Pathophysiological effects of the excess of sodium in renal and vascular tissues

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Silvana Lorena Della Penna

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PhD Thesis

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General Introduction
&
Aim of this Thesis

Salt consumption and diseases related to excess of salt

Salt has played a vital role since the beginning of time. It has served as a spiritual icon and to preserve and improve the taste of food. Roman soldiers were paid partly in salt, their salarium, today's "salary". Around 2.700 years B.C., the earliest known treatise on pharmacology was published in China, where a major portion of this writing was devoted to descriptions of more than 40 kinds of salt, including methods of extracting and putting it in usable form[1]. Nowadays, the average individual dietary salt intake worldwide largely exceeds its physiological need. According to the new guidelines on sodium intake issued by the World Health Organization, adults are advised to consume less than 2 grams of sodium (Na) or 5 grams of salt (NaCl) per day. Sodium is found naturally in a variety of foods, for example milk and cream (50 mg Na/100g approx.). It is also found in much higher concentrations in processed foods such as bacon (1.500mg.$100g^{-1}$ approx.), snacks (e.g. pretzels, cheese puffs, popcorn) and condiments[2]. The body uses Na to control blood pressure and to maintain circulating volume. The ingestion of excess dietary salt is strongly correlated with morbidity, mortality of cardiovascular diseases and atherosclerosis[3]. Excess salt intake is also regarded as a major contributing factor to the pathogenesis of hypertension and kidney diseases by increasing water and sodium retention, which results in blood volume expansion, vascular abnormalities and neurogenically-mediated increase in peripheral resistance[3-4].

Aquaporins in Water Balance Disorders

Water constitutes around 70% of our body mass, and every day our kidneys filter and reabsorb around 180 litres of water. Thus, the appropriate distribution of water is required to maintain fluid balance within different anatomic compartments[5]. In contrast to the classic view of simple diffusion through the lipid bilayer of cell membranes, several studies have shown the existence of a membrane-protein-mediated water movement in certain membranes, called Aquaporins (AQPs)[5]. AQPs are transmembrane proteins formed by 6 α-helixes which have a narrow opening in the interior from where water molecules can pass. Four of these proteins (subunits) are arranged in parallel forming a fifth pore in the centre of the tetramer (Fig. 1). These specialized proteins do not allow anions and the majority of big cations to pass through. There are cationic amino acids acting as "doors", preventing the passage of small cations like H_3O^+. Although recent experiments have indicated conductance of some ions such as K^+, Cs^+, Na^+ and tetramethylammonium, through

the central pore of the AQP-1 tetramer. It has also been suggested that the central pore could conduct certain gases (G) such as CO_2 or NO[6] in certain AQPs.

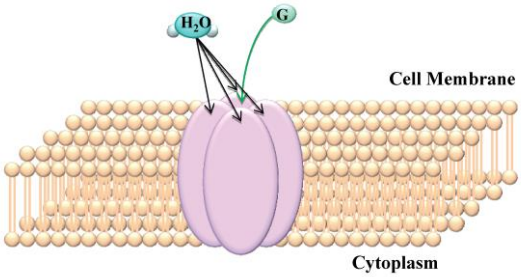

Figure 1. Simplified figure of an Aquaporin. Aquaporins (AQPs) are transmembrane proteins formed by 4 subunits forming tetramers. Each subunit has 6 α-helixes which have a narrow opening in the interior from where water molecules can pass. These tetramers form a central pore, which may conduct certain gases (G) such as CO_2 or NO.

To date, 13 AQPs have been identified in different mammal tissues (AQP-0 to AQP-12)[7]. AQP water permeability is essential in several situations, such as in kidney collecting duct, for example, when water movement is driven across a barrier by a continuous osmotic gradient, or for active, near-isosmolar fluid absorption/secretion, as in kidney proximal tubule[8]. The main AQPs expressed in kidney are: i) AQP-1 in the luminal and basolateral membranes of the proximal tubules, in the epithelial cells of the thin descending limb of Henle and in endothelial cells of the descending vasa recta[6-7], which reabsorbs 80% of the glomerular filtrate; and ii) AQP-2, a vasopressin-regulated water channel, found in collecting-duct apical membrane and intracellular vesicles, which reabsorbs the 20% left of the tubular fluid[9]. Unlike ion channels and solute transporters, the AQPs do not show gating, saturation, or membrane potential-dependence behaviour[9]. The importance of AQPs resides in the responses to physiological or pathological changes, as they can be activated or deactivated by different regulatory mechanisms. For example, vasopressin (antidiuretic hormone) translocates AQP-2 from the intracellular vesicles to the apical plasmatic membrane in the renal tubular cells, and stimulates the transcription of AQP-2 gene (Fig. 2)[10], resulting in water reabsorption. Moreover, it has been recently demonstrated that Angiotensin II (Ang II) regulates the activity of the main AQPs in the kidney[11]. It has also been shown that Ang II could regulate the addressing of AQP-2 to the plasmatic membrane in the cells of the collecting duct of the internal membrane, through the activation of its receptor AT1[12], and that the peptide also potentiates the effects of vasopressin on this channel[13].

