RESEARCH ARTICLE

Inhibition of Endothelin system during the postnatal nephrogenic period in the rat. Its relationship with hypertension and renal disease in adulthood

María Florencia Albertoni Borghese1,2, María del Carmen Ortiz1, Rocío C. Marinoni1, Lucas H. Oronel1, Milena Palamidesi1, Carolina A. Yarza1, Nicolás Di Siervi3, Carlos Davio3,4, Mónica P. Majowicz1,*

1 Departamento de Ciencias Biológicas, Cátedra de Biología Celular y Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, 2 Departamento de Ciencias Biológicas, Cátedra de Biología Celular y Molecular, CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, 3 CONICET, Facultad de Farmacia y Bioquímica, Instituto de Investigaciones Farmacológicas (ININFA), Universidad de Buenos Aires, Buenos Aires, Argentina, 4 Departamento de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

* mmajow@ffyb.uba.ar

Abstract

The aim of this work was to study the effect of a high sodium (HS) diet on blood pressure and renal function in male adult rats that have been treated with a dual Endothelin receptor antagonist (ERA) during their early postnatal period (day 1 to 20 of life). Male Sprague-Dawley rats were divided in four groups: CNS: control rats with normosodic diet; ERA: treated rats with normosodic diet; CHS: control rats with high sodium diet; ERAHS: ERA-treated rats with HS diet. Systolic blood pressure (SBP) was recorded before and after the diet and 24-hour metabolic cage studies were performed. AQP2 and αENaC expressions were measured by western blot and real time PCR in the renal medulla. Vasopressin (AVP) pathway was evaluated by measuring V2 receptor and adenylyl cyclase 6 (AC6) expression and cAMP production in the renal medulla. Pre-pro ET-1mRNA was also evaluated in the renal medulla. Only rats that had been treated with an ERA during their postnatal period increased their SBP after consumption of a HS diet, showing an impaired capacity to excrete sodium and water, i.e. developing salt sensitivity. This salt sensitivity would be mediated by an increase in renomedullary expression and activity of AQP2 and αENaC as a consequence of increased AC6 expression and cAMP production and/or a decreased ET-1 production in the renal medulla. The knowledge of the molecular mechanisms underlying the perinatal programming of salt sensitive hypertension will allow the development of reproprogramming strategies in order to avoid this pathology.
Introduction

The developing embryo and/or fetus is highly sensitive to perturbations of the maternal environment. Adverse environmental factors (nutritional factors, physiologic or psychological stress, endocrine imbalance, ingestion or exposition to drugs among others) can disturb the processes of cell proliferation and differentiation, leading to changes in the normal developmental pathways of mature organs and tissues [1–3]. The developing organs can mount an adaptive response in order to ensure survival and the maintenance of critical functions of the tissues. However, these adaptive responses may represent an increased risk for diseases later in life; a process known as “Disease programming” [3]. The double hit hypothesis proposes that a genetic or environmental first hit during critical periods of development makes an individual more susceptible to a second hit later in life [4].

Bearing in mind that renal development in rodents continues along the early postnatal period, not only the fetus is at a risk for developmental disease programming, but also the neonate. Endothelin (ET) has a relevant role during embryonic development since KO animals for any component of ET system have a lethal phenotype [5–8]. However, the role of ET system during the postnatal period is not completely understood. We have shown previously that the inhibition of ET system in the rat with a dual ET receptor antagonist (ERA) during the early postnatal period affects both renal structure and function, decreasing the number of glomeruli, the juxtamedullary filtration surface area and the glomerular filtration rate and increasing the proteinuria, being these effects more pronounced in male rats [9]. It is widely accepted that a reduced glomerular number predisposes to hypertension and to kidney disease in the adulthood [10–12]. Brenner and colleagues postulated that reduced filtration surface area associated with a low nephron number would lead to sodium retention and development of systemic hypertension as a compensatory response to maintain sodium homeostasis [10, 13, 14].

The final control of sodium and water reabsorption takes place in the collecting duct (CD) through the amiloride-sensitive epithelial sodium channel (ENaC) and aquaporin-2 (AQP2) water channel respectively [15]. Both transporters are regulated by vasopressin (AVP) through V2 receptors [16]. Considering that ENaC is responsible for the fine-tuning of sodium reabsorption in the last nephron segment, the role of this channel in sodium reabsorption in the kidney is critical to maintain sodium and volume homeostasis and to control arterial blood pressure [17–20]. Excessive AVP-dependent ENaC stimulation could be a risk factor for sodium retention, leading to an increase in blood pressure [21].

ENaC expression and activity is tightly regulated by both aldosterone-dependent and aldosterone-independent mechanisms [22, 23]; endocrine as well as local autocrine and paracrine factors play a critical role in the modulation of ENaC, such as ET and purinergic system [17, 24]. Mice with CD-specific knockout for ET-1 are hypertensive and had reduced sodium excretion in response to sodium loading [25]. ET-1 inhibits AVP action at both cortical and medullary CD level, being this effect mediated, at least in part, by PKC-sensitive inhibition of adenylyl cyclase (AC) activity [26–28]. Moreover, CD-specific knockout of ET-1 resulted in increased sensitivity to the hydroosmotic and cAMP-stimulating effects of AVP [29] and is associated with an increase in AC6 protein abundance [27], the protein that mediates AVP-stimulated ENaC activity in the kidney [30].

On these bases, the aim of this work was to study the effect of a high sodium diet in adult rats that have been treated with a dual ERA during their early postnatal period (day 1 to 20 of life). Our hypothesis was that the renal alterations produced by ET system inhibition during the postnatal period predispose to hypertension during adulthood, especially after a second adverse impact, in this case a high sodium diet. This salt sensitivity would be mediated by an increased expression and/or activity of AQP2 and α-ENaC in the animals treated with an ERA.
during their early postnatal life as a consequence of an exacerbated AVP pathway and/or a decreased renal medullary ET production.

