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ORIGINAL PAPER

Role of angiotensin II and oxidative stress on renal aquaporins expression in hypernatremic rats

Silvana L. Della Penna · Gabriel Cao · Nicolás M. Kouyoumdzian · Lorena Sarati · Andrea Fellet · Ana M. Balaszczuk · Marcelo R. Choi · Elsa Zotta · Susana Gorzalczany · Marcela Pandolfo · Jorge E. Toblli · María I. Rosón · Belisario E. Fernández

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Abstract The aim of this study was to assess whether endogenous Ang II and oxidative stress produced by acute hypertonic sodium overload may regulate the expression of aquaporin-1 (AQP-1) and aquaporin-2 (AQP-2) in the kidney. Groups of anesthetized male Sprague–Dawley rats were infused with isotonic saline solution (control) or with hypertonic saline solution (Na group, 1 M NaCl), either alone or with losartan (10 mg kg^{-1}) or tempol (0.5 mg min^{-1} kg^{-1}) during 2 h. Renal function parameters were measured. Groups of unanesthetized animals were injected intraperitoneally with hypertonic saline solution, with or without free access to water intake, Na+W, and Na−W, respectively. The expression of AQP-1, AQP-2, Ang II, eNOS, and NF-kB were evaluated in the kidney by Western blot and immunohistochemistry. AQP-2 distribution was assessed by immunofluorescence. Na group showed increased natriuresis and diuresis, and Ang II and NF-kB expression, but decreased eNOS expression. Losartan or tempol enhanced further the diuresis, and AQP-2 and eNOS expression, as well as decreased Ang II and NF-kB expression. Confocal immunofluorescence imaging revealed labeling of AQP-2 in the apical plasma membrane with less labeling in the intracellular vesicles than the apical membrane in kidney medullary collecting duct principal cells both in C and Na groups. Importantly, our data also show that losartan and tempol induces a predominantly accumulation of AQP-2 in intracellular vesicles. In unanesthetized rats, Na+W group presented increased diuresis, natriuresis, and AQP-2 expression (112±25 vs 64±16; $*p<0.05$). Water deprivation increased plasma sodium and diuresis but decreased AQP-2 (46±22 vs 112±25; $\S p<0.05$) and eNOS expression in the kidney. This study is a novel demonstration that renal endogenous Ang II–oxidative stress, induced in vivo in hypernatremic rats by an acute sodium overload, regulates AQP-2 expression.

Keywords Sodium overload · Aquaporin · Angiotensin II · Kidney · Losartan · Tempol

S. L. Della Penna · N. M. Kouyoumdzian · L. Sarati · A. Fellet · A. M. Balaszczuk · M. R. Choi · E. Zotta · M. I. Rosón · B. E. Fernández
Department of Pathophysiology, University of Buenos Aires, INFIBIOC-CONICET, Buenos Aires, Argentina

S. Gorzalczany
Department of Pharmacology, University of Buenos Aires, INFIBIOC-CONICET, Buenos Aires, Argentina

M. Pandolfo
Department of Clinical Biochemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, INFIBIOC-CONICET, Buenos Aires, Argentina

G. Cao · J. E. Toblli
Laboratory of Experimental Medicine, Hospital Alemán, Buenos Aires, Argentina

S. L. Della Penna (✉)
Cátedra de Fisiopatología, Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires 1113, Argentina
e-mail: sdellapenna@ffyb.uba.ar

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Introduction

Under normal conditions, plasma sodium levels are regulated within a physiologic range despite the large variations in daily sodium and water intake. On the other hand, in states of hypernatremia, such as after acute sodium overload, the brain and the kidney contribute both in concert to restore plasma sodium homeostasis [22]. Brain and kidney responses include changes in water intake and renal excretion. In the kidney, aquaporin-1 (AQP-1) channels are responsible for the 80–90 % of the fluid reabsorption of the glomerular filtrate [14], and their main expression appear in the luminal and basolateral membranes of the proximal tubules, in the epithelial cells of the thin descending limb of Henle's loop, and in the endothelial cells of descending vasa recta [8, 15, 19, 21]. Aquaporin-2 (AQP-2) channels reabsorb most of the 10–20 % remaining fluid and are mainly expressed in the principal cells of the collecting ducts [5, 23]. The vasopressin hormone (AVP), which is released from the pituitary into the bloodstream in cases of hypernatremia, enhanced collecting duct water permeability due to AQP-2 accumulation at the cell surface and increased AQP-2 protein abundance [6]. However, it has been shown that AQP-2 expression levels decreased shortly (>3 h) following hypertonic challenge implying another regulation mechanism AVP non-dependent [7]. In this order, the hyperosmolality regulates, AVP independently, the expression, targeting, stability, and degradation of AQP-2 in the medullary collecting duct [11]. In this order, it has been shown that the addition of hypertonic NaCl upregulated AQP-1 and AQP-2 expression in cultured renal epithelial cells, by activating the transcription factor associated with hypertonicity (TonEBP) [2, 10, 24]. However, studies carried out in vitro using cultured principal cells of collecting duct have shown that the activation of NF-kB, a main transcription factor related to renal inflammation, decreased AQP-2 mRNA and protein levels, as a result of the binding of NF-kB complexes, consisting of p50 and/or p52, to specific kB elements of the AQP-2 promoter [3, 20]. In this order, a wide range of stimuli closely linked to kidney inflammation, including the increase of renal angiotensin II and the oxidative stress, activates NF-kB and simultaneously reduces the NO-cGMP signaling [13, 16]. In previous studies, we have observed that renal NF-kB and angiotensin II (Ang II), as well as the oxidative stress, were also increased in rats with hypernatremia provoked by an acute sodium overload [17]. Based on these antecedents, it was hypothesized that a hypernatremia produced by sodium overload could early suppress, rather than increase renal AQP-2 expression through the activation of Ang II–oxidative stress pathway. Thus, the aim of this study was, first, to investigate in vivo, AQP-1 and AQP-2 protein expression levels in renal tissues of hypernatremic rats induced by acute hypertonic sodium overload, and secondly, to analyze their regulation by renin–Ang II system and oxidative stress.

Materials and methods

Animal preparation

Male Sprague–Dawley rats (10–12 weeks old; 270–350 g body weight) were housed at controlled temperature (23±2 °C) and exposed to a daily 12-h light–dark cycle (lights on from 07:00 a.m. to 07:00 p.m.), with free access to tap water and standard rat chow (Cooperación SRL, Argentina). Experiments were conducted in accordance with the institutional guidelines for the care and use of research animals of Universidad de Buenos Aires and protocols were approved by Universidad de Buenos Aires (UBACYT B113) and the National Scientific and Technical Research Council (CONICET, PIP 1337/09).

