.

Aiming to restore the sodium balance and the body volume after depletion, all groups of animals previously tested were placed in individual cages with free access to water and 0.3 M NaCl in 0.1 mL graduated glass burettes. After 2 h of fluids intake, the repleted rats were re-

connected to the pressor transducer, undergone an exploratory period and the cardiovascular parameters and injection of drugs were executed as previously described. The visual representation of the experimental protocols is presented in Fig. S1 in the SI.

5. Results

5.1. Changes in VE, VT and fR in rats depleted of sodium with furosemide and 24 h on a low-sodium diet

Sodium depletion increased VE (708 ± 71 mL/min/kg body wt, vs. normovolemic rats: 478 ± 40 mL/min/kg body wt) [$F_{(2,24)} = 5.807$; $p < 0.05$] and VT (7 ± 0.4 mL/kg body wt, vs. normovolemic rats: 5 ± 0.4 mL/kg body wt, $p < 0.05$) [$F_{(2,24)} = 4.326$; $p < 0.05$], without changing the fR (99 ± 5 breaths/min vs. normovolemic: 85 ± 4 breaths/min) (Figure 1), baseline MAP (104 ± 4 mmHg, vs. normovolemic rats: 105 ± 4 mmHg) and HR (334 ± 20 bpm, vs. normovolemic rats: 379 ± 13 bpm) (Figure 2). Even after fluid repletion (2 h after the access to 0.3 M NaCl and water), VE (640 ± 22 mL/min/kg body wt) of rats submitted to sodium depletion did not return to normovolemic levels (Figure 1). Rats treated with s.c. injection of vehicle of furosemide did not change baseline MAP (108 ± 3 mmHg, vs. normovolemic rats: 105 ± 2 mmHg) [$F_{(2,21)} = 0.222$; $p > 0.05$], HR (419 ± 7 bpm, vs. normovolemic rats: 390 ± 13 bpm) [$F_{(2,21)} = 1.997$; $p > 0.05$], VE (405 ± 27 mL/min/kg body wt, vs. normovolemic rats: 383 ± 27 mL/min/kg body wt) [$F_{(2,21)} = 0.834$; $p > 0.05$].

5.2. Cumulative 0.3 M NaCl and water intake

Table 1 shows 2 h of 0.3 M NaCl and water intake in sodium depleted rats (24 h after injection of furosemide s.c. and low sodium diet) and in vehicle-treated rats (24 h after injection of vehicle s.c.) to restore fluid balance.

5.3. Blood analysis in normovolemic, sodium depleted and repleted rats

Table 2 shows that sodium depletion decreased plasma sodium and potassium concentration (143 ± 1.1 and 3.2 ± 0.1 meq/l, vs. normovolemic: 146 ± 1.2 and 4 ± 0.2 meq/l, respectively) [$F_{(2,63)} = 4.733$; $p = 0.012$] and [$F_{(2,63)} = 13.42$; $p < 0.001$], respectively, and increase plasma protein concentration (7.3 ± 0.1 g/ml, vs. normovolemic: 6.5 ± 0.1 g/ml) [$F_{(2,63)} = 13.42$; $p < 0.001$]. Plasma sodium and protein, but not plasma potassium, were returned to normovolemic levels after to restore fluid balance by 2 h water and 0.3 M NaCl intake. The treatment with vehicle did not change plasma sodium (149 ± 1.3 vs. normovolemic: 147 ± 1.1) potassium (3.8 ± 0.1 meq/l, vs. normovolemic: 4.1 ± 0.1 meq/l) and protein concentration (6.5 ± 0.3 g/ml, vs. normovolemic: 6.7 ± 0.3 g/ml). There were no differences in osmolality in the treatment with vehicle or furosemide.

5.4. Arterial blood gases and pHa

Table 3 shows that sodium depletion induced increase in pHa and HCO_3^- compared with normovolemic condition (7.53 ± 0.01, 35.6 ± 0.8 mmol/l, vs. normovolemic: 7.48 ± 0.01, 30.6 ± 0.6 mmol/l, respectively) [$F_{(2,65)} = 8.645$; $p < 0.001$] and [$F_{(2,65)} = 28.367$; $p < 0.001$], without changes in blood gases ($PaCO_2$ and PaO_2). After restore fluid balance, sodium repleted rats had pHa adjusted (7.47 ± 0.01) [$F_{(2,65)} = 8.645$; $p < 0.001$] and decreased in $PaCO_2$ (34.8 ± 1.3 mmHg, vs. normovolemic: 40.6 ± 1.0 mmHg) [$F_{(2,65)} = 7.432$; $p = 0.001$], HCO_3^- (25.5 ± 0.7 mmol/l, vs. normovolemic: 30.6 ± 0.6 mmol/l) [$F_{(2,65)} = 28.367$; $p < 0.001$] and hematocrit ($31.2 \pm 1.5\%$, vs. normovolemic: $35.2 \pm 1.0\%$) [$F_{(2,65)} = 7.812$; $p < 0.001$]. Vehicle s.c. did not affect pHa, blood gases and hematocrit but decrease HCO_3^- (27.1 ± 0.6 mmol/l, vs. normovolemic: 30.4 ± 0.5 mmol/l) [$F_{(2,65)} = 28.367$; $p < 0.001$] this values kept low after 2 h of fluid access (26.2 ± 0.4 mmol/l).

5.5. Effect of peripheral ANG II AT1 receptors blockage with losartan (ANG II AT1 receptor antagonist) in cardiovascular responses in rats depleted of sodium with furosemide and 24 h on a low-sodium diet

The i.v. losartan injection provoked differences in MAP between control groups treated with vehicle of furosemide (vehicle + losartan: $\Delta = -9 \pm 2$ mmHg vs. vehicle + saline: $\Delta = -0.1 \pm 0.9$ mmHg, $p < 0.05$, Figure 3A) and between sodium depleted groups (furosemide + losartan: $\Delta = -16 \pm 2$ mmHg vs. furosemide + saline: $\Delta = -0.1 \pm 0.4$ mmHg, $p < 0.05$, Figure 3A). Moreover, i.v. losartan injection also caused variation in HR between control groups treated with vehicle of furosemide (vehicle + losartan: $\Delta = 18 \pm 7.7$ mmHg vs. vehicle + saline: $\Delta = -6 \pm 3$ mmHg, $p < 0.05$, Figure 4A) and between sodium depleted groups (furosemide + losartan: $\Delta = 43 \pm 5$ mmHg vs. furosemide + saline: $\Delta = 4 \pm 1$ mmHg, $p < 0.05$, Figure 3A). It is important to highlight that i.v. losartan injection produced a greater difference in MAP in the depleted group when compared to the vehicle group (furosemide + losartan: $\Delta = -16 \pm 2$ mmHg, vs. vehicle + losartan: $\Delta = -8 \pm 2$ mmHg, $p < 0.05$, Figure 3A). The same was observed with HR (furosemide + losartan: $\Delta = 43 \pm 5$ mmHg vs. vehicle + losartan: $\Delta = 18 \pm 8$ mmHg, $p < 0.05$, Figure 3A).