**Materials and methods**

**Animals and treatments**

Sprague Dawley (SD) rats were purchased from the School of Pharmacy and Biochemistry from the University of Buenos Aires. Protocols were designed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the American Physiological Society “Guiding Principles in the Care and Use of Animals” and to the 6344/96 regulation of Argentinean National Drug Food and Medical Technology Administration (ANMAT) and were approved by the Institutional Committee for Use and Care of Laboratory Animals from the School of Pharmacy and Biochemistry (Cudap N˚78096/18; Res D 1388). All rats were housed in rooms with controlled temperature (24˚C) and 12 h. dark-light cycle. Food and water were supplied ad libitum. Adult female SD rats (approx. 250 g body weight) were mated by exposure to a fertile SD male during 1 week. After birth, litter size was fixed in 10±1. Litters with less than 9 pups were excluded. Newborn rats were treated daily from postnatal day 1 to postnatal day 20 with vehicle (distilled water) or with Bosentan (Actelion, 20 mg/kg/day), a dual ERA, which was administered orally with a micropipette. Blockade of ET receptors was performed during the first 20 days of life, comprising all the lactation period, since in rats growth and maturation of the kidney also continue after the completion of nephrogenesis and it has been considered that nephrons reach terminal differentiation at the time of weaning [9].

After weaning, the animals were allowed to grow up to 65–70 days old and at that point they received a normal sodium diet (NS; 0,3% ClNa) or a high sodium diet (HS; 8% ClNa) [31–33] for 8 days. In this study we will only show results corresponding to male groups, so, 4 groups were conformed: C<sub>NS</sub>: control rats with normosodic diet; ERA<sub>NS</sub>: ERA-treated rats with normosodic diet; C<sub>HS</sub>: control rats with high sodium diet; ERA<sub>HS</sub>: ERA-treated rats with high sodium diet.

Food and water consumption was measured daily. Arterial blood pressure was determined before and after the administration of the diets. At the end of the experiment the animals were anesthetized with ketamine/xylacine (100 and 10 mg/kg respectively), blood samples were obtained by cardiac puncture, and the kidneys were immediately excised, weighed and processed.

**Determinations in the 24-hour metabolic cage studies**

Twenty four-hour urine samples were collected using metabolic cages. Animals were allowed to acclimatize to metabolic cages for two days and then fasted for 24 h before the collection of urine. Urine samples were analyzed for total protein using a kit provided by Wiener (Proti U/LCR; Wiener Lab., Rosario, Argentina). Urinary and plasmatic sodium and potassium concentrations were evaluated using an ion analyzer (Tecnolab; Mod T-412). Kinetic determinations of serum and urinary creatinine concentrations were evaluated using a kit provided by Wiener (Wiener Lab., Rosario, Argentina). Urine volume was measured gravimetrically.

**Determination of systolic blood pressure**

Systolic BP was recorded by triplicate before and after the administration of the normosodic or high sodium diet in conscious rats by tail plethysmography (ADInstruments PowerLab 8/30 and NIBP Controller ML125).
Histological and histochemical evaluation

The left kidneys were decapsulated and cut longitudinally, fixed in phosphate buffered 10% formaldehyde, pH 7.4, embedded in paraffin wax and cut to a thickness of 5μm. Renal tissue sections were stained with hematoxylin and eosin (H-E) for histological evaluation. Kidney sections were subjected to Masson’s trichrome and Sirius red staining to determine the presence of early fibrosis. At least ten fields of each renal zone from three animals of each group were analyzed.

Histochemistry. Masson’s trichrome staining. Masson’s trichrome staining was carried out and the proportion of blue-stained fibrotic area in the different renal zones of each section was graded semiquantitatively (0: ≤5%, 1: 5% to 25%, 2: 25% to 50%, 3: 50% to 75%, 4: ≥75%). These examinations were performed blindly by two researchers and the mean values were calculated [34].

Sirius red staining. Collagen accumulation was examined in the renal sections with the collagen-specific stain picrosirius red (Sirius Red 3 in a saturated aqueous solution of picric acid and fast green as a counterstain). Sirius Red staining is a method for collagen determination, enabling quantitative morphometric measurements to be performed in locally defined tissue areas [35]. Staining was scored as 0 (normal and slight staining surrounding the tubular, glomerular, and vascular structures), 1 (weak staining that doubles the normal label surrounding the tubular, glomerular, and vascular structures), 2 (moderate staining in the peritubular interstitium and inside the glomeruli), 3 (strong staining that replaces the glomerular and tubular structures, comprising <25% of the cortical area), or 4 (strong staining that replaces the glomerular and tubular structures, compromising >25% of the cortical area).

Image capture and analysis. Images from histological and histochemical sections were captured using a Nikon Alphaphot-2 YS2 light microscope (Nikon Instrument Group, Melville, NY), coupled to a Sony color video camera digital (Model N° SSC-DC50A). All determinations were performed blindly and under similar light, gain and offset conditions by the same researcher.

Tissue processing for Western blot analysis. Immediately after the animals were sacrificed, their kidneys were isolated and the renal medulla was dissected and homogenized at 3,000 rpm in an appropriate buffer (250 mmol/l sucrose, 1 mmol/l EDTA, 0.1 mmol/l PMSF and 10 mmol/l Tris-ClH), pH 7.6. Large tissue debris and nuclear fragments were removed by a low-speed spin (1000 g, 10 min, 4˚C). Protein concentration was measured using BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Absorbances for protein concentration measurements were read using a RT-2100C microplate reader (Rayto, China) at 560 nm.

Western blots for AQP2 and α-ENaC. Western blot analysis was used to identify AQP2 and α-ENaC. We evaluated α-ENaC because this is the rate-limiting subunit to form the functional channel [23]. Blots were incubated overnight at 4˚C with the AQP2 antibody (mouse monoclonal anti-rat IgG1 AQP2 [sc 515770]; Santa Cruz Biotechnology, Inc., CA, USA) diluted in blocking solution (1:200), or with α-ENaC antibody (rabbit anti-rat; diluted 1:500; Santa Cruz Biotechnology, Inc. California, USA). Beta-tubulin was used as loading control (rabbit anti-rat beta-tubulin; Abcam Inc., Cambridge, MA, USA). The membranes were then incubated with a donkey anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (1:3000) (Abcam Inc., Cambridge, MA) for α-ENaC and tubulin and were incubated with mlGx BP-HRP (sc-516102) HRP conjugated for AQP2 blots (1:2000); Santa Cruz Biotechnology, Inc. California, USA. Blots were visualized using Super SignalTM West Pico Plus chemiluminescent substrate (Thermo Scientific; Rockford, IL, USA).

The relative protein levels were determined by analyzing the bands with Gel Pro Analyzer 3.1 for Windows and relative protein expression was calculated as the ratio protein of interest/
β-tubulin. The AQP2 antibody recognizes a 28-kDa band corresponding to unglycosylated AQP2 and bands between 35–40 kDa representing glycosylated forms of the protein. The α-ENaC antibody recognizes a 78-kDa band and the β-tubulin antibody recognizes a 50kDa band.