Experimental protocols

Anesthetized rats

Animals were anesthetized with urethane (1.2 g kg^{-1}, intra-peritoneal, i.p.) and the anesthesia plane was kept on a surgical level by repeatedly testing the absence of corneal reflex every 15 min throughout the surgical procedure. A tracheotomy was then performed and a PE-90 tube (3 cm long) was inserted into the trachea to maintain an open airway. The left femoral vein was catheterized with a Silastic cannula (0.12 mm i.d.) for continuous infusion. The right carotid artery was also catheterized with a T4 tube for blood sampling and arterial pressure recording using a Statham GOULD P23ID transducer coupled to a Grass Polygraph 79D. The bladder was cannulated with a PE-75 cannula for urine collection. During a 45-min stabilization period, the animals were infused with isotonic saline solution

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(0.15 M NaCl) at a rate of 0.04 mL min^{-1} (Syringe Infusion Pump, SageTM, Orion) for diuresis to reach a steady state and allow urine collection (basal records). Infusion continued at the same rate for 120 min (experimental period). Urine was collected every 30 min for sodium measurement and urine flow rate, and a blood sample was collected at the end of the experimental period. Mean arterial pressure (MAP) was recorded at the end of the experimental period.

The animals were randomized into six experimental groups:

1. C group ($n=5$): infused with isotonic saline solution for 120 min (experimental period).
2. C-Los group ($n=5$): injected with losartan (10 mg mL^{-1} in *iv* bolus, at a dose of 10 mg kg^{-1}) at the beginning of the experimental period. Thereafter, animals were infused with isotonic saline solution for 120 min.
3. C-Temp group ($n=5$): infused with isotonic saline solution plus tempol (0.5 mg min^{-1} kg^{-1}) during the 120 min of the experimental period.
4. Na group ($n=5$): infused with of hypertonic saline solution (NaCl 1.0 M) for 120 min.
5. Na-Los group ($n=5$): injected with losartan (10 mg mL^{-1} in *iv* bolus, at a dose of 10 mg kg^{-1}) at the beginning of the experimental period. Thereafter, animals were infused with hypertonic saline solution (NaCl 1.0 M) for 120 min.
6. Na-Temp group ($n=5$): infused with hypertonic saline solution (NaCl 1.0 M) plus tempol (0.5 mg min^{-1} kg^{-1}) during the 120 min of the experimental period.

Unanesthetized rats

The aim of the second set of experiments was to determine if the hypertonicity or the sodium overload were each one *per se* the stimulus for the inflammatory response that we have observed in renal tissues in the first set of experiments.

In this order, another set of experiments was carried out in unanesthetized rats subjected to an intraperitoneal (i.p.) injection of hypertonic saline solution and without access to water intake. The control group consisted in rats subjected to a hypertonic saline solution, but their plasma isotonicity was maintained by allowing free access to drinking water. Unanesthetized animals were housed in metabolic cages during 48 h before the experiments with ad libitum water intake. At the day of the experiment, the following groups of animals were slowly injected with saline solution (2 ml/100 g of body weight, i.p.): control group: C group (0.15 mol/L NaCl) and experimental group: Na group (0.8 mol/L NaCl). The animals were immediately returned to the metabolic cages for the urine collection and to record water intake for 90 min, with (+W) or without (−W) free access to drinking water. Then, they were anesthetized with urethane (1.2 g kg^{-1}, i.p.) and 1 ml of blood samples were obtained from the abdominal aorta. After blood centrifugation, plasma Na^+ and K^+ were measured by standard methods by means of an auto-analyzer. In order to determine AQP-2 and eNOS protein expression, the kidneys were isolated, rapidly excised, decapsulated, longitudinally cut, and harvested for Western blot and immunohistochemistry as described below.

Urine and blood measurements

Urine flow rate (UV), urine and plasma sodium and plasma potassium (respectively U_{Na}, PL_{Na}, and PL_K), were measured by standard methods using an autoanalyzer. Urinary osmolality was determined by freezing-point depression. FE_{Na} were calculated according to standard formula. Urine flow is expressed as ml/min, urinary and plasmatic U_{Na}, PL_{Na}, PL_K as mEq/L, urinary osmolarity (U_{Osm}) as mOsmol Kg^{-1}, FE_{Na} as the percentage (%) of filtrated sodium. UV is expressed as μL min^{-1}, U_{Na}, PL_{Na}, and PL_K as mEq L^{-1}.

Preparation of renal homogenates for Western blot

The right kidney from all groups was extracted and the renal cortex and medulla were immediately dissected and separated. Tissue samples were homogenized on ice with a Tissue Tearor (Biospec Products Inc) in a buffer mixture (50 mmol L^{-1} Tris, 0.1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} EGTA, 1 % Triton, 1 mmol L^{-1} PMSF, 1 μmol L^{-1} pepstatin, and 2 μmol L^{-1} leupeptin, 1× protease inhibitor cocktail (Roche Diagnostics). Protein concentration in the Triton-soluble supernatant was determined by the Lowry technique [12].

Western blot analysis for AQP-1, AQP-2, and eNOS

Pooled samples of cortex and medulla from five animals of each group and containing similar amounts of protein

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(100 μg protein/lane) were separated by electrophoresis in 7.5 % SDS-polyacrylamide gels (Bio-Rad) and then transferred to a nitrocellulose membrane (Bio-Rad) and incubated with rabbit polyclonal anti-AQP-1 (Santa Cruz Biotechnology, Inc. 1:250 dilution), rabbit polyclonal-anti-AQP-2 (Santa Cruz Biotechnology, Inc.; 1:200 dilution), or rabbit polyclonal anti-eNOS (Santa Cruz Biotechnology, Inc.; 1:200). The polyclonal AQP-1 antibody recognized the 29 and 38 kDa forms corresponding to non-glycosylated and glycosylated AQP-1, respectively. A secondary immunoreaction followed with a goat anti-rabbit IgG (H+L) conjugated with horseradish peroxidase (dilution of 1:5,000). A third streptavidin conjugated with horseradish peroxidase was used (GE Healthcare Life Sciences; dilution of 1:1,000). The samples were revealed by chemiluminescence using ECL reagent (Amersham Pharmacia Biotech) for 2–4 min. The density of the respective bands was quantified by densitometric scanning of Western blots using a Hewlett-Packard scanner and Totallab analyzer software (Biodynamics Corp.). To avoid inaccuracies in protein loading, beta-actin was measured as internal standard (anti-beta actin, clone EP1123Y, rabbit monoclonal antibody) for each blot and protein levels were calculated and expressed as the ratio between the optical densities of the bands corresponding to AQP-1, AQP-2, or eNOS and β-actin.