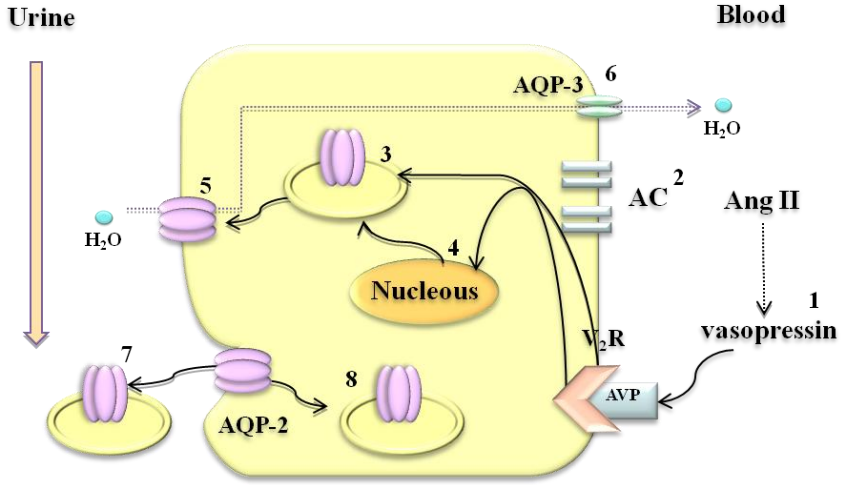

Figure 2. Simplified schematic regulation of AQP-2 trafficking and expression in collecting duct principal cells. Vasopressin (AVP) acts on V2 receptors (V2R) in the basolateral plasma membrane (1). Angiotensin II (Ang II) is able to stimulate the release of aldosterone and thus inducing this step. Adenylyl cyclase (AC) is activated, which accelerates the production of cAMP, activating the catalytic subunit of protein kinase A (PKA) (not shown). PKA phosphorylates AQP-2 in intracellular vesicles (3) and in the nuclei, activating transcription factors (4), thus increasing gene transcription of AQP-2, synthesis and trafficking to the apical plasma membrane (3 and 5). In parallel, AQP-3 synthesis and trafficking to the basolateral plasma membrane takes place (6). In this way, water is reabsorbed. AQP-2 is then excreted into urine (7) or recycled from the apical plasma membrane (8).

AQPs are involved in several human disease conditions involving fluid transport such as brain edema, lung edema, dry eye, congestive heart failure and nephrogenic diabetes insipidus (NDI)[8]. NDI caused by AQP-2 mutation is characterized by severe polyuria and polydipsia, refractory to antidiuretic hormone. People who lack functional AQP-1 are phenotypically normal but manifest defective urinary concentrating function when deprived of water, similar to AQP-1-null mice. AQPs are involved in cell migration as well, having implications in tumor angiogenesis, local invasion, and metastasis. AQP-facilitated cell migration appears to be a general phenomenon relevant not only to angiogenesis but also to wound healing or tumor spread, glial scarring, and likely other phenomena including immune-cell chemotaxis[10].

The kidney and the Renin-Angiotensin-Aldosterone-System

The main hormonal regulator of sodium homeostasis is the Renin-Angiotensin-Aldosterone System (RAAS) which contributes to a large extent to the regulation of blood pressure. The juxtaglomerular (JG) cells of the kidney release renin into the circulation stimulated by changes in renal perfusion pressure, sodium content in the tubules, and the renal sympathetic nerve activity. The liver produces angiotensinogen which is cleaved in the blood by renin to an inactive decapeptide named Angiotensin I (Ang I). Then, the angiotensin-converting enzyme (ACE) converts Ang I into the active form of Ang II. The main source of ACE is the pulmonary endothelium, although it exists in several other tissues such as the kidney, the vessel walls, heart and brain (Fig. 3).