Real-time PCR for AQP2, α-ENaC, V2 receptor, adenyl cyclase-6 and Pre-pro-ET-1. Total RNA was isolated using the SV total RNA Isolation System (Promega, Madison, WI, USA). Total RNA was reverse transcribed to cDNA using a high capacity reverse transcription kit (A&B, CA, USA). For real-time detection of AQP2 transcripts and the reference gene (GAPDH), MezclaReal (Real Time PCR commercial mixture from Biodynamics, Argentina) and specific primers were used [36, 37].

The normalized gene expression method ($2^{ΔΔCT}$) for the relative quantification of gene expression was used. The difference in the cycle threshold (CT) for AQP2 and GAPDH for the control untreated rats was subtracted from the difference in the CT for AQP2 and GAPDH for each of the experimental groups [38]. The following formula was applied:

$ΔΔCT = (CT_{AQP2} - CT_{GAPDH})_{experimental} - (CT_{AQP2} - CT_{GAPDH})_{control\ untreated\ rats}$.

The real-time PCR started at 94˚C for 2 min and was followed by 35 thermal cycles at 94˚C for 15 s, 58˚C for 35 s and 72˚C for 30 s.

cAMP measurements. cAMP was measured in the renal medulla. Approximately 100 mg of renal medulla was homogenized in ice-cold absolute ethanol and centrifuged for 15 min at 1200g. The supernatant was dried, and the remaining residue was suspended for cAMP determination by competition of [3H]-cAMP for PKA [39]. Results were expressed as pmoles cAMP/mg of protein.

Statistics. Two-way ANOVA with Bonferroni’s post-test for multiple comparisons was performed using Graph Pad Prism version 5.0 for Windows.

Results
Characterization of the experimental model
As can be seen in Fig 1, systolic blood pressure (SBP), expressed as SBP percentage difference, only increased significantly (20% increment) in ERA_HS vs both C_HS and ERA_NS (p<0.01).

Table 1 shows weights and consumptions for the different experimental groups. There were no significant changes in body weight (b.w.) or femur length among experimental groups. However, there was a significant increase in renal weight expressed as g/100g of body weight in both C_HS and ERA_HS when compared with their respective NS controls (p<0.01 in both cases). Food intake, expressed per 100g b.w. was similar in all groups of rats, suggesting that the differences seen in blood pressure were not due to different sodium intakes. As expected, water intake significantly increased in the groups that received HS diet, being this increment of the same magnitude in both groups (p<0.001 vs NS groups).

Renal and plasmatic functional parameters
Diuresis significantly increased in both groups that received HS diet when compared with their respective NS groups (‘p<0.05 C_HS vs C_NS; #p<0.05 ERA_HS vs ERA_NS). However, this increment was of lower magnitude in ERA_HS than in C_HS rats. Fractional sodium excretion significantly increased in both groups that received HS diet when compared with their respective NS groups (‘‘p<0.001 vs C_NS; ‘‘#p<0.001 vs ERA_NS). However, this increment was significantly lower (‘&p<0.05 vs C_NS) in ERA_HS than in C_HS rats. Kaliuresis was significantly lower in both HS groups when compared with their respective NS controls (‘‘p<0.01 C_HS vs
Creatinine clearance significantly increased in both HS groups when compared with their respective NS controls (\(p<0.05 \text{ C}_{\text{HS}} \text{ vs } \text{ C}_{\text{NS}}\); \(\#p<0.01 \text{ vs } \text{ ERA}_{\text{NS}}\)). Proteinuria significantly increased in both groups that received HS diet vs their respective controls with NS diet (\(p<0.05\) in both cases). Note that the values of proteinuria were higher (although not significant; \(p=0.1182\)) in the rats treated postnatally with the ERA when compared with both groups of control rats. Natremia increased in \(\text{ERA}_{\text{HS}}\) when

\[
\begin{array}{l}
\text{C}_{\text{HS}} \# \#p<0.01 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}.
\end{array}
\]

Creatinine clearance significantly increased in both HS groups when compared with their respective NS controls (\(p<0.05 \text{ C}_{\text{HS}} \text{ vs } \text{ C}_{\text{NS}}\); \(\#p<0.05 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}\)). Proteinuria significantly increased in both groups that received HS diet vs their respective controls with NS diet (\(p<0.05\) in both cases). Note that the values of proteinuria were higher (although not significant; \(p=0.1182\)) in the rats treated postnatally with the ERA when compared with both groups of control rats. Natremia increased in \(\text{ERA}_{\text{HS}}\) when

\[
\begin{array}{l}
\text{C}_{\text{NS}} \# \#p<0.01 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}.
\end{array}
\]

Creatinine clearance significantly increased in both HS groups when compared with their respective NS controls (\(p<0.05 \text{ C}_{\text{HS}} \text{ vs } \text{ C}_{\text{NS}}\); \(\#p<0.05 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}\)). Proteinuria significantly increased in both groups that received HS diet vs their respective controls with NS diet (\(p<0.05\) in both cases). Note that the values of proteinuria were higher (although not significant; \(p=0.1182\)) in the rats treated postnatally with the ERA when compared with both groups of control rats. Natremia increased in \(\text{ERA}_{\text{HS}}\) when

\[
\begin{array}{l}
\text{C}_{\text{NS}} \# \#p<0.01 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}.
\end{array}
\]

Creatinine clearance significantly increased in both HS groups when compared with their respective NS controls (\(p<0.05 \text{ C}_{\text{HS}} \text{ vs } \text{ C}_{\text{NS}}\); \(\#p<0.05 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}\)). Proteinuria significantly increased in both groups that received HS diet vs their respective controls with NS diet (\(p<0.05\) in both cases). Note that the values of proteinuria were higher (although not significant; \(p=0.1182\)) in the rats treated postnatally with the ERA when compared with both groups of control rats. Natremia increased in \(\text{ERA}_{\text{HS}}\) when

\[
\begin{array}{l}
\text{C}_{\text{NS}} \# \#p<0.01 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}.
\end{array}
\]

Creatinine clearance significantly increased in both HS groups when compared with their respective NS controls (\(p<0.05 \text{ C}_{\text{HS}} \text{ vs } \text{ C}_{\text{NS}}\); \(\#p<0.05 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}\)). Proteinuria significantly increased in both groups that received HS diet vs their respective controls with NS diet (\(p<0.05\) in both cases). Note that the values of proteinuria were higher (although not significant; \(p=0.1182\)) in the rats treated postnatally with the ERA when compared with both groups of control rats. Natremia increased in \(\text{ERA}_{\text{HS}}\) when

\[
\begin{array}{l}
\text{C}_{\text{NS}} \# \#p<0.01 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}.
\end{array}
\]