Kidney processing for immunohistochemistry

In order to determine the effect of sodium overload on AQP-1, AQP-2, Ang II, eNOS, and NF-kB immunostaining, left kidneys were isolated, rapidly excised, decapsulated, incised in the midline longitudinal plane to divide them into two similar halves, and harvested for immunohistochemical studies. Tissues were fixed in 10 % phosphate-buffered formaldehyde (pH 7.20) and embedded in paraffin. Paraffin-embedded tissue sections of 3-μm were deparaffined and dehydrated. Endogenous peroxide activity was blocked by treatment with 0.5 % H_2O_2 in methanol for 30 min. Immunohistochemical assays were conducted as reported previously [13]. Immunostaining was detected using the following specific antibodies: rabbit polyclonal anti-AQP-1 (Santa Cruz Biotechnology, Inc. 1:200 dilution), polyclonal rabbit anti-AQP-2 (Santa Cruz Biotechnology, Inc.; 1:200 dilution), monoclonal human anti-Ang II (Peninsula, 1:500 dilution), polyclonal rabbit anti-eNOS (C-20, Santa Cruz Biotechnology Inc., 1:200 dilution), and rabbit anti-NF-κB p65 (Santa Cruz Biotechnology Inc., 1:200 dilution). Immunostaining was performed using a commercial modified avidin–biotin–peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories). Immunostaining was expressed as a percentage (%) of positive stained area ± standard error media (SEM).

Quantitative imaging and morphological analysis

All tissue samples were evaluated independently by two investigators without prior knowledge of the group to which the rats belonged (inter-observer variability was 0.89). All the measurements were carried out using an image analyzer, Image-Pro Plus version 4.5 for windows (Media Cybernetics, LP, Silver Spring, Maryland, USA). Morphological analyses were performed on ten consecutive fields at a magnification of ×400 and the data were averaged. In every kidney, the positive immunostaining for AQP-1, AQP-2, Ang II, eNOS, and NF-kB were expressed as density per square millimeter and the mean percentage value was then calculated for each rat.

Immunofluorescence of AQP-2 expression in renal tissue

Rats in each group were transcardially perfused with fixative containing 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The kidneys were removed and stored overnight in the same fixative. They were infiltrated with 30 % sucrose solution and kept at 4 °C. The specimens were rapidly frozen and sectioned with a vibratome (5 μm sections) on a cryostat. Fluorescence labeling was performed. After washing in PBS (pH 7.4), sections were incubated for 30 min at room temperature in 10 % serum diluted in PBS.They were then incubated for 24 h at 4 °C with antisera against AQP-2 diluted 1:200 in PBS. After washing in PBS, they were incubated for 1 h at room temperature with a antisera against IgG of rabbit conjugated to rodhamine B (AP307R Chemicon) diluted 1:100 in PBS. The sections were mounted on glass slides. Image analysis of the immunofluorescent labeling was performed by a confocal laser scanning microscope (Olympus FV300/BX61).

Statistical analysis

All results are expressed as mean ± SEM. Gaussian distribution was evaluated by the Kolmogorov and

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Smirnov method and comparisons between groups were made using ANOVA followed by the Bonferroni test. p values <0.05 were considered significant.

Results

Studies in anesthetized rats

Mean arterial pressure

As reported previously [17], MAP did not increase after 2 h of acute sodium overload (Na group) compared to basal levels. Moreover, no differences in MAP were observed when losartan or tempol were administered to sodium overloaded animals (Na-Los and Na-Temp groups; Table 1).

Urine and blood measurements

Table 1 shows biochemical results for each group. As expected, the animals subjected to an acute sodium overload showed higher levels of PL_{Na} respect to the control group. This increase was not modified by the co-administration of losartan or tempol with the sodium overload. Plasmatic potassium concentration was not statistically modified. The group with acute sodium overload showed higher levels of FE_{Na} and lower levels of U_{Osm} compared to the control group infused with isotonic solution. The co-administration of losartan or tempol with sodium overload increased further FE_{Na} and increased U_{Osm} as compared to the Na group. The administration of losartan or tempol with isotonic saline solution did not show significant changes in both parameters.

Figure 1 shows results of UV (panel a) and U_{Na} (panel b). As reported previously [17], the group with acute sodium overload showed higher levels of UV and U_{Na} compared to the control group infused with isotonic solution. The co-administration of losartan or tempol with sodium overload increased further UV but did not modify U_{Na} as compared to the Na group. The administration of losartan or tempol with isotonic saline solution did not show significant changes in both parameters.

Western blot and immunohistochemistry analysis of AQPs expression in renal tissue

Figure 2 shows AQPs immunostaining in renal tubules. As indicated in panel a, AQP-1 staining in the control group was not statistically modified either by the sodium overload or by losartan or tempol administration. As shown in panel b, AQP-2 staining was similar in Na and control groups. Nonetheless, losartan or tempol co-administration with the sodium overload increased AQP-2 immunostaining, as compared with Na and control groups.

Figure 3 shows the Western blot analysis of both AQPs, carried out in homogenates of renal tissues. The sodium overload did not modify AQP-1 protein levels in renal cortex (panel a). Additionally, losartan and tempol did not modify AQP-1 staining area, compared with control and sodium overload groups. The sodium overload did not modify AQP-2 protein levels in renal medulla (panel b). However, both losartan and tempol

Table 1 Electrolytes, urinary osmolality, and mean arterial pressure in anesthetized rats

	MAP (mmHg)	PL_{Na} (mEq L^{-1})	PL_K (mEq L^{-1})	FE_{Na} (%)	U_{Osm} (mOsm Kg^{-1})
C	92±3	141±1	3.6±0.1	0.28±0.08	1,764±49
C-Los	65±3*	139±1	3.5±0.2	0.36±0.09	1,630±35
C-Temp	87±1	140±1	3.6±0.1	0.45±0.09	1,835±23
Na	88±3	156±1*	3.7±0.2	8.38±0.75*	721±29*
Na-Los	84±4	156±1*	3.5±0.2	11.60±1.25*§	1,012±15*§
Na-Temp	94±4	154±1*	3.6±0.1	13.74±1.37*§	1,070±22*§

All values are mean ± SEM (n=5–6 per group)

Abbreviations: *MAP* mean arterial pressure, PL_{Na} sodium plasmatic concentration, PL_K potassium plasmatic concentration, FE_{Na} fractional excretion sodium, U_{Osm} urinary osmolality, *C* control group infused with isotonic saline solution, *C-Los* infused with isotonic saline solution plus losartan, *C-Temp* infused with isotonic saline solution plus tempol, *Na* infused with sodium overload, *Na-Los* infused with sodium overload plus losartan, *Na-Temp* infused with sodium overload plus tempol