After repletion with NaCl 0.3 M and water for 2 h, the pre-treatment with losartan also provoked differences in MAP between control groups treated with vehicle of furosemide (vehicle + losartan: $\Delta = -5 \pm 1$ mmHg, vs. vehicle + saline: $\Delta = 0.2 \pm 0.7$ mmHg, $p < 0.05$, Figure 3A) and between sodium depleted rats (furosemide + losartan: $\Delta = -5 \pm 1$ mmHg, vs. furosemide + saline: $\Delta = -0.5 \pm 0.8$ mmHg, $p < 0.05$, Figure 3A). Additionally, the i.v. losartan injection caused variation in HR between control groups treated with vehicle of furosemide (vehicle + losartan: $\Delta = 16 \pm 7$ mmHg vs. vehicle + saline: $\Delta = -4 \pm 2$ mmHg, $p < 0.05$, Figure 3A).

To test the effectiveness of ANG II AT1 receptor blockade after losartan treatment, the same animals received i.v. ANG II (50 ng/0.1 mL) 10 min after losartan or its vehicle (saline), before and after 2 h of NaCl 0.3 M repletion and water. The pre-treatment with losartan blocked the pressor response induced by i.v. ANG II injection in control rats (vehicle + losartan + ANG II: $\Delta = -0.3 \pm 1$ mmHg vs. vehicle + saline + ANG II: $\Delta = 43 \pm 2$ mmHg, $p < 0.05$, Figure 3B), and blocked bradycardia (vehicle + losartan + ANG II: $\Delta = -4 \pm 4$ bpm, vs. vehicle + saline + ANG II: $\Delta = -75 \pm 11$, $p < 0.05$, Figure 3B). In sodium depleted rats, the pre-treatment with losartan also blocked the ANG II response (furosemide + losartan + ANG II: $\Delta = 0.2 \pm 1$ mmHg vs. furosemide + saline + ANG II: $\Delta = 31 \pm 3$ mmHg, $p < 0.05$, Figure 3B), as well as bradycardia (furosemide + losartan + ANG II: $\Delta = -1 \pm 4$ bpm vs. furosemide + saline + ANG II: $\Delta = -48 \pm 14$ bpm, $p < 0.05$, Figure 3B). It is important to highlight that the pressor response and bradycardia resulting from i.v. ANG II treatment were attenuated in the depleted group (furosemide + saline + ANG II group: $\Delta MAP = 31 \pm 3$ mmHg and $\Delta HR = -48 \pm 14$ bpm, $p < 0.05$, Figure 3B) when compared with its