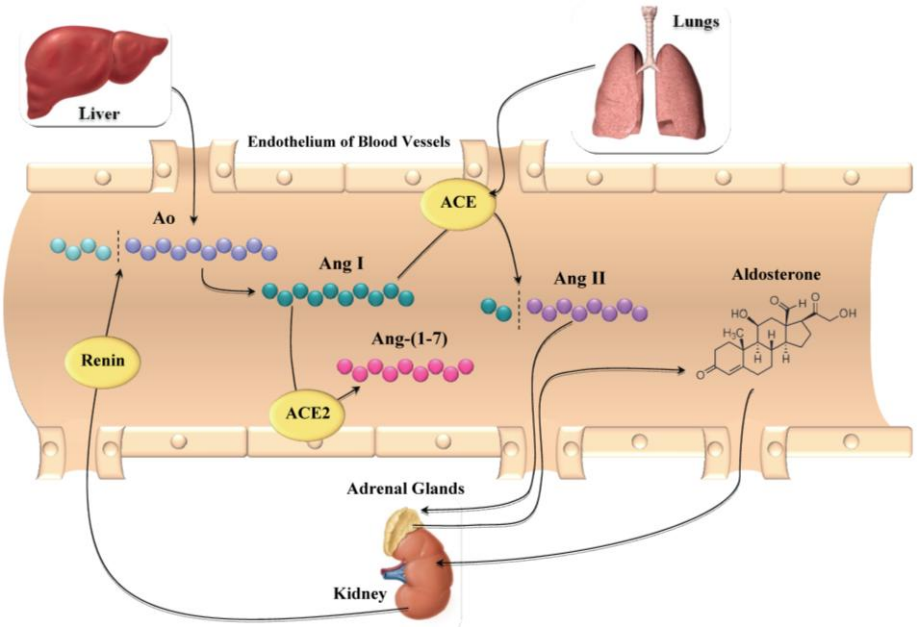

Figure 3. Angiotensin peptide formation. Renin, released by the kidney cleaves the precursor protein angiotensinogen (Ao) to angiotensin I (Ang I), which is further processed to the biologically active peptides Angiotensin II (Ang II) by angiotensin converting enzyme (ACE) and Angiotensin-(1-7) by ACE2. Ang II stimulates the adrenal glands to release Aldosterone, thus increasing sodium retention in the kidneys.

Ang II signals are mediated by the stimulation of two types of receptors: Ang II type 1 (AT1R) and Ang II type 2 (AT2R). The RAAS mediates the majority of its effects via Ang II and its AT1R, conferring the most classical actions, such as vasoconstriction, aldosterone release from the adrenal gland, salt retention in the renal proximal tubules, and stimulation of the sympathetic nervous system in the brain. In addition, there is the ACE2–Ang-(1–7)–Mas axis. A homolog of ACE, ACE2, degrades Ang II into Ang-(1-7) which binds to the Mas receptor which counter-regulates the actions of the classical RAAS (Fig. 4)[14]. Apart from the systemic RAAS, there is a local RAAS present in the nephrons of the kidneys. Ang II is locally converted and secreted, reaching a concentration 100-fold higher in the lumen compared with the plasma[15-17]. In this way, the Ang II formed in the kidney could have an important role in sodium retention and blood pressure regulation[16]. In pathological conditions, Ang II can contribute to renal diseases by inducing sodium retention, inflammation, fibrosis[18-19], and down-regulation of water channels in the kidney (aquaporins, AQPs)[20-21]. These processes can occur in spite of the suppression of the systemic RAAS and even before a rise in blood pressure takes place[15, 21].

In the kidney, AT1R mediates vasoconstriction of glomerular microvasculature, modifying the glomerular filtration rate, the tubuloglomerular feedback and cell growth[15]. Moreover, Ang II modifies through this receptor, the activity of different transporters in the kidney such as Na^+/H^+ interchanger (NHE), ENaCs, NKCC2 and NCC co-transporters[15, 22]. Overall, these actions contribute to an increased capacity of the kidney to preserve sodium and keep blood pressure within normal values. AT2R is localized in glomerular epithelial cells, proximal tubules, collecting ducts and renal vasculature. It is considered as a functional antagonist of AT1R and is associated with vasodilation, apoptosis, antiproliferation, and increase of natriuresis, by stimulation of nitric oxyde/cGMP/protein-kinase G pathway[23] (Fig. 4).