*p<0.05 vs C group; §p<0.05 vs. Na group

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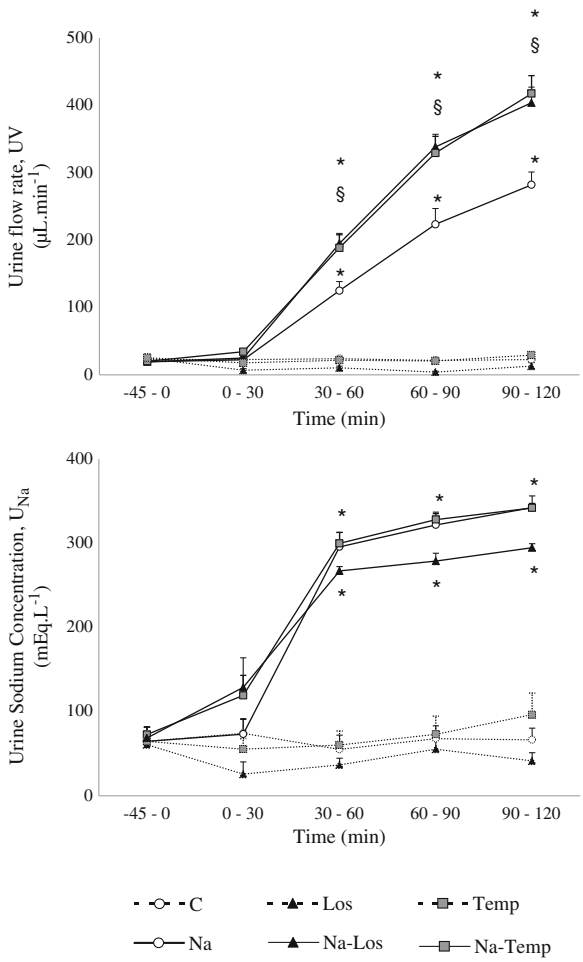

Fig. 1 Urinary flow (UV, µL min^{-1} kg^{-1}; **a**) and urinary sodium concentration (U_{Na}, mEq L^{-1}; **b**). Control group infused with isotonic saline solution (*C*) and experimental groups infused with sodium overload (*Na*), isotonic saline solution plus losartan (*Los*), isotonic saline solution plus tempol (*Temp*), sodium overload plus losartan (*Na-Los*), and sodium overload plus tempol (*Na-Temp*). Values are expressed as mean ± SEM; n=5–8. *p<0.05 vs. C group, §p<0.05 vs. Na group, at the time of infusion

enhanced AQP-2 protein expression in sodium-overloaded rats, without changes in control rats. Thus, AQPs expressions measured by Western blot and by inmmunohistochemistry showed a similar fashion.

Immunoflourescence (Fig. 4) labeling of AQP-2 using sections of the kidney revealed similar AQP-2 labeling in collecting duct of control kidney compared with Na group (Fig. 4, C and Na). Moreover, AQP-2 labeling in kidney was predominantly associated with apical plasma membrane domains of IMCD principal cells (Fig. 4, C and Na), suggesting AQP-2 trafficking from intracellular vesicles to the apical plasma membrane was maintained. In contrast, there was an increased abundance of AQP-2 and it was markedly redistributed into the intracellular compartment, in medulla of groups Na-Los and Na-Temp (Fig. 4, Na-Los and Na-Temp).

Table 2 shows Ang II, NF-kB, and eNOS immunostaining in renal tubules. Ang II and NF-kB immunostaining in collecting ducts increased in the Na group, compared with the C group, where losartan and tempol treatments prevented this increase. In addition, eNOS immunostaining in renal medulla in the Na group was significantly lower than in the C group, being the decrease prevented by losartan and tempol administration. As an illustration of the observed changes, Fig. 5 shows immunostaining images of NF-kB nuclear factor.

Studies in unanesthetized rats

Table 3 shows the results of urine flow rate, urinary and plasmatic electrolytes, and water intake. Water deprivation (C−W) did not alter PL_{Na} in i.p. isotonic saline injected animals compared to those who had free access to drinking water (C+W). On the other hand, the hypertonic saline solution did not modify PL_{Na} in (+W) rats as it was expected, but it was increased in water deprived animals (−W). Plasmatic potassium values remained unchanged in all studied groups. Both groups i.p. injected with sodium overload (+W and −W) had higher levels of UV and U_{Na} as compared with their respective control group (+W or −W), injected with isotonic solution. Additionally, the urine flow rate increased further after the hypertonic saline injection in (−W) rats respect to (+W) rats. The water intake in Na+W group was significantly higher as compared with the respective C+W group.

Figure 6 shows that AQP-2 staining increased in Na+W group as compared with isotonic infused rats with free access to water (C+W group) in renal cortex (left panel) and medulla (right panel). On the other hand, water deprivation decreased AQP-2 staining in sodium overloaded rats (Na−W group) compared with hypertonic infused rats with free access to water (Na+W group).

Figure 7 illustrates renal protein expression of AQP-2 (panel a) and eNOS expression (panel b) in cortex and medulla from unanesthetized rats as measured by

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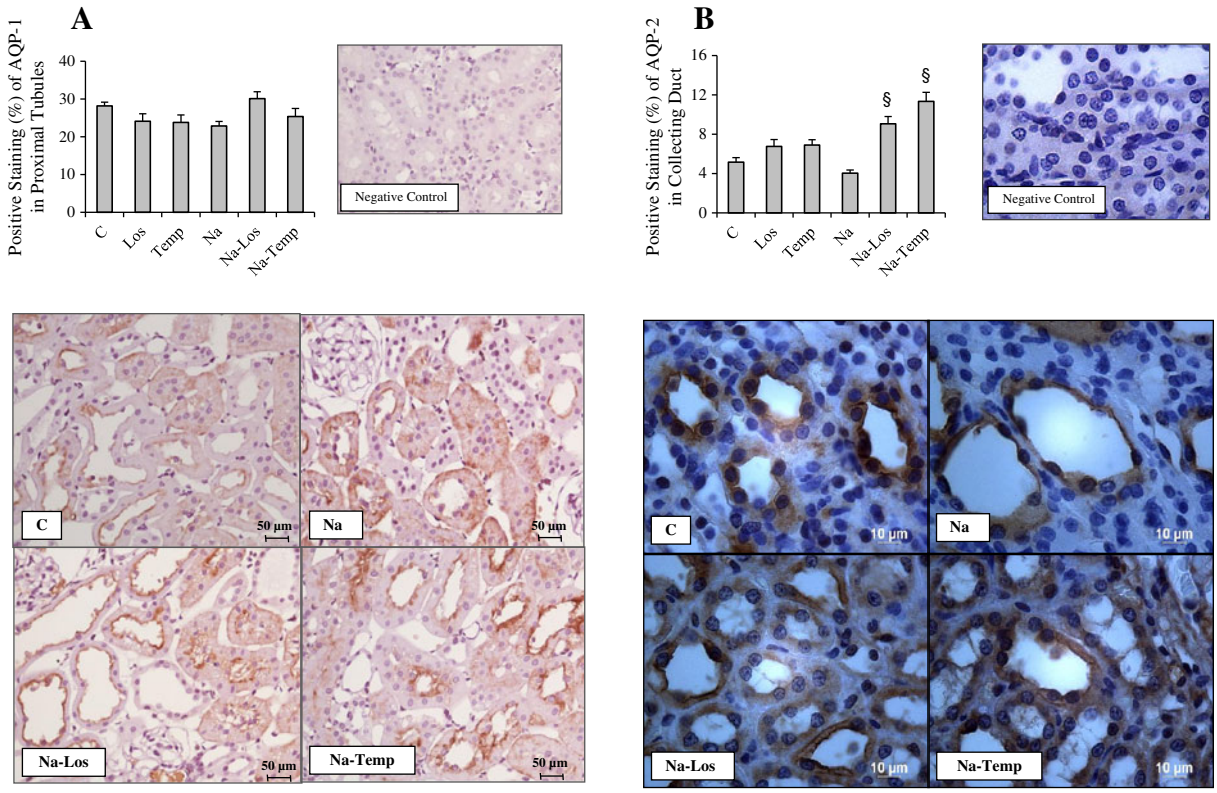