control group (vehicle + saline + ANG II: $\Delta MAP = 43 \pm 2$ mmHg and $\Delta HR = -75 \pm 11$ bpm, Figure 3B) before repletion. After repletion with NaCl 0.3 M and water for 2 h, the pre-treatment with losartan blocked the pressor response induced by i.v. ANG II in control groups treated with vehicle of furosemide (vehicle + losartan + ANG II: $\Delta = 1 \pm 1$ mmHg vs. vehicle + saline + ANG II: $\Delta = 43 \pm 2$ mmHg, $p < 0.05$, Figure 3B) and bradycardia (vehicle + losartan + ANG II: $\Delta = 1 \pm 3$ bpm vs. vehicle + saline + ANG II: $\Delta = -66 \pm 5$ bpm, $p < 0.05$, Figure 3B), as well as in depleted groups (furosemide + losartan + ANG II: $\Delta = -0,1 \pm 1$ mmHg vs. furosemide + saline + ANG II: $\Delta = 38 \pm 4$ mmHg, $p < 0.05$, Figure 3B) and bradycardia (furosemide + losartan + ANG II: $\Delta = 2 \pm 4$ bpm vs. furosemide + saline + ANG II: $\Delta = -64 \pm 9$ bpm, $p < 0.05$, Figure 3B). However, the pressor response and bradycardia of the depleted group (furosemide + saline + ANG II: $\Delta MAP = 38 \pm 4$ mmHg e $\Delta HR = -64 \pm 9$ bpm) and its control group (vehicle + saline + ANG II: $\Delta MAP = 43 \pm 2$ mmHg and $\Delta HR = -66 \pm 5$ bpm) were not statistically different (Figure 3B).
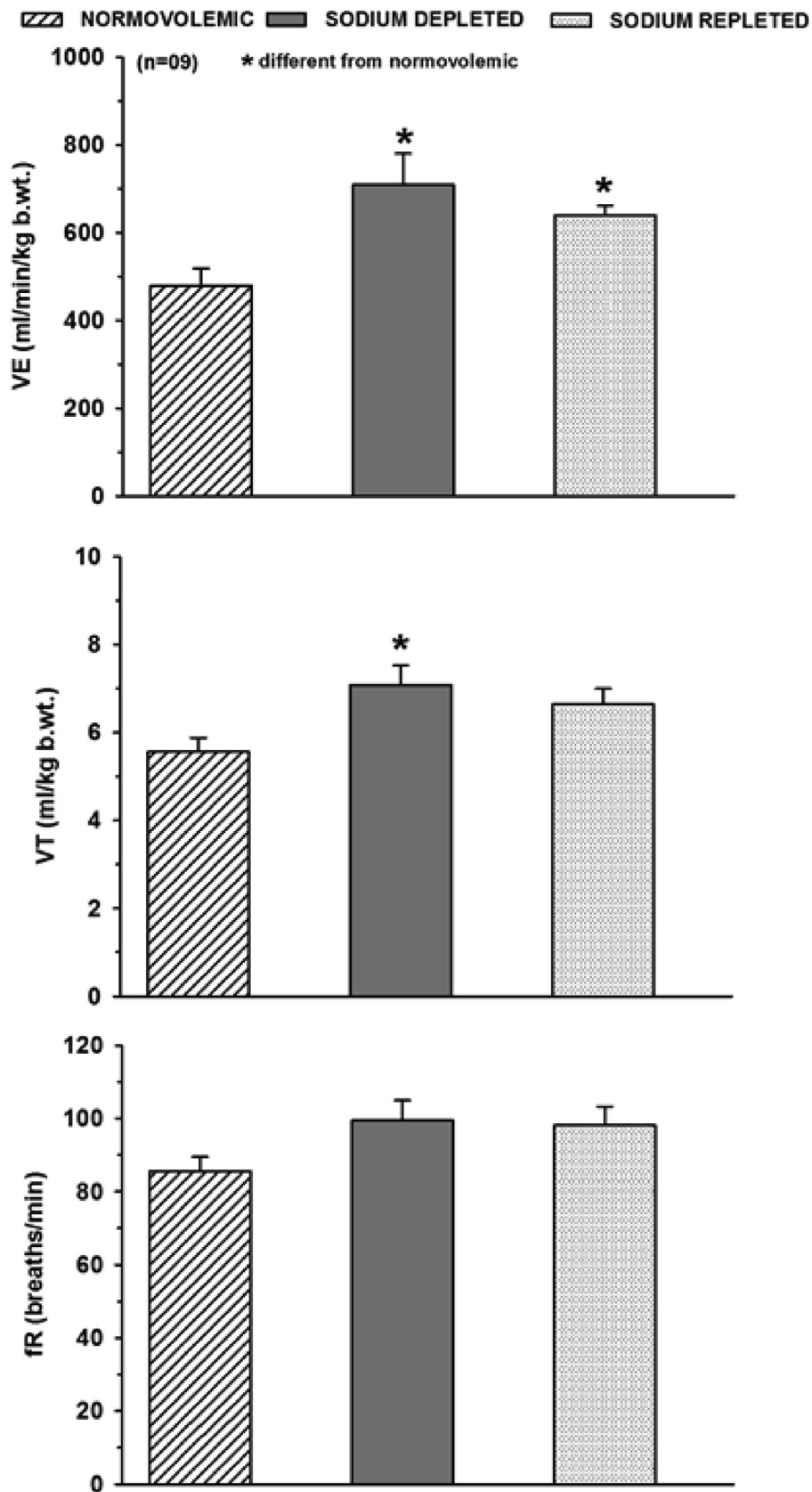
Figure 1. Baseline values of ventilation (VE), tidal volume (VT), and respiratory frequency (fR) in normovolemic (before sodium depletion), sodium depleted (24 h after injection of furosemide s.c. and low sodium diet) and sodium repleted rats (after 2 h of cumulative 0.3 M NaCl and water intake). Results are expressed as means ± S.E.M. n = number of animals. * different from normovolemic.
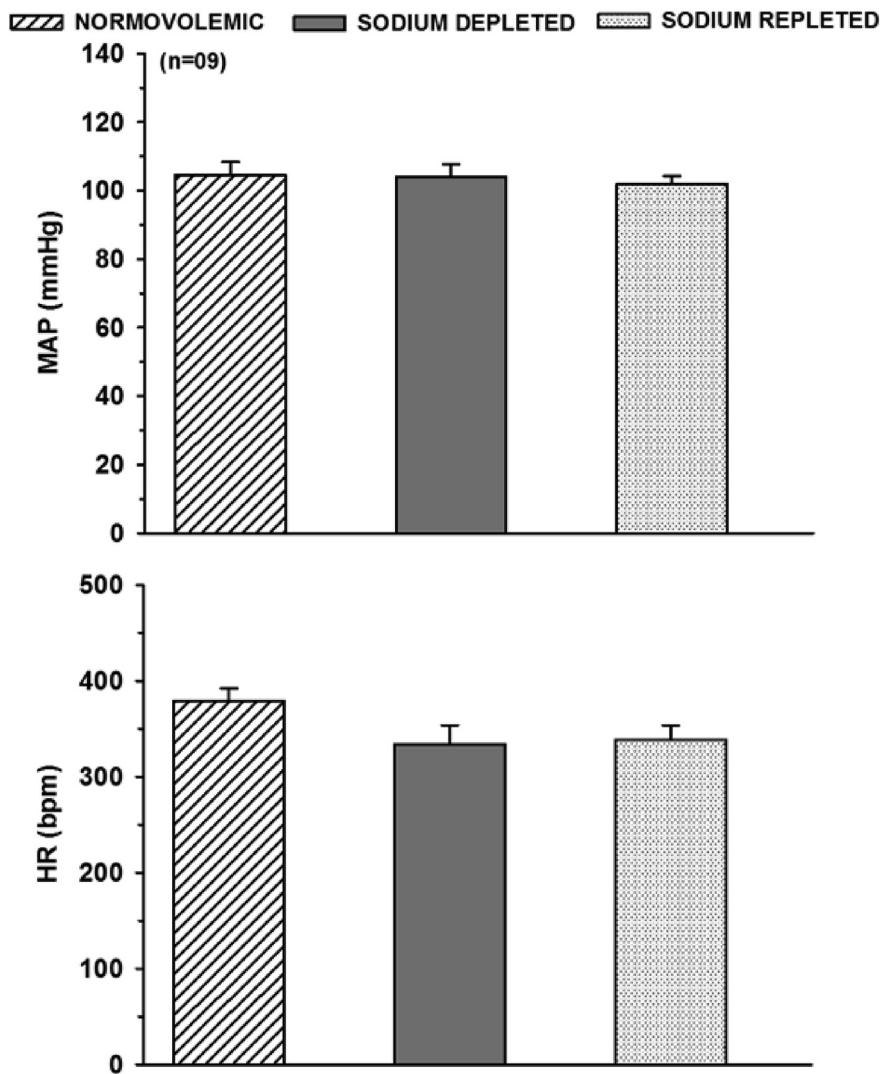
Figure 2. Baseline values of MAP and HR in normovolemic (before sodium depletion), sodium depleted (24 h after injection of furosemide s.c. and low sodium diet) and sodium repleted rats (after 2 h of cumulative 0.3 M NaCl and water intake). Results are expressed as means ± S.E.M. n = number of animals.