Creatinine clearance significantly increased in both HS groups when compared with their respective NS controls (\(p<0.05 \text{ C}_{\text{HS}} \text{ vs } \text{ C}_{\text{NS}}\); \(\#p<0.05 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}\)). Proteinuria significantly increased in both groups that received HS diet vs their respective controls with NS diet (\(p<0.05\) in both cases). Note that the values of proteinuria were higher (although not significant; \(p=0.1182\)) in the rats treated postnatally with the ERA when compared with both groups of control rats. Natremia increased in \(\text{ERA}_{\text{HS}}\) when

\[
\begin{array}{l}
\text{C}_{\text{NS}} \# \#p<0.01 \text{ ERA}_{\text{HS}} \text{ vs } \text{ ERA}_{\text{NS}}.
\end{array}
\]
compared with C_HS rats (\(p<0.05\)) while kalemia had a tendency to increase in both HS groups vs their respective NS controls (\(p = 0.1137\)). The results correspondents to renal and plasmatic parameters are shown in Table 2.

**Histological and histochemical evaluation**

The histological structure of rat kidneys in the H-E sections seemed to be unaffected (S1 Fig). The score for both Masson’s trichrome and Sirius red staining was <1 for all the groups in the different renal zones (Table 3); it means a normal and slight staining surrounding tubular, glomerular and vascular structures. There was a significant effect of the HS diet on Masson’s trichrome staining in the renal cortical and juxtamedullary areas. However, the scores were <1. Representative images are shown in S2, S3, S4, S5 and S6 Figs.

**AQP2 and α-ENaC expression**

AQP2 mRNA expression significantly decreased in C_HS group when compared with C_NSN (\(p<0.05\)), meanwhile failed to decrease in ERA_HSN. Moreover, AQP2 protein expression significantly decreased in C_HS group when compared with C_NSN (\(p<0.01\)) and in ERA_HSN group when compared with ERA_NSN (\(p<0.01\)). However, the expression of AQP2 protein was significantly higher in ERA_HSN when compared with C_HS (\(p<0.05\)). These results can be seen in Fig 2A and 2B.

α-ENaC mRNA expression significantly decreased in C_HS group when compared with C_NSN (\(p<0.05\)), meanwhile failed to decrease in ERA_HSN. Moreover, α-ENaC protein expression significantly decreased in C_HS group when compared with C_NSN (\(p<0.05\)) and in ERA_HSN group when compared with ERA_NSN (\(p<0.05\)). However, the expression of α-ENaC protein was higher although not significant (\(p = 0.1138\)) in ERA_HSN (0.72 ± 0.05) when compared with C_HS (0.54 ± 0.03). In fact, the administration of HS diet decreased 45.5% α-ENaC protein expression in control rats while decreased 25% α-ENaC protein expression in ERA rats. These results can be seen in Fig 3A and 3B.

The results related to AQP2 and α-ENaC in combination with those obtained when we evaluated renal parameters suggest that ERA_HSN rats have an impaired ability to excrete water and sodium.

### Table 2. Renal and plasma functional parameters.

| Parameter                        | C_NSN | ERA_NSN | C_HSN | ERA_HSN |
|----------------------------------|-------|---------|-------|---------|
| Diuresis (ml/24 h/100g b.w.)     | 1.9±0.5 | 2.2±0.4 | 3.5±0.6* | 2.9±0.5# |
| Fractional Na⁺ excretion (FENa %) | 0.43±0.03 | 0.36±0.03 | 1.37±0.15*** | 1.10±0.12### & |
| Kaliuresis (meq/24 h/100g b.w.)  | 0.38±0.02 | 0.51±0.13 | 0.24±0.07** | 0.23±0.06## |
| Creatinine clearance (ml/min/100g b.w.) | 0.28±0.04 | 0.37±0.04 | 0.42±0.05* | 0.53±0.06# |
| Proteinuria (mg/24 h/100g b.w.)  | 2.8±0.5 | 3.6±0.5 | 4.0±0.5* | 4.9±0.5# |
| Natremia (meq/L)                 | 143±1 | 145±1 | 141±2 | 148±2& |
| Kalemia (meq/L)                  | 4.2±0.3 | 4.3±0.4 | 4.6±0.8 | 5.2±0.3 |

ERA: Endothelin receptor antagonist. C_NSN: control rats with normosodic diet; ERA_NSN: ERA-treated rats with normosodic diet; C_HSN: control rats with high sodium diet; ERA_HSN: ERA-treated rats with high sodium diet. Data were analyzed using two-way ANOVA followed by Bonferroni posttest.

\(p<0.05\) vs C_NSN
\(p<0.01\) vs C_NSN
\(p<0.001\) vs C_NSN
\(p<0.05\) vs ERA_NSN
\(p<0.01\) vs ERA_NSN
\(p<0.001\) vs ERA_NSN
\(p<0.05\) vs C_HSN.

Data are mean ± SEM of 9 independent determinations.

https://doi.org/10.1371/journal.pone.0229756.t002
Participation of AVP pathway on AQP2 and α-ENaC altered expression in ERA HS

Bearing in mind that AVP regulates both AQP2 and α-ENaC [16], we decided to evaluate if this pathway was implicated in our experimental model, so we evaluated V2 receptor mRNA expression, AC6 mRNA expression and renomedullary cAMP production.

AVP-V2 receptor expression

V2 receptor mRNA expression was significantly lower in ERA NS vs C NS (p < 0.05) and in ERA HS vs C HS (p < 0.05), suggesting that the increased expression of AQP2 and α-ENaC in ERA HS rats would not be due to a greater level of V2 receptor expression. This result can be seen in Fig 4.

AC6 mRNA expression

AC6 mRNA expression increased in ERA HS vs C HS group (p < 0.05). Besides, ERA NS group had a higher level of AC6 expression than C NS (p < 0.05). This result can be seen in Fig 5A.

Renomedullary cAMP production

There were no significant differences between groups in renomedullary cAMP production. However, there was a tendency to increase cAMP production (expressed as pmoles cAMP/g protein) in ERA HS when compared with ERA NS (p = 0.1655) rats meanwhile that tendency was not seen in C HS when compared with C NS (p = 0.8204). Although cAMP production in ERA HS was not significantly different from the value obtained for C HS, it was 25% higher. Besides, cAMP production in ERA HS was 51% higher than in ERA NS (C NS: 2.93 ± 0.36; ERA NS: 2.47 ± 0.69; C HS: 2.82 ± 0.29; ERA HS: 3.74 ± 0.49). These results can be seen in Fig 5B.