Fig. 2 Histograms illustrate the values of AQP-1 expression (*top*, **a**) in proximal tubules of renal cortex and AQP-2 expression (*top*, **b**) in medullar collecting duct. Values are expressed as a percentage (%) of positive stained area ± SEM; $n=5$; §$p<0.01$ vs. Na group. Representative immunohistochemical images of negative staining control, positive staining of AQP-1 in renal cortex (*bottom*, **a**) and AQP-2 in renal medulla (*bottom*, **b**) of control group infused with isotonic saline solution (*C*) and experimental groups infused with sodium overload (*Na*), sodium overload plus losartan (*Na-Los*), and sodium overload plus tempol (*Na-Temp*). Original magnification ×400

Western-blot. AQP-2 expression increased in Na+W group respect to C+W group. In addition, AQP-2 and eNOS protein levels in renal medulla of the Na−W group were significantly lower than those in Na+W group. Moreover, eNOS levels in renal cortex remained unchanged in all groups.

Discussion

The present results indicate that increased endogenous Ang II and oxidative stress in response to hypernatremia produced by acute sodium overload could early regulate AQP-2 expression in collecting duct. The sodium-overloaded animals had increased diuresis, U_{Na} and FE_{Na}, decreased urinary osmolality, together with increased Ang II and NF-kB expression, decreased eNOS and unchanged AQP-1 and AQP-2 expression. In previous reports, we demonstrated that these animals also had increased glomerular filtration rate and sodium tubular reabsorption, favoring greater flow and sodium transport, which produces oxidative stress [17]. The administration of losartan or tempol to these animals increased further the diuresis and FE_{Na}, without changes in U_{Na} and restored partially the urinary osmolality. This was accompanied by a significantly higher expression of AQP-2, and eNOS, and decreased Ang II and NF-kB expression. We have demonstrated that the abundance of collecting duct water channels AQP-2 in Na group was not changed but was maintained at control levels. Immunoperoxidase and immunofluorescence microscopy further demonstrated trafficking of AQP-2 to the apical plasma membrane, with less labeling of intracellular vesicles. In contrast, losartan and tempol increased the abundance of AQP-2, which was more abundantly present in intracellular

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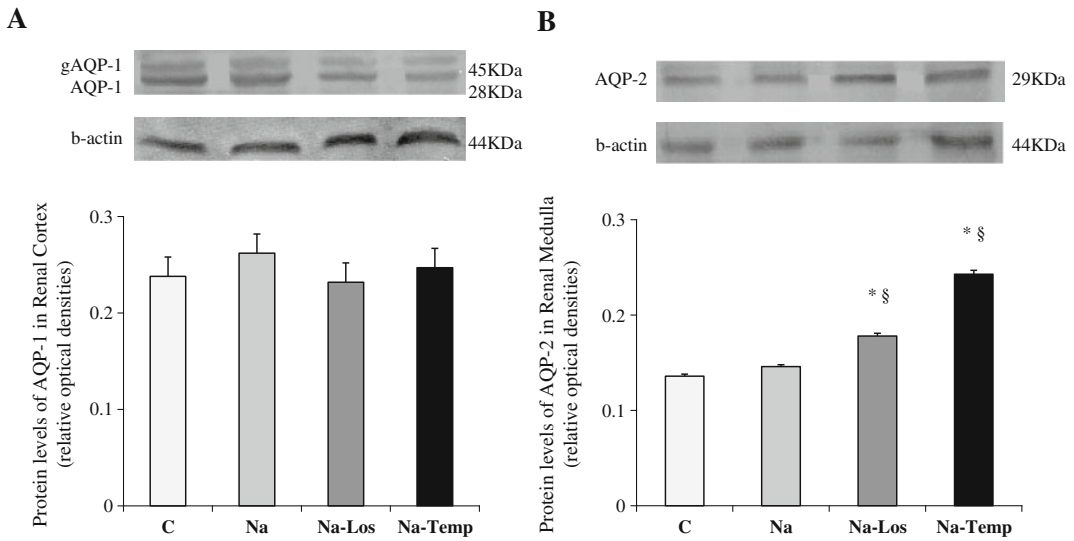

Fig. 3 Representative Western blot analysis of AQP-1 in renal cortex (**a**) and AQP-2 in renal medulla (**b**) of control group infused with isotonic saline solution (*C*) and experimental groups infused with sodium overload (*Na*), sodium overload plus losartan (*Na-Los*), and sodium overload plus tempol (*Na-Temp*). Histograms illustrate the values of protein expression of AQP-1 and AQP-2 for every group. Each blot was normalized to the expression of β-actin from the same gel. Data are mean ± SEM, expressed as relative optical densities; $n=5$. *$p<0.05$ vs. C group; §$p<0.05$ vs. Na group

vesicles and slightly in the apical plasma membrane of collecting duct.

Urinary osmolality diminishes in the hypertonic group, as a result of the osmotic diuresis produced by the sodium overload but partially increased in groups Na-Los and Na-Temp. The increased AQP-2 expression in these groups is reflected in changes of urinary osmolality but not of U_{Na}. Whereas, the maximum capacity of concentrating sodium is already achieved in Na groups, and thus losartan and tempol cannot increase the value of U_{Na} over such group, as we have already described in a previous work [17]. Considering that plasmatic hypertonicity independently of AVP increases AQP-1 and AQP-2 expression in the kidney through TonEBP, it would be expected that the rats with hypernatremia produced by an acute sodium overload had increased the expression of these aquaporins. However, we observed that the expression of both aquaporins did not change in the kidney of rats with sodium overload compared with the control group, but when sodium overloaded rats were treated with losartan or tempol, in both circumstances, the AT1 receptor blockade or the inhibition of the oxidative stress, produced an increase in AQP-2 expression in renal tubular cells. As we have stated in the introduction, AVP released from the pituitary into the bloodstream in cases of hypernatremia, as in our animal model, enhanced collecting duct water permeability due to AQP-2 accumulation at the cell surface and increased AQP-2 protein abundance [6]. Although AVP is augmented, our results did not show stimulation of AQP-2. Then, the issue that concerns us is to know which other regulatory factor can be involved, counteracting the increase of AQP-2 protein by AVP, and if it could be a consequence of an inflammatory process. For this reason we propose, according to our results, that AQP-2 could be inhibited at the genetic level by hypertonicity through the transcription factor NF-kB.