Table 1. Cumulative 0.3 M sodium and water intake in sodium depleted rats (24 h after injection of furosemide s.c. and low sodium diet) or vehicle treatment (24 h after injection of vehicle s.c.), n = number of animals.

	Sodium intake			
	30 min	60 min	90 min	120 min
Vehicle-treated (n = 08)	2.1 ± 0.4	2.7 ± 0.6	3.8 ± 0.6	4.1 ± 0.6 (ml)
Furosemide-treated (n = 09)	0.3 ± 0.3	4.0 ± 1.7	8.6 ± 2.2	15.2 ± 3.0(ml)
	Water intake			
	30 min	60 min	90 min	120 min
Vehicle-treated (n = 08)	1.2 ± 0.3	1.3 ± 0.2	1.5 ± 0.2	1.8 ± 0.4 (ml)
Furosemide-treated (n = 09)	0.1 ± 0.08	0.9 ± 0.3	2.7 ± 0.9	5.8 ± 0.8 (ml)

Values are means ± S.E.M.

5.6. Effect of central ANG II AT1 receptors blockage with losartan (ANG II AT1 receptor antagonist) on cardiovascular responses in rats depleted of sodium with furosemide and 24 h on a low-sodium diet

The i.c.v. losartan injection did not provoke a significant differences in MAP between control groups treated with vehicle of furosemide (vehicle + losartan: Δ = −4 ± 2 mmHg vs. vehicle + saline: Δ = −1 ± 1 mmHg), sodium depleted groups (furosemide + losartan: Δ = −4 ± 2 vs.

furosemide + saline: Δ = 2 ± 2 mmHg), neither caused a significant variation in HR in any of the studied groups (Figure 4A). After repletion with NaCl 0.3 M and water for 2 h, i.c.v. losartan maintained MAP unaltered not just in control groups treated with vehicle of furosemide (vehicle + losartan: Δ = −3 ± 2 mmHg vs. vehicle + saline: Δ = 3 ± 1 mmHg, Figure 4A), but also in sodium depleted rats (furosemide + losartan: Δ = −0.1 ± 1 mmHg vs. furosemide + saline: Δ = 0.1 ± 0.9 mmHg, Figure 4A). There were also no significative changes in HR among the studied groups, Figure 4A.

To test the effectiveness of ANG II AT1 receptor blockade after losartan treatment, the same animals received i.c.v. ANG II (50 ng/μL) 10 min after losartan or vehicle treatment, before and after 2 h of NaCl 0.3 M repletion and water. The pre-treatment with losartan blocked the pressor response induced by i.c.v. ANG II injection in control groups treated with vehicle of furosemide (vehicle + losartan + ANGII: Δ = −3 ± 2 mmHg, vs. vehicle + saline + ANG II: Δ = 13 ± 3 mmHg, p < 0.05, Figure 4B). Interestingly, the pre-treatment with losartan did not block the pressor response induced by i.c.v. ANG II injection in depleted groups (furosemide + losartan + ANG II: Δ = 5 ± 3 mmHg vs. furosemide + saline + ANG II: Δ = 17 ± 2 mmHg, Figure 4B), however data showed a considerable difference between these two groups. Central losartan treatment also did not block bradycardia in control groups treated with vehicle of furosemide (vehicle + losartan + ANG II: Δ = −23 ± 7 bpm, vs.

Table 2. Plasma concentration of sodium (PNa$^+$), potassium (PK$^+$), total protein (Pt), osmolality (Posm) of control rats (vehicle s.c.) n = 08, and sodium depleted rats (furosemide s.c.) n = 15, n = number of animals.

	CONTROL (vehicle s.c.)			SODIUM DEPLETION (furosemide s.c.)		
	Normovolemic	Vehicle-treated	2 h after fluid access	Normovolemic	Sodium depleted	Sodium repleted
P$_{Na}^+$(meq/l)	147 ± 1.1	149 ± 1.3	146.5 ± 0.1	146 ± 1.2	143 ± 1.1*#	147.5 ± 0.8 ^{δ}
P$_K^+$(meq/l)	4.1 ± 0.1	3.8 ± 0.1*	3.5 ± 0.1*	4.0 ± 0.2	3.2 ± 0.1*#	3.2 ± 0.1*
Pt (g/100 mL)	6.7 ± 0.31	6.5 ± 0.29	6.0 ± 0.29	6.5 ± 0.17	7.3 ± 0.16*#	6.0 ± 0.18 ^{δ}
P$_{Osm}$(mOsm/kg)	293.6 ± 3.9	299.3 ± 2.6	297.6 ± 1.7	294.2 ± 3.0	293.2 ± 1.7	300.6 ± 2.2

*different from normovolemic in the same group, # different from vehicle-treated, δ different from sodium depleted in the same group.

Table 3. Values of arterial pH (pHa), arterial carbon dioxide partial pressure (PaCO$_2$), oxygen partial pressure (PaO$_2$), plasma bicarbonate (HCO$_3^-$) and hematocrit (Hct) of control rats (vehicle s.c.), n = 08 and sodium depleted rats (furosemide s.c.), n = 16, n = number of animals.