Regarding AC6 expression in our experimental model, it was significantly increased in both groups of ERA-treated rats. However, renomedullary cAMP had a tendency to increase only in ERA HS rats, suggesting a greater activity of AC6 in this group. Thus it is probably that HS diet differently regulates AC6 activity in ERA-treated rats than in control rats. We must consider that 8 days is an acute period of time; possibly a chronic salt consumption will show more significant changes.

Table 3. Histochemical evaluation: Masson’s trichrome and Sirius red staining.

|                      | C NS | C HS | ERA NS | ERA HS |
|----------------------|------|------|--------|--------|
| Masson’s trichrome score (CA) | 0.70±0.11 | 0.95±0.05* | 0.80±0.09 | 0.90±0.07# |
| Masson’s trichrome score (JA) | 0.75±0.10 | 0.96±0.05* | 0.81±0.09 | 0.91±0.07# |
| Masson’s trichrome score (Medulla) | 0.65±0.11 | 0.70±0.11 | 0.75±0.10 | 0.80±0.09 |
| Masson’s trichrome score (Papilla) | 0.33±0.13 | 0.41±0.12 | 0.46±0.14 | 0.67±0.14 |
| Sirius red score (CA) | 0.35±0.11 | 0.50±0.11 | 0.45±0.11 | 0.43±0.11 |
| Sirius red score (JA) | 0.80±0.09 | 0.90±0.10 | 0.85±0.08 | 0.90±0.07 |
| Sirius red score (Medulla) | 0.55±0.11 | 0.59±0.11 | 0.55±0.11 | 0.65±0.11 |
| Sirius red score (Papilla) | 0.33±0.13 | 0.44±0.13 | 0.30±0.15 | 0.71±0.13 |

ERA: Endothelin receptor antagonist. C NS: control rats with normosodic diet; ERA NS: ERA-treated rats with normosodic diet; C HS: control rats with high sodium diet; ERA HS: ERA-treated rats with high sodium diet. Data were analyzed using two-way ANOVA followed by Bonferroni posttest.

*p < 0.05 vs C NS; #p < 0.05 vs ERA NS. Data are expressed as mean ± SEM. n = 3 rats/group and at least 10 fields/animal were analyzed.

https://doi.org/10.1371/journal.pone.0229756.t003
Pre-pro ET-1 production is decreased in the renal medulla of ERA_{HS} rats

Bearing in mind that medullary ET-1 is fundamentally important in physiologic regulation of renal sodium and water excretion and maintenance of normal systemic blood pressure, we measured mRNA pre-pro ET-1 by real time PCR. As expected, pre-pro ET-1 significantly...
Fig 4. Vasopressin V2 receptor expression in the renal medulla. Vasopressin V2 receptor mRNA levels are expressed as relative values from C\textsubscript{NS} rats. The following formula was applied: ΔΔCT = (CT\textsubscript{V2 receptor}-CT\textsubscript{GAPDH}) \text{experimental} – (CT\textsubscript{V2 receptor}-CT\textsubscript{GAPDH}) \text{C\textsubscript{NS} rats}. ERA: Endothelin receptor antagonist; NS: normosodic diet; HS: high sodium diet (8% NaCl). Data were analyzed using Two-way ANOVA followed by Bonferroni posttest. *p<0.05 vs C\textsubscript{NS}; &p<0.05 vs C\textsubscript{HS}. Data are mean ± SEM of five independent determinations.

https://doi.org/10.1371/journal.pone.0229756.g004

Fig 5. Adenylyl cyclase 6 expression and cAMP production in the renal medulla. A. Adenylyl cyclase 6 (AC6) mRNA levels are expressed as relative values from C\textsubscript{NS} rats. The following formula was applied: ΔΔCT = (CT\textsubscript{AC6}-CT\textsubscript{GAPDH}) \text{experimental} – (CT\textsubscript{AC6 receptor}-CT\textsubscript{GAPDH}) \text{C\textsubscript{NS} rats}. ERA: Endothelin receptor antagonist; NS: normosodic diet; HS: high sodium diet (8% NaCl). Data were analyzed using Two-way ANOVA followed by Bonferroni posttest. *p<0.05 vs C\textsubscript{NS}; &p<0.05 vs C\textsubscript{HS}. Data are mean ± SEM of four independent determinations. B. cAMP production assessed by competition of [3H]-cAMP for PKA. ERA: Endothelin receptor antagonist; NS: normosodic diet; HS: high sodium diet (8% NaCl). Data were analyzed using Two-way ANOVA followed by Bonferroni posttest. Data are mean ± SEM of five independent determinations.

https://doi.org/10.1371/journal.pone.0229756.g005
increased in C_{HS} vs C_{NS} rats (p<0.05). On the other hand, ERA_{NS} showed significantly lower expression of pre-pro ET-1 when compared with C_{NS} rats (p<0.05) and besides, ERA_{HS} failed to increase pre-pro ET-1 expression when compared with ERA_{NS} rats. These results can be observed in Fig 6.

**Discussion**

In this paper we show that adult male SD rats that had been treated with a dual ERA during their early postnatal period have a 20% increase in their blood pressure after 8 days consuming a high sodium diet. We had shown in a previous paper that the inhibition of the ET system with a dual ERA produces a decrease in nephron number [9]. Although low nephron number is not always associated with hypertension, offspring with diminished nephron number are more susceptible to a second insult or adverse impact [40]. The increase in blood pressure seen in ERA_{HS} rats was not due to higher food intake because there were no significant differences in this parameter among the different experimental groups. Besides, there was neither significant difference in body weight nor in femur length among the different experimental groups. High salt intake increased proteinuria in both control and ERA-treated rats in a similar magnitude (p<0.05) but ERA-treated rats on a NS diet already had a proteinuria 28% higher (although not significant; p = 0.1182) than control rats on a NS diet. Thus it is possible that the composition and/or the function of the glomerular filtration barrier had been affected during postnatal development in ERA-treated rats. In fact, it has been shown that both glomerular endothelial cells and podocytes express ET receptors and synthetize ET-1 and there is a cross-talk between these two cell types that may be pathologic if there is an imbalance in the ET system [41, 42].