In order to confirm that the results observed in Na group were caused by the hypernatremia and not by the sodium overload per se, we designed another experimental model of hypernatremia, carried out in unanesthetized rats subjected to the i.p. administration of a hypertonic saline solution and with no access to drinking water. Water deprivation did not modify any parameter in controls subjected to isotonic saline solution intraperitoneally injected. The i.p. administration of hypertonic saline solution to animals with free access to drinking water did not change plasma sodium and potassium levels and renal eNOS expression but increased the diuresis, natriuresis, and renal AQP-2 expression. On the other hand, water deprivation in these animals, increased plasma sodium levels, increased the diuresis but not shown enhancement of AQP-2 and eNOS

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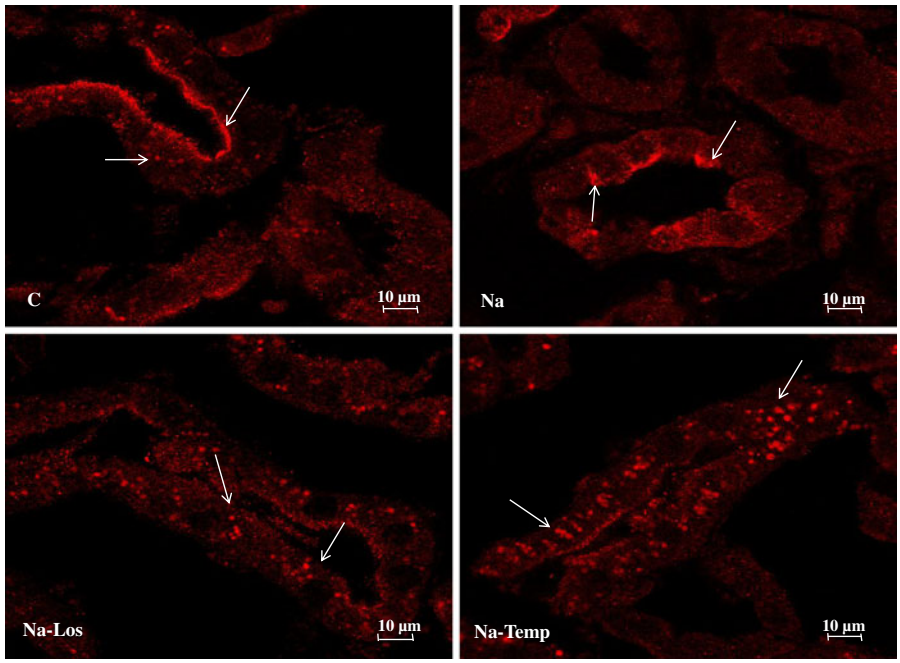

Fig. 4 Representative confocal laser scanning microscopy using immunofluorescence labeling of AQP-2 (*red*) in cryo-sections of kidney control group infused with isotonic saline solution (*C*) and experimental groups infused with sodium overload (*Na*), sodium overload plus losartan (*Na-Los*), and sodium overload plus tempol (*Na-Temp*). AQP-2 labeling was associated with apical plasma membrane domains (*arrows*) and intracellular vesicles in the medullary collecting duct principal cells from control rats. In Na group, there was a similar abundance in overall AQP-2 labeling, and it was associated with apical plasma membrane domains and intracellular vesicles in the medullary collecting duct principal cells (*arrows*). In Na-Los group, there was an increased abundance in overall AQP-2 labeling that was associated with apical plasma membrane domains and increased intracellular vesicles in the medullary collecting duct principal cells (*arrows*). In Na-Temp group, there was an increased abundance in overall AQP-2 labeling that was associated with apical plasma membrane domains and increased intracellular vesicles in the medullary collecting duct principal cells (*arrows*). Original magnification ×600+zoom ×2.5

expression in renal medulla tissues. These results support the hypothesis that, whereas AQP-1 was not modified, AQP-2 expression could be initially inhibited rather than activated by hypernatremia. In summary, in both models of hypernatremia produced by sodium overload, observed decreased or no changes in the increase of AQP2 expression, suggesting another regulatory mechanism. Therefore, we speculate that the increase of AQP-2 expression in the kidney tubules induced by hypertonicity may be masked by the overexpression of Ang II and the development of oxidative stress and this may be the reason why its expression increased after the treatment with losartan and tempol.

Table 2 Ang II, eNOS, and NF-kB immunostaining in renal medulla of anesthetized rats

	Ang II (%)	eNOS (%)	NF-kB (%)
C	13.9±0.6	6.18±0.20	6.4±0.5
Na	27.8±0.4*	2.87±0.13*	10.8±0.9*
Na-Los	12.1±0.5	6.18±0.2	7.0±0.1
Na-Temp	8.9±0.7	7.49±0.49	2.6±0.2*

All values are mean ± SEM (n=5–6 per group)

Abbreviations: *C* control group infused with isotonic saline solution, *Na* infused with sodium overload, *Na-Los* infused with sodium overload plus losartan, *Na-Temp* infused with sodium overload plus tempol

*p<0.05 vs respective control group

It is known that NF-kB downregulates AQP-2 expression by binding to its promoter, which contains two highly conserved kB elements [4]. On the other hand, it has been reported that the intrarenal Ang II, through nuclear AT1 receptor binding, stimulates renal tubular functions through a redox-sensitive pathway and that it inhibits eNOS expression through the increase of superoxide production [20]. Our group has previously reported the presence of an imbalance between Ang II and eNOS expression in sodium overloaded rats [18]. This imbalance was now confirmed, as shown in the

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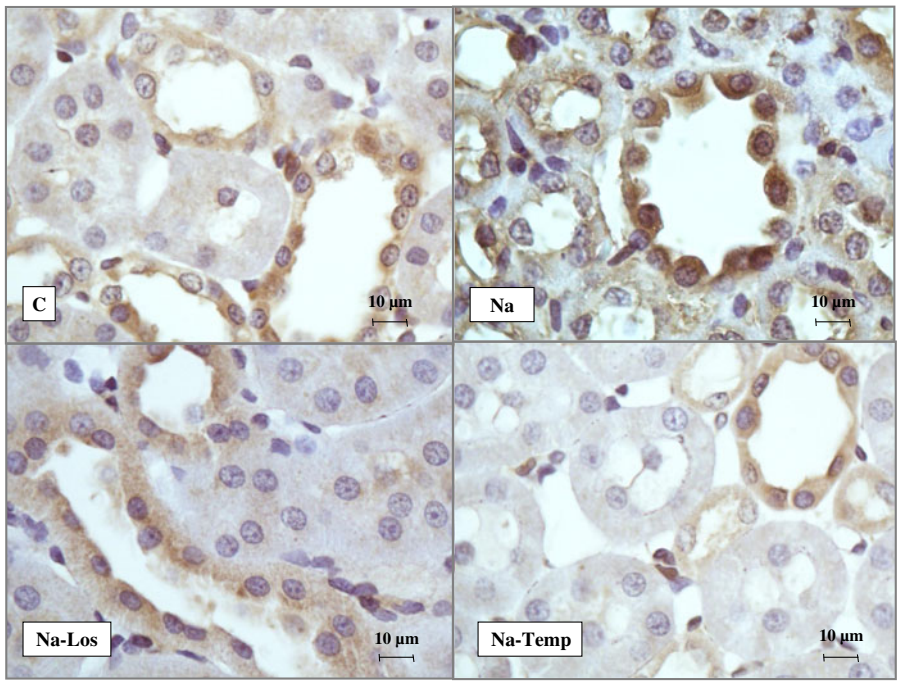