	CONTROL (vehicle s.c.)			SODIUM DEPLETION (furosemide s.c.)		
	Normovolemic	Vehicle-treated	2 h after fluid access	Normovolemic	Sodium depleted	Sodium repleted
pHa	7.48 ± 0.017	7.45 ± 0.011	7.46 ± 0.007	7.48 ± 0.01	7.53 ± 0.01*#	7.47 ± 0.01 ^{δ}
PaCO$_2$ (mmHg) 39.8 ± 1.8	38.2 ± 0.9	36.0 ± 0.9	40.6 ± 1.0	41.8 ± 1.3	34.8 ± 1.3*^{δ}	
PaO$_2$ (mmHg)	73.7 ± 2.9	74.5 ± 2.3	74.5 ± 2.2	73.1 ± 1.0	70 ± 0.9	72.2 ± 1.9
HCO$_3^-$(mmol/l)	30.4 ± 0.5	27.1 ± 0.6	26.2 ± 0.4*	30.6 ± 0.6	35.6 ± 0.8*#	25.5 ± 0.7*^{δ}
Hct (%)	37 ± 0.5	37.6 ± 0.8	33.5 ± 1.6	35.2 ± 1.0	37.3 ± 1.1	31.2 ± 1.5*^{δ}

*different from normovolemic in the same group, # different from vehicle, δ different from sodium depleted in the same group.
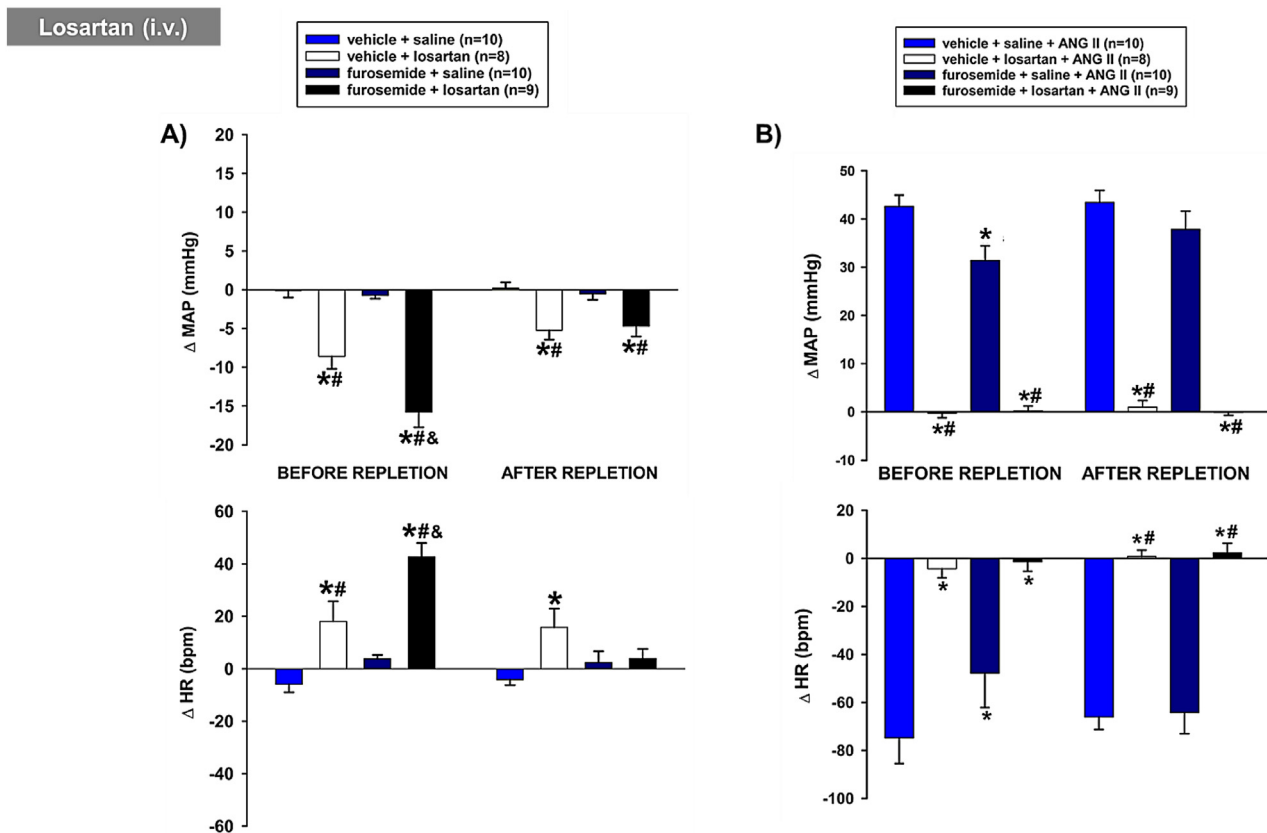
Figure 3. A) Variation in mean arterial pressure (ΔMAP) and heart rate (ΔHR) in rats treated with vehicle or furosemide s.c., combined with i.v. injection of saline or losartan (10 mg/kg body wt) before and after sodium repletion (2 h of water and sodium 0.3 M intake). B) Variation in ΔMAP and ΔHR in rats after i.v. injection of ANG II (50 ng/0,1 mL) 10 min after saline or losartan injection (10 mg/kg). The groups were treated with vehicle or furosemide s.c. and submitted to sodium repletion (2 h of water and sodium 0.3 M intake). Results expressed as mean ± SEM. n = number of animals. Two-way ANOVA. "Before Depletion" and "After Repletion" situations were analysed separately. The charts with the p values for significant differences between the groups are presented in Figures S3 and S4 in the SI. * different from vehicle + saline and vehicle + saline + ANG II. # different from furosemide + saline and furosemide + saline + ANG II. & different from vehicle + losartan and vehicle + losartan + ANG II.
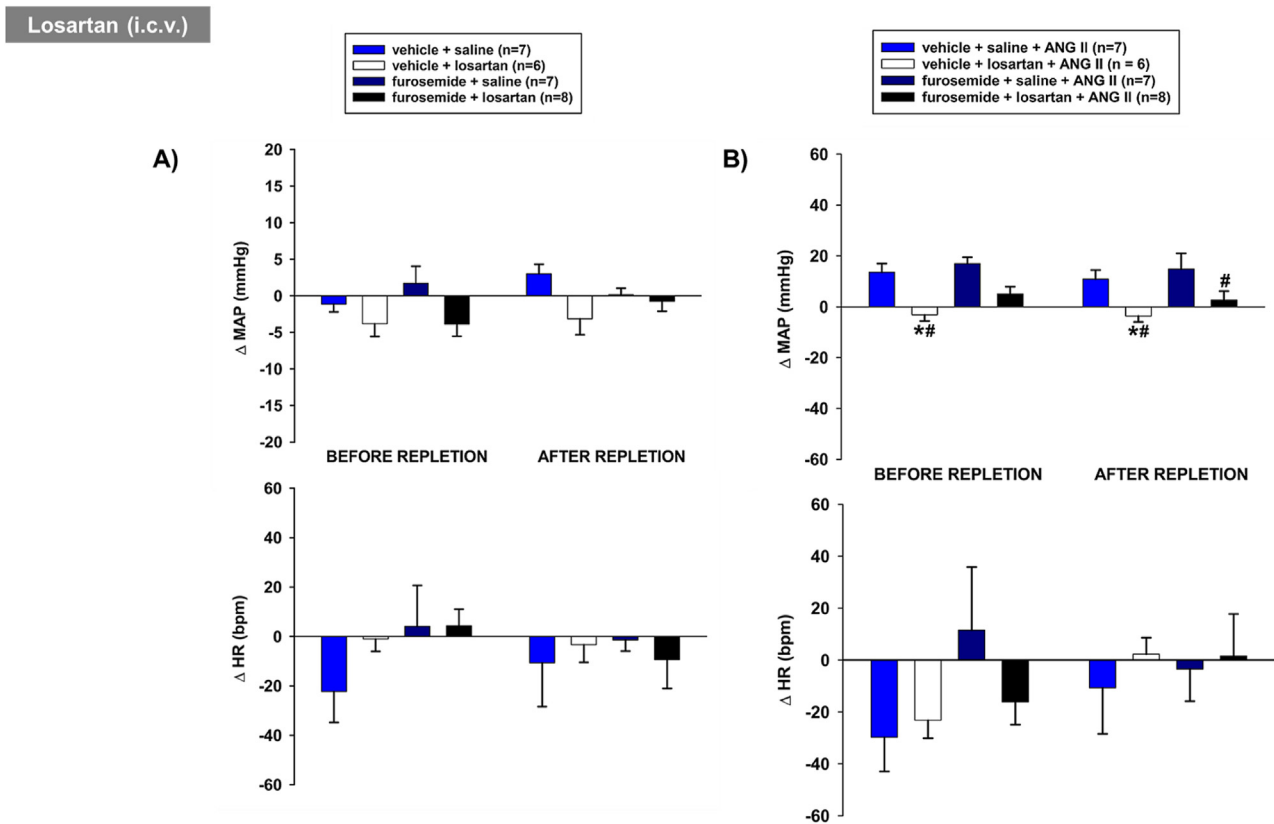
Figure 4. A) Variation in mean arterial pressure (ΔMAP) and heart rate (ΔHR) in rats treated with vehicle or furosemide s.c. (20 mg/kg body wt) combined with i.c.v. injection of saline or losartan (1 μg/μl) before and after sodium repletion (2 h of water and sodium 0.3 M intake) B) Variation in ΔMAP and ΔHR in rats after i.c.v. injection of ANG II (50 ng/μL) 10 min after saline or losartan injection (3.3 μg/kg body wt). The groups were treated with vehicle or furosemide s.c. and submitted to sodium repletion (2 h of water and sodium 0.3 M intake). Results expressed as mean ± SEM. n = number of animals. Two-way ANOVA. "Before Depletion" and "After Repletion" situations were analysed separately. The chart with the p values for significant differences between the groups is presented in Fig. S5 in the SI. * different from vehicle + saline + ANG II. # different from furosemide + saline + ANG II.