On the other hand, ERA_{HS} rats showed a decreased ability to eliminate sodium and water when compared with C_{HS} group. This decreased ability to excrete sodium and water is in line with the higher plasmatic sodium levels and concomitantly with the higher blood pressure in ERA_{HS} group.

Bearing in mind that the final control of sodium and water reabsorption takes place in the CD through ENaC and AQP2 respectively [15], we evaluated the expression of these transporters. As expected, the expression of both α-ENaC and AQP2 mRNA and proteins decreased in C_{HS} vs C_{NS} rats but failed to decrease or decreased at a lower extent in ERA_{HS} rats. These and the above mentioned results suggest that ERA_{HS} rats have a decreased ability to excrete sodium and water during a sodium overload due to a higher level expression of sodium and water transporters ENaC and AQP2. In fact, ERA_{HS} showed a lower diuresis and fractional sodium excretion than C_{HS} rats. The lower diuresis and fractional sodium excretion in ERA_{HS} was not due to a decreased glomerular filtration rate because creatinine clearance was even higher in both HS groups when compared with their respective controls. Thus the decreased ability to excrete sodium and water during a sodium overload in ERA_{HS} rats would be due to increased sodium and water reabsorption mediated by ENaC and AQP2 respectively at CD level. Another result of the current study is that rats treated with a high sodium diet have a decreased ability to excrete K⁺. This result is in accordance with Jensen et al, who provide experimental data showing that ENaC activity is a rate-limiting element for powerful K⁺ excretion, hindering K⁺ excretion during high Na⁺ conditions [43].

It is well known that AVP, through V2-receptors, is the main regulator of AQP2, stimulating both its expression and translocation and thus promoting water reabsorption at CD level [44]. However, in the last years, it has re-emerged the concept that the ability of AVP to stimulate water reabsorption is possible by promoting discretionary sodium reabsorption via ENaC along the distal nephron and consequently decreasing sodium excretion [16]. Thus AVP uses V2
receptors coupled to Gs and stimulation of AC and production of cAMP as a common signaling pathway to increase both ENaC and AQP2 expression and activity [16]. In the current study we show that V2 receptor mRNA expression was decreased in ERA HS rats, thus the increased α-ENaC expression and decreased ability to excrete sodium was not consequence of an increased V2 receptor expression in our experimental model. However, we found that AC6 expression was increased and renomedullary cAMP had a marked tendency to increase in ERA HS rats when compared with C NS, so these results suggest that the increased ENaC and AQP2 expression seen in ERA HS rats would be a consequence of increased AC6 and concomitantly increased cAMP production in the renal medulla. In fact, AC6 in the CD regulates renal water excretion, most likely through control of AVP-stimulated cAMP accumulation and AQP2 [45] and it was recently shown that AC6 mediates AVP-stimulated ENaC activity in the kidney [30].

Another interesting result of the current study is that renomedullary Pre-pro-ET-1 production was decreased in ERA HS when compared with C HS. In fact, as expected, we found an increment in renomedullary Pre-pro-ET-1 production in control rats but we failed to find this increment in ERA rats after the administration of the high sodium diet. It is well-known that medullary ET-1 is fundamentally important in physiologic regulation of renal sodium and water excretion and maintenance of normal systemic blood pressure [46]. Mice with CD-specific knockout of the ET-1 gene have impaired sodium excretion in response to sodium loading and have hypertension

---

**Fig 6. Pre-pro ET-1 expression in the renal medulla.** Pre-pro ET-1 receptor mRNA levels are expressed as relative values from C NS rats. The following formula was applied: ΔΔCT = (CT Pre-pro ET-1 -CT GAPDH) experimental−(CT Pre-pro ET-1 receptor-CT GAPDH) C NS rats. ERA: Endothelin receptor antagonist; NS: normosodic diet; HS: high sodium diet (8% NaCl). Data were analyzed using Two-way ANOVA followed by Bonferroni posttest. *p<0.05 vs C NS. Data are mean ± SEM of four independent determinations.
which worsens with high salt intake [25]. Strait et al showed that CD ET-1 KO IMCD had greater sensitivity to forskolin than did control IMCD, suggesting that neither the V2 receptor nor G proteins can account for the increased cAMP levels in CD ET-1 KO mice, supporting a primary change in AC activity per se. They concluded that due to the known acute inhibitory effect of ET-1 on AVP-stimulated cAMP accumulation CD-derived ET-1 might exert a diuretic effect through both acute modulation of AC activity and chronic down-regulation of AC protein content [27]. Therefore, the enhanced AVP pathway in the ERA-treated rats that received a HS diet may be a consequence of the decreased renomedullary ET-1 production.

Conclusions
The results of the present study provide evidence that the inhibition of ET system during the early postnatal period in rodents could predispose to salt sensitive hypertension during adulthood. This salt sensitivity would be mediated by an increased renomedullary expression and activity of AQP2 and α-ENaC as a consequence of increased AC6 expression and cAMP production and/or a decreased ET-1 production in the renal medulla.

The knowledge of the molecular mechanisms underlying the perinatal programming of salt sensitive hypertension will allow the development of reprogramming strategies in order to prevent this pathology.

Supporting information
S1 Raw images.
(PDF)

S1 Fig. Hematoxylin-eosin staining of the renal cortex. Representative images for Hematoxylin-eosin staining of the renal cortex. A = C_NS (CA); B = C_HS (CA); C = ERA_NS (CA); D = ERA_HS (CA); E = C_NS(JA); F = C_HS (JA); G = ERA_NS (JA); H = ERA_HS (JA). C: control; ERA: Endothelin receptor antagonist. NS: normosodic diet; HS: high sodium diet. CA: cortical area; JA: juxtamedullary area. Total magnification: 400x.
(TIF)

S2 Fig. Hematoxylin-eosin staining of the renal medulla and papilla. Representative images for Hematoxylin-eosin staining of the renal medulla (A-D) and the renal papilla (E-H). A = C_NS; B = C_HS; C = ERA_NS; D = ERA_HS; E = C_NS; F = C_HS; G = ERA_NS; H = ERA_HS. C: control; ERA: Endothelin receptor antagonist. NS: normosodic diet; HS: high sodium diet. Total magnification: 400x.
(TIF)

S3 Fig. Masson’s trichrome staining of the renal cortex. Representative images for Masson’s trichrome staining of the renal cortex. A = C_NS (CA); B = C_HS (CA); C = ERA_NS (CA); D = ERA_HS (CA); E = C_NS(JA); F = C_HS (JA); G = ERA_NS (JA); H = ERA_HS (JA). C: control; ERA: Endothelin receptor antagonist. NS: normosodic diet; HS: high sodium diet. CA: cortical area; JA: juxtamedullary area. Total magnification: 400x.
(TIF)