Fig. 5 Representative immunohistochemical images of positive staining of NF-kB in renal medulla of control group infused with isotonic saline solution (*C*) and experimental groups infused with sodium overload (*Na*), sodium overload plus losartan (*Na-Los*), and sodium overload plus tempol (*Na-Temp*). Original magnification ×400

"Results" section, and may favor the increase of superoxide anion production and lower nitric oxide availability, two circumstances that are able to activate the NF-kB transcription factor [1, 25]. Thus, the imbalance between increased Ang II and decreased eNOS expressions, would contribute to enhance NF-kB activation, and therefore to the inhibition of AQP-2 expression. In order to investigate the participation of Ang II–NF-kB signal on AQPs expression, we studied the intrarenal expression of Ang II, eNOS, and NF-kB in rats with hypernatremia and the effects of the inhibition of Ang II by the AT1 receptor blocker losartan or the oxidative stress by the administration of tempol. The results showed that the rats with hypernatremia by the sodium overload had an increase in NF-kB expression in renal tubules and the above commented imbalance between intrarenal expressions of Ang II and eNOS. In addition, the administration of losartan as well as tempol prevented the increase of NF-kB and Ang II expression, restored that of eNOS to control levels, and increased

Table 3 Electrolytes, urine flow, and water intake in unanesthetized rats

	PL_{Na} (mEq L^{-1})	PL_K (mEq L^{-1})	UV (µL min^{-1} kg^{-1})	U_{Na} (mEq L^{-1})	WI (mL min^{-1} kg^{-1})
C+W	139.0±0.6	4.1±0.2	32.2±10.1	36.6±11.1	0.81±0.13
Na+W	141.4±0.4	4.2±0.1	186.0±14.5*	273.2±8.2*	4.03±0.26*
C−W	139.6±0.5	4.0±0.2	37.2±12.2	37.7±10.6	–
Na−W	145.8±0.7*§	4.3±0.1	263.3±12.1*§	282.7±5.6*	–

All values are mean ± SEM (n=5–6 per group)

Abbreviations: PL_{Na} sodium plasmatic concentration, PL_K potassium plasmatic concentration, *UV* urinary volume, U_{Na} urinary sodium concentration, *WI* water intake, *C* control, *Na* infused with sodium overload, with (+W) or without (−W) access to drinking water

*p<0.05 vs. respective control group; §p<0.05 vs. respective group with access to drinking water

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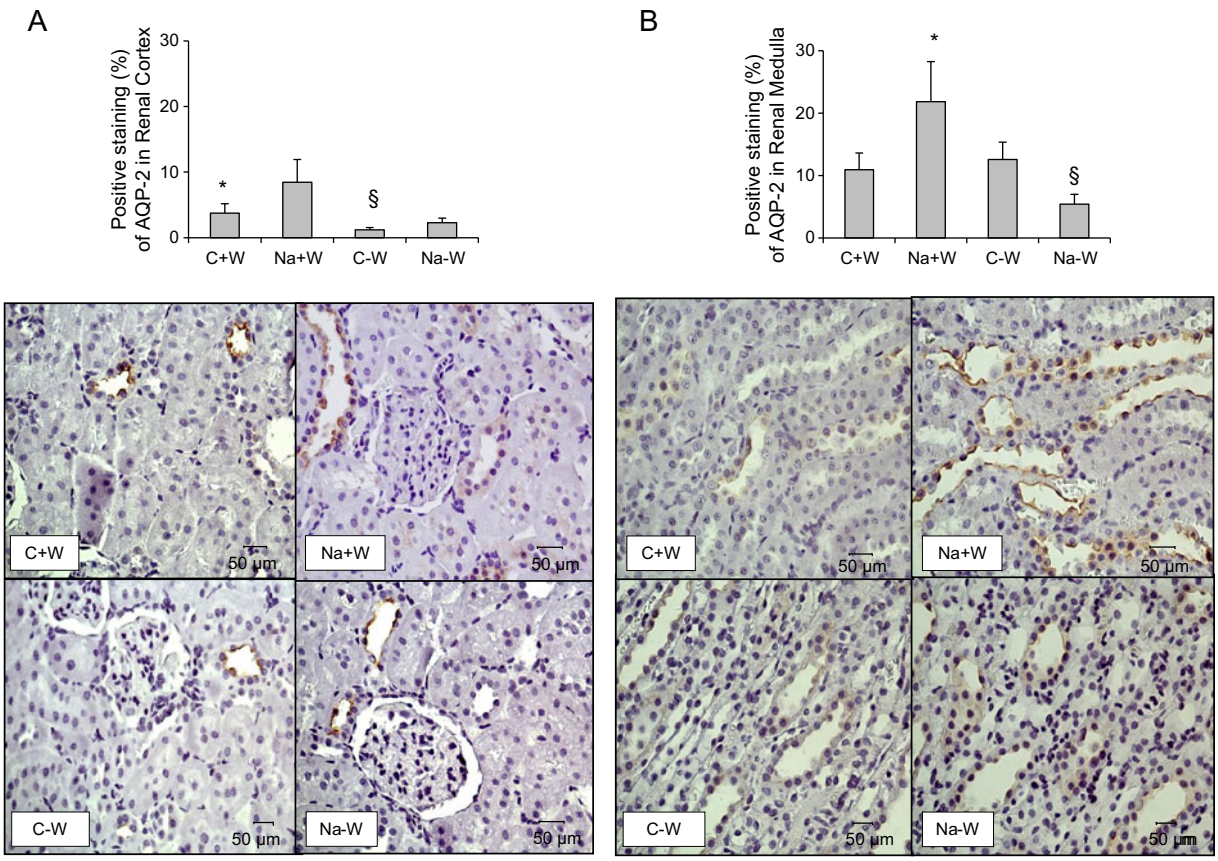

Fig. 6 Histograms illustrate the values of AQP-2 expression in renal cortex (*top*, **a**) and renal medulla (*top*, **b**) of unanesthetized rats intraperitoneally injected with saline solution in two concentrations: C group (control, 0.15 mol L^{-1} NaCl) and experimental group: Na group (0.8 mol L^{-1} NaCl), with (+W) or without (−W) access to drinking water. Values are expressed as a percentage (%) of positive stained area ± SEM; $n=5$; *$p<0.01$ vs. C group, §$p<0.01$ vs. Na group. Representative images of positive staining of AQP-2 in renal cortex (*bottom*, **a**) and medulla (*bottom*, **b**). Original magnification ×400

AQP-2 expression, with no changes in AQP-1 in renal cortex. Consequently, our results could indicate that the Ang II–oxidative stress–NF-kB pathway may be a repressor mechanism to diminish AQP-2 expression in rats with hypernatremia. This possibility is also supported by previous findings in vitro using renal tubule cells obtained from rat kidney slices. They showed that NF-kB activation, stimulated by a hypertonic medium, decreased AQP-2 mRNA and protein levels as a result of the binding of NF-kB complexes to specific kB elements of the AQP-2 promoter [4]. Therefore, one explanation of the present results would be that NF-kB could decrease AQP-2 gene transcription, while the blockage of AT1 receptors by losartan and the inhibition of oxidative stress by tempol could prevent NF-kB activation and cause an acute increase of AQP-2 expression.