vehicle + saline + ANG II: Δ = −30 ± 13 bpm, Figure 4B) and in depleted groups (furosemide + losartan + ANG II: Δ = −16 ± 9 bpm, vs. furosemide + saline + ANG II: Δ = 11 ± 24 bpm, Figure 4B). After repletion with NaCl 0.3 M and water, the pre-treatment with losartan blocked the pressor response induced by i.c.v. ANG II injection in control groups treated with vehicle of furosemide (vehicle + losartan + ANG II: Δ = −3 ± 2 mmHg vs. vehicle + saline + ANG II: Δ = 11 ± 4 mmHg, p < 0.05, Figure 4B), without significant variation in bradycardia (vehicle + losartan + ANG II: Δ = 2 ± 6 bpm vs. vehicle + saline + ANG II: Δ = −11 ± 18 bpm, Figure 4B). Pre-treatment with losartan was also effective in blocking the pressor response induced by i.c.v ANG II injection in depleted groups (furosemide + losartan + ANG II: Δ = 3 ± 3 mmHg, vs. furosemide + saline + ANG II: Δ = 15 ± 6 mmHg, p < 0.05, Figure 4), although it did not alter bradycardia (furosemide + losartan + ANG II: Δ = 2 ± 16 bpm vs. furosemide + saline + ANG II: Δ = −3 ± 12 bpm, Figure 4B).

5.7. Effect of vasopressinergic system blockage with Manning compound AVPX (vasopressin V1 receptor antagonist) on cardiovascular responses in rats depleted of sodium with furosemide and 24 h on a low-sodium diet

The i.v. AVPX injection did not provoke significant differences in MAP between control groups treated with vehicle of furosemide (vehicle + AVPX: Δ = −5 ± 3 mmHg vs. vehicle + saline: Δ = 0.9 ± 0.6 mmHg, Figure 5A) and between depleted groups (furosemide + AVPX: Δ = −0.3 ± 1 mmHg, vs. furosemide + saline: Δ = −3 ± 0.8 mmHg, Figure 5A). Nevertheless, there was a significant variation in MAP in the vehicle + AVPX group (Δ = −8 ± 7 mmHg) when compared to the furosemide +

AVPX group (Δ = −1.3 ± 13 mmHg), p < 0.05, Figure 5A. The AVPX treatment did not produce a significant changes in HR in any of the groups, Figure 5A. After repletion with NaCl 0.3 M and water for 2 h, the pre-treatment with AVPX maintained MAP unaltered not just between control groups (vehicle + AVPX: Δ = −2 ± 2 mmHg vs. vehicle + saline: Δ = −0.8 ± 0.4 mmHg, Figure 5A) but also between sodium depleted groups (furosemide + AVPX: Δ = 0.2 ± 1 mmHg vs. furosemide + saline: Δ = −0.8 ± 0.4 mmHg, Figure 5A). There were also no significant changes in HR among the studied groups, Figure 5A.