S4 Fig. Masson’s trichrome staining of the renal medulla and papilla. Representative images for Masson trichrome staining of the renal medulla (A-D) and the renal papilla (E-H). A = C_NS; B = C_HS; C = ERA_NS; D = ERA_HS; E = C_NS; F = C_HS; G = ERA_NS; H = ERA_HS. C: control; ERA: Endothelin receptor antagonist. NS: normosodic diet; HS: high sodium diet. Total magnification: 400x.
(TIF)
S5 Fig. Sirius red staining of the renal cortex. Representative images for Sirius red staining of the renal cortex. A = C_{NS} (CA); B = C_{HS} (CA); C = ERA_{NS} (CA); D = ERA_{HS} (CA); E = C_{NS} (JA); F = C_{HS} (JA); G = ERA_{NS} (JA); H = ERA_{HS} (JA). C: control; ERA: Endothelin receptor antagonist. NS: normosodic diet; HS: high sodium diet. CA: cortical area; JA: juxtamedullary area. Total magnification: 400x.

S6 Fig. Sirius red staining of the renal medulla and papilla. Representative images for Sirius red staining of the renal medulla (A-D) and the renal papilla (E-H). A = C_{NS}; B = C_{HS}; C = ERA_{NS}; D = ERA_{HS}; E = C_{NS}; F = C_{HS}; G = ERA_{NS}; H = ERA_{HS}. C: control; ERA: Endothelin receptor antagonist. NS: normosodic diet; HS: high sodium diet. Total magnification: 400x.

Acknowledgments

We thank Actelion Pharmaceuticals Ltd. for the kind gift of Bosentan. We thank Sabrina Balonga, Ana Laura Filipuzzi and Agustina Lavagna for their technical assistance in the first stages of the study.

Author Contributions

Conceptualization: Mónica P. Majowicz.

Formal analysis: María Florencia Albertoni Borghese, María del Carmen Ortiz, Rocío C. Marinoni, Lucas H. Oronel, Milena Palamidessi, Carolina A. Yarza, Nicolás Di Siervi, Carlos Davio, Mónica P. Majowicz.

Funding acquisition: Mónica P. Majowicz.

Investigation: María Florencia Albertoni Borghese, María del Carmen Ortiz, Rocío C. Marinoni, Lucas H. Oronel, Milena Palamidessi, Carolina A. Yarza, Mónica P. Majowicz.

Methodology: María Florencia Albertoni Borghese, María del Carmen Ortiz, Rocío C. Marinoni, Lucas H. Oronel, Milena Palamidessi, Carolina A. Yarza, Nicolás Di Siervi, Carlos Davio.

Project administration: Mónica P. Majowicz.

Resources: María Florencia Albertoni Borghese, Carlos Davio.

Supervision: Mónica P. Majowicz.

Writing – original draft: María Florencia Albertoni Borghese, María del Carmen Ortiz, Mónica P. Majowicz.

Writing – review & editing: María Florencia Albertoni Borghese, María del Carmen Ortiz, Rocío C. Marinoni, Lucas H. Oronel, Milena Palamidessi, Carolina A. Yarza, Carlos Davio, Mónica P. Majowicz.

References

1. Puddu M, Fanos V, Podda F, Zaffanello M. The kidney from prenatal to adult life: perinatal programming and reduction of number of nephrons during development. Am J Nephrol. 2009; 30(2):162–70. https://doi.org/10.1159/000211324 PMID: 19339773

2. Tomat AL, Costa Mde L, Arranz CT. Zinc restriction during different periods of life: influence in renal and cardiovascular diseases. Nutrition. 2011; 27(4):392–8. https://doi.org/10.1016/j.nut.2010.09.010 PMID: 21074972
3. Langley-Evans SC, McMullen S. Developmental origins of adult disease. Med Princ Pract. 2010; 19 (2):87–98. https://doi.org/10.1159/000273066 PMID: 20134170
4. Maynard TM, Sikich L, Lieberman JA, LaMantia AS. Neural development, cell-cell signaling, and the "two-hit" hypothesis of schizophrenia. Schizophr Bull. 2001; 27(3):457–76. https://doi.org/10.1093/schbul/a06887 PMID: 11596847
5. Barni T, Fantoni G, Gloria L, Maggi M, Peri A, Balsi E, et al. Role of endothelin in the human craniofacial morphogenesis. J Craniofac Genet Dev Biol. 1998; 18(4):183–94. PMID: 10100047
6. Clouthier DE, Williams SC, Yanagisawa H, Wieduwilt M, Richardson JA, Yanagisawa M. Signaling pathways crucial for craniofacial development revealed by endothelin-A receptor-deficient mice. Dev Biol. 2000; 217(1):10–24. https://doi.org/10.1006/dbio.1999.9527 PMID: 10625532
7. Yanagisawa H, Yanagisawa M, Kapur RP, Richardson JA, Baynash AG, Cheung JC, Giaid A, et al. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell. 1994; 79(7):1267–76. https://doi.org/10.1016/0092-8674(94)90017-5 PMID: 8001159
8. Albertoni Borghese MF, Ortiz MC, Balonga S, Moreira Szokalo R, Majowicz MP. The Role of Endothelin System in Renal Structure and Function during the Postnatal Development of the Rat Kidney. PLoS One. 2016; 11(2):e0148866. https://doi.org/10.1371/journal.pone.0148866 PMID: 26872270 PMCID: PMC4752218
9. Brenner BM, Garcia DL, Anderson S. Glomeruli and blood pressure. Less of one, more the other? Am J Hypertens. 1988; 1(4 Pt 1):335–47. https://doi.org/10.1093/ajh/1.4.335 PMID: 3063284
10. Brenner BM, Mackenzie HS. Nephron mass as a risk factor for progression of renal disease. Kidney Int Suppl. 1997; 63:S124–7. PMID: 9407439
11. Brenner BM, Milford EL. Nephron underdosing: a programmed cause of chronic renal allograft failure. Am J Kidney Dis. 1993; 21(Suppl 2):66–72. https://doi.org/10.1016/0272-6386(93)70097-i PMID: 8494022
12. Singh RR, Denton KM. Role of the kidney in the fetal programming of adult cardiovascular disease: an update. Curr Opin Pharmacol. 2015; 21:53–9. https://doi.org/10.1016/j.coph.2014.12.010 PMID: 25588322
13. Luyckx VA, Bertram JF, Brenner BM, Fall C, Hoy WE, Ozanne SE, et al. Effect of fetal and child health on kidney development and long-term risk of hypertension and kidney disease. Lancet. 2013; 382 (9888):273–83. https://doi.org/10.1016/S0140-6736(13)60311-6 PMID: 23727166
14. Sorokin A, Staruschenko A, Pochynyuk O. Direct activation of ENaC by angiotensin II: recent advances and new insights. Curr Hypertens Rep. 2013; 15(1):17–24. https://doi.org/10.1007/s11906-012-0316-1 PMID: 23180052 PMCID: PMC3545060
15. Bankir L, Bichet DG, Bouby N. Vasopressin V2 receptors, ENaC, and sodium reabsorption: a risk factor for hypertension? Am J Physiol Renal Physiol. 2010; 299(5):F917–28. https://doi.org/10.1152/ajprenal.00413.2010 PMID: 20826569
16. Zaika O, Mamenko M, Staruschenko A, Pochynyuk O. Direct activation of ENaC by angiotensin II: recent advances and new insights. Curr Hypertens Rep. 2013; 15(1):17–24. https://doi.org/10.1007/s11906-012-0316-1 PMID: 23180052 PMCID: PMC3545060
17. Snyder PM. Minireview: regulation of epithelial Na+ channel trafficking. Endocrinology. 2005; 146 (12):5079–85. https://doi.org/10.1210/en.2005-0894 PMID: 16150899
24. Mironova E, Boiko N, Bugaj V, Kucher V, Stockand JD. Regulation of Na+ excretion and arterial blood pressure by purinergic signalling intrinsic to the distal nephron: consequences and mechanisms. Acta Physiol (Oxf). 2015; 213(1):213–21. https://doi.org/10.1111/apha.12372 PMID: 25154328