Alternatively, inhibited eNOS expression by sodium overload could also decrease AQP-2 levels by another mechanism, in this case, NF-kB non-dependent. A recent report showed that the hypertonicity-induced activation of the NFATc factor may also increase AQP-2 transcription [3]. Furthermore, it has been demonstrated that NO can enhance the nuclear import of NFATc and decrease its export via PKG, thus enhancing NFATc nuclear accumulation and transcriptional activity, supporting a novel mechanism by which NO could regulate AQP-2 expression via NFATc [1]. In agreement with this finding, the present data also show that sodium overload lowered eNOS expression, while losartan and tempol administration caused a marked increase. Furthermore, the unanesthetized rats subjected to i.p. sodium overload and deprived of water drinking had lower AQP-2 expression in renal medulla and

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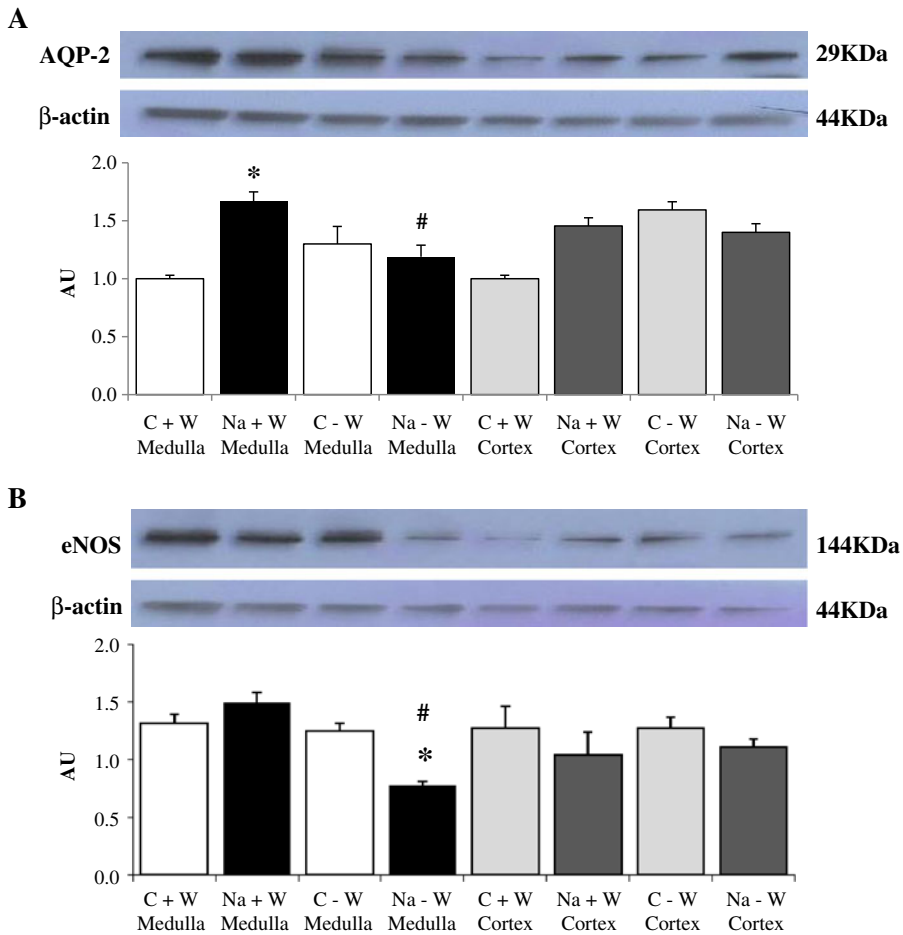

Fig. 7 Representative Western blot analysis of AQP-2 (**a**) and eNOS (**b**) in renal cortex and medulla of unanesthetized rats injected intraperitoneally with saline solution at two concentrations: C group (control, 0.15 mol L^{-1} NaCl) and experimental group: Na group (0.8 mol L^{-1} NaCl), with (+W) or without (−W) access to drinking water. Histograms illustrate the values of protein expression of AQP-2 and eNOS for every group. Each blot was normalized to the expression of β-actin from the same gel. *AU* arbitrary units. Data are mean ± SEM; $n=5$

simultaneously, lowered eNOS expression. Therefore, although the mechanism for the up-regulation of AQP-2 expression in rats with renal Ang II blockade and oxidative stress inhibition remains unclear, it is possible to suggest that the NO system may be involved in this process.

On the other hand, the results show that the urinary excretion in the present work increased in the sodium overloaded group, and losartan and tempol administration increased further the urine flow rate despite the rise of renal AQP-2 expression. Moreover, in the unanesthetized rats subjected to i.p. sodium overload and deprived of water intake, AQP-2 expression decreased, but urine flow rate increased. The reason for this different behavior is unknown. It must be mentioned that when a NO donor as sodium nitroprusside interacts with the Cys 189 of AQP-1 water channel, it suppresses water permeability of renal cell membranes [9]. Thus, a possible explanation to support our results is that NO could act as an inhibitor of the water transport through AQP-1 channels. Then, the enhanced eNOS expression observed after losartan and tempol administration would facilitate the improvement of NO availability. Enhanced NO, in turn, may show opposed effects on the diuresis by different pathways: (1) NO may inhibit AQP-1 water channel functions and increase water excretion and (2) NO may increase AQP-2 expression and inhibit tubular water re-absorption, being this effect masked by AQP-1 inhibition.

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Conclusions

AQP-2 levels in renal tubular cells are acutely regulated in rats subjected to sodium overload. The inflammatory response including imbalance between Ang II and eNOS expression levels in renal medulla may be one of the repressor mechanisms on AQP expression, through NF-kB pathway. The higher levels of eNOS and lower Ang II expression observed after losartan or tempol administration may result in increased NO availability, which could regulate AQP-2 expression, through NF-kB pathway. Accordingly, a better understanding of the interaction between NO and AQPs in the kidney would clarify the mechanisms involved in water balance disorders. Since NF-kB plays anti-apoptotic roles, it is therefore reasonable to consider that the appropriate downregulation of AQP-2 expression may be crucial for the survival of cells expressing this AQP isoform following acute hypertonic stress.

The present study constitutes a novel demonstration that the hypernatremia induced in vivo by an acute sodium overload in rats may display an early inhibitory effect on AQP-2 expression in the kidney, through local Ang II–oxidative stress pathway.

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Conflict of interests All authors declare no conflict of interests.

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