To test the effectiveness of vasopressin V1 receptors blockade after AVPX treatment, the same animals received AVP (12.5 ng/0.1 mL) 10 min after AVPX or vehicle treatment, before and after 2 h of NaCl 0.3 M repletion and water (Figure 5B). The pre-treatment with AVPX blocked the pressor response induced by the i.v. injection of AVP in control groups treated with vehicle of furosemide, (vehicle + AVPX + AVP: Δ = 1 ± 1 mmHg vs. vehicle + saline + AVP: Δ = 22 ± 3 mmHg, p < 0.05, Figure 5B) and blocked the bradycardia (vehicle + AVPX + AVP: Δ = 4 ± 4 bpm vs. vehicle + saline + AVP: −40 ± 7 bpm, p < 0.05, Figure 5B). That also blocked the pressor response induced by i.v. AVP injection in depleted groups (furosemide + AVPX + AVP: Δ = 1 ± 1 mmHg, vs. furosemide + saline + AVP: 25 ± 4 mmHg, p < 0.05, Figure 5B) and bradycardia (furosemide + AVPX + AVP: Δ = −1 ± 4 bpm, vs. furosemide + saline + AVP: Δ = −42 ± 14 bpm, p < 0.05, Figure 5B). After repletion with NaCl 0.3 M and water, the pre-treatment with AVPX blocked the pressor response induced by i.v. injection of AVP in control groups treated with furosemide (vehicle + AVPX + AVP: Δ = −1 ± 1 mmHg vs. vehicle + saline + AVP: Δ = 23 ± 2 mmHg, p < 0.05, Figure 5B) and bradycardia (vehicle + AVPX + AVP: Δ = −3 ± 5 bpm vs.
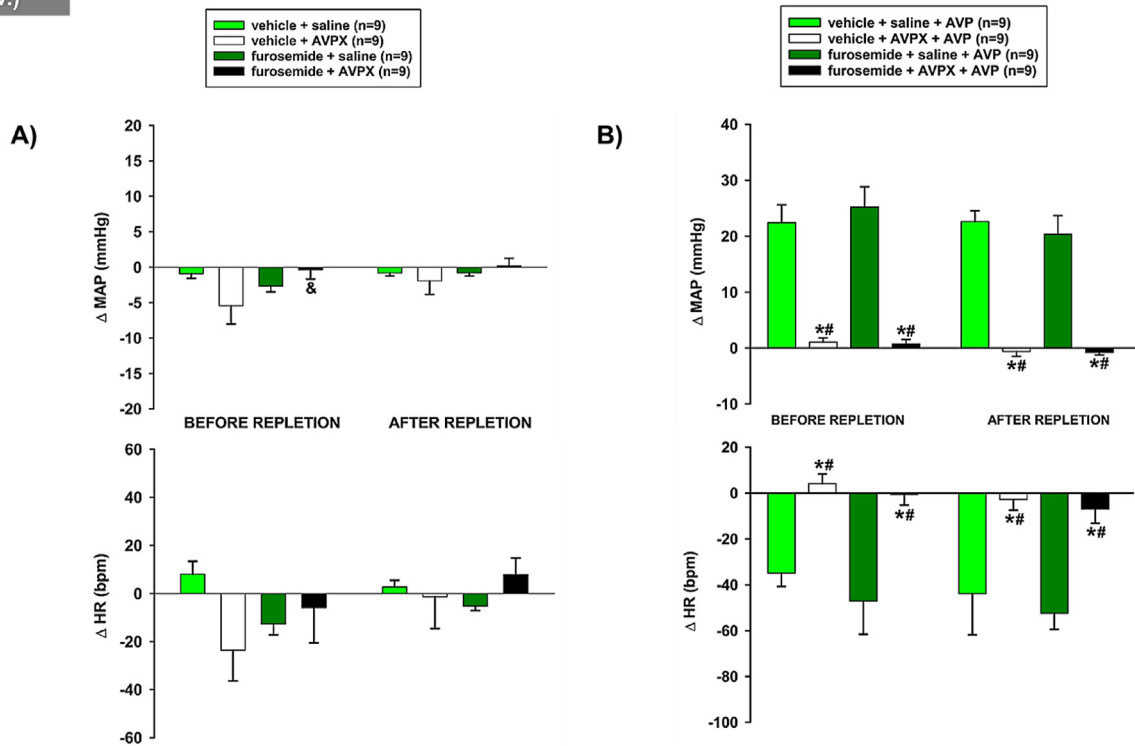
Figure 5. A) Variation in mean arterial pressure (ΔMAP) and heart rate (ΔHR) in rats treated with vehicle or furosemide s.c., combined with i.v. injection of saline or AVPX (10 μg/kg body wt) before and after sodium repletion (2 h of water and sodium 0.3 M intake). B) Variation in ΔMAP and ΔHR in rats after i.v. injection of AVP (12.5 ng/0.1 mL/per rat) 10 min after saline or AVPX injection (10 μg/kg body wt). The groups were treated with vehicle or furosemide s.c. and submitted to sodium repletion (2 h of water and sodium 0.3 M intake). Results are expressed as mean ± SEM. n = number of animals. Two-way ANOVA. "Before Depletion" and "After Repletion" situations were analysed separately. The charts with the p values for significant differences between the groups are presented in Figures S6 and S7 in the SI. * different from vehicle + saline + AVP. # different from furosemide + saline + AVP. & different from vehicle + AVPX.

vehicle + saline + AVP: Δ = −51 ± 16 bpm, p < 0.05, Figure 5B), as well as in depleted groups (furosemide + AVPX + AVP: Δ = −1 ± 1 mmHg vs. furosemide + saline + AVP: Δ = 20 ± 3 mmHg, p < 0.05, Figure 5B) and bradycardia (furosemide + AVPX + AVP: Δ = −7 ± 6 bpm vs. furosemide + saline + AVP: 45–45 ± 10 bpm, p < 0.05, Figure 5B).