25. Ahn D, Ge Y, Stricklett PK, Gill P, Taylor D, Hughes AK, et al. Collecting duct-specific knockout of endothelin-1 causes hypertension and sodium retention. J Clin Invest. 2004; 114(4):504–11. https://doi.org/10.1172/JCI21064 PMID: 15314687 PMCID: PMC503768

26. Oishi R, Nonoguchi H, Tomita K, Marumo F. Endothelin-1 inhibits AVP-stimulated osmotic water permeability in rat inner medullary collecting duct. Am J Physiol. 1991; 261(6 Pt 2):F951–6. https://doi.org/10.1152/ajprenal.1991.261.6.F951 PMID: 1661085

27. Strait KA, Stricklett PK, Kohan DE. Altered collecting duct adenylyl cyclase content in collecting duct endothelin-1 knockout mice. BMC Nephrol. 2007; 8:8. https://doi.org/10.1186/1471-2369-8-8 PMID: 17521429 PMCID: PMC1894628

28. Ahn D, Ge Y, Stricklett PK, Gill P, Taylor D, Hughes AK, et al. Collecting duct-specific knockout of endothelin-1 causes hypertension and sodium retention. J Clin Invest. 2004; 114(4):504–11. https://doi.org/10.1172/JCI21064 PMID: 15314687 PMCID: PMC503768

29. Ge Y, Ahn D, Stricklett PK, Hughes AK, Yanagisawa M, Verbalis JG, et al. Collecting duct-specific knockout of endothelin-1 alters vasopressin regulation of urine osmolality. Am J Physiol Renal Physiol. 2005; 288(5):F912–20. https://doi.org/10.1152/ajprenal.00432.2004 PMID: 15632412 PMCID: PMC296672

30. Roos KP, Bugaj V, Mironova E, Stockand JD, Ramkumar N, Rees S, et al. Adenylyl cyclase VI mediates vasopressin-regulated ENaC activity. J Am Soc Nephrol. 2013; 24(2):218–27. https://doi.org/10.1681/ASN.2012050449 PMID: 23264685 PMCID: PMC3559481

31. Crestani S, Gasparotto Junior A, Marques MC, Sullivan JC, Webb RC, da Silva-Santos JE. Enhanced angiotensin-converting enzyme activity and systemic reactivity to angiotensin II in normotensive rats exposed to a high-sodium diet. Vascul Pharmacol. 2014; 60(2):67–74. https://doi.org/10.1016/j.vph.2013.12.001 PMID: 24321189 PMCID: PMC5560024

32. Farjah M, Washington TL, Roxas BP, Geenen DL, Danziger RS. Dietary NaCl regulates renal aminopeptidase N: relevance to hypertension in the Dahl rat. Hypertension. 2004; 43(2):282–5. https://doi.org/10.1161/01.HYP.0000115841.28096.6c PMID: 14718364

33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609
42. Qi H, Casalena G, Shi S, Yu L, Ebefors K, Sun Y, et al. Glomerular Endothelial Mitochondrial Dysfunction Is Essential and Characteristic of Diabetic Kidney Disease Susceptibility. Diabetes. 2017 Mar; 66 (3):763–778. https://doi.org/10.2337/db16-0695 PMID: 27899487 PMCID: PMC5319717

43. Jensen IS, Larsen CK, Leipziger J, Sorensen MV. Na(+) dependence of K(+) -induced natriuresis, kaliuresis and Na(+) /Cl(-) cotransporter dephosphorylation. Acta Physiol (Oxf). 2016; 218(1):49–61. https://doi.org/10.1111/apha.12707 PMID: 27172453

44. Kwon TH, Frokiaer J, Nielsen S. Regulation of aquaporin-2 in the kidney: A molecular mechanism of body-water homeostasis. Kidney Res Clin Pract. 2013; 32(3):96–102. https://doi.org/10.1016/j.krcp.2013.07.005 PMID: 26877923 PMCID: PMC4714093

45. Roos KP, Strait KA, Raphael KL, Blount MA, Kohan DE. Collecting duct-specific knockout of adenylyl cyclase type VI causes a urinary concentration defect in mice. Am J Physiol Renal Physiol. 2012; 302 (1):F78–84. https://doi.org/10.1152/ajprenal.00397.2011 PMID: 21937603 PMCID: PMC3251343

46. Kohan DE. The renal medullary endothelin system in control of sodium and water excretion and systemic blood pressure. Curr Opin Nephrol Hypertens. 2006; 15(1):34–40. https://doi.org/10.1097/01.mnh.0000186852.15889.1a PMID: 16340664