6. Discussion

Our results, as well as previous results from our laboratory [6] demonstrate that sodium depletion did not modify baseline MAP, HR. However, this treatment increased baseline VE, due the increase in baseline VT, without significant changes in fR. Blood analysis showed no change in arterial PO_2 or PCO_2 and a slight alkalosis together with increased blood HCO_3^- in sodium depleted rats, which might be the result of the treatment with diuretic [27]. Nevertheless, alkalosis might also result from an increased loss of CO_2 due to the hyperventilation, however, arterial CO_2 was not modified in sodium depleted rats, suggesting that the alkalosis is probably more related to the effects of diuretic treatment. The consequence of a metabolic alkalosis like that produced by diuretic treatment would be a hypoventilation and not the hyperventilation found in sodium depleted rats. Sodium and fluid repletion reduced arterial PCO_2 and HCO_3^- and normalized arterial pH without changes in arterial PO_2. In spite of the differences in blood gases and pH compared to sodium depletion, hyperventilation was maintained after sodium/fluid repletion, which suggests that the hyperventilation is probably not related to changes in blood gases. The results showed also that plasma sodium, total protein and osmolarity, except plasma potassium, returned to normal by sodium/fluid repletion. Therefore, only plasma potassium was low in sodium depleted and sodium/fluid repleted

condition, which suggests that low plasma potassium might be a reason for the hyperventilation.

Peripheral injections of losartan caused an attenuation in MAP not only in control rats, but also in sodium depleted rats, results already reported by De Luca et al. (1996) [6]. However, our results demonstrate that the reduction of MAP observed with losartan injection in sodium depleted rats (furosemide + losartan) was more accentuated when compared with control animals (vehicle + losartan). After sodium repletion, peripheral injection of losartan also attenuated MAP in control (vehicle + losartan) and depleted (furosemide + losartan) animals, but the significant difference between these groups disappeared. In fact, more studies are needed to investigate whether previous losartan treatment, given to this group before repletion, induced this impaired decrease in blood pressure or 0.3 M NaCl repletion and water intake re-stablished the ANG II activity. In addition, the peripheral treatment with losartan also produced differences in HR in control and sodium depleted rats, however, the HR elevation observed in the latter group was greater when compared with control animals. The peripheral treatment with losartan was effective in blocking the pressor responses of ANG II before and after repletion. Before sodium repletion, groups that did not receive losartan showed the classic ANG II response (hypertension and bradycardia). Although, the sodium depleted group (furosemide + saline) presented an attenuated response to ANG II when compared to the control group (vehicle + saline), a behaviour already reported by Colombari *et al.* (1992) [15].

For the central antagonism of ANG II receptors with losartan, there was no variation in MAP between control and sodium depleted rats with furosemide, before or after sodium repletion. Additionally, there were also no significant changes in HR. These results suggest that the central

mechanisms that regulate blood pressure in rats with sodium depletion may not be dependent on RAS. Before water and sodium repletion, the central treatment with losartan was effective in blocking the pressor responses of ANG II in control groups; however, there was no significant difference between depleted groups. After water and sodium repletion, the pressor response of ANG II was attenuated in both depleted and control groups. Our results also showed differences between vehicle + losartan + ANG II and furosemide + saline + ANG II groups, before and after repletion, showing the central losartan dose of 3.3 μg/kg of body wt was effective in attenuating the ANG II pressure response. According to De Luca *et al.* (1996) [6], the AT1 antagonist losartan promotes an increase in MAP in doses of 6, 25, 12,5, 25, 50, 100 e 200 nmol/2 μL when injected in the 3rd ventricule of sodium depleted and repleted rats and in the 4th ventricule of sodium repleted rats. It is uncertain whether the observed hypertensive effect is related to the agonist effects of ANG II receptors in the brain [7]. Furthermore, this hypertensive effect contrasts with our central pressor results, as the dose of 3.3 μg/kg of body wt performed into the LV, a region very close to the primary target for ANG II's central circulating actions, the subfornical organ [28], did not produce any pressor response. In addition to the renin-angiotensin system, another factor that could be contributing to the maintenance of blood pressure in sodium-depleted animals is the sympathetic nervous activity, however, unpublished studies from our laboratory carried out in anesthetized animals (2–3% isoflurane) showed that renal sympathetic nerve activity was similar and that the treatment with the ganglionic blocker (hexamethonium, 10 mg/kg of body weight, i.v) promoted a similar decrease in BP of depleted animals compared to controls, suggesting that sympathetic activity would be the same in both groups. Though, anesthesia is a factor that depresses sympathetic activity and it would be interesting to perform these experiments in non-anesthetized animals.

The antagonism association of vasopressin V1 receptors with injected Manning compound peripherally i.v. did not produce variations in MAP or HR baselines in control or depleted groups. Similar results were published by Jover *et al.* (1987), who demonstrated that the intraventricular infusion treatment with [d(CH&Tyr(Me)AVP) (AVPA, also a receptor V1 antagonist), did not show a relevant effect on blood pressure, heart rate, cardiac output, total peripheral resistance or regional blood flow in sodium-depleted animals treated during 6 days with low sodium diet [10]. Nevertheless, MAP in furosemide + AVPX group was lower when compared to vehicle + AVPX, before repletion. Although, groups that received Manning compound had their i.v. AVP responses blocked, showing significant difference when compared with groups that did not receive the treatment (vehicle + saline and furosemide + saline). The former groups presented the classic response to i.v. AVP injection (hypertension and bradycardia), with no differences between control and depleted groups. The same behavior was observed following sodium repletion, in accordance with Jover *et al* (1987), who postulates that endogenous vasopressin acts as a vasoconstrictor hormone, especially in the kidney in sodium depletion, when the two systems, either renin-angiotensin or α-adrenergic, are inhibited [10]. Thus, variations in MAP and HR triggered by losartan i.v. may indicate that peripheral RAS acts independently of endogenous AVP in the maintenance of cardiovascular parameters in sodium depleted rats. Due to lack of peripheral cardiovascular response, we decided not to proceed with experiments on the central vasopressin system.

7. Conclusion

Our results suggest that changes produced by sodium depletion affect cardiorespiratory control increasing baseline VT and ventilation. Moreover, peripheral but not central angiotensinergic mechanisms are relevant for maintaining cardiovascular parameters in sodium depleted rats and vasopressin V1 receptors do not participate in the mechanisms of maintenance of MAP in these animals.

Declarations

Author contribution statement

Fernanda Cardoso: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Michele Thaís Fávero, Nathalia Vieira Veríssimo: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Miguel Furtado Menezes: Performed the experiments.

José Vanderlei Menani: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Patricia Maria de Paula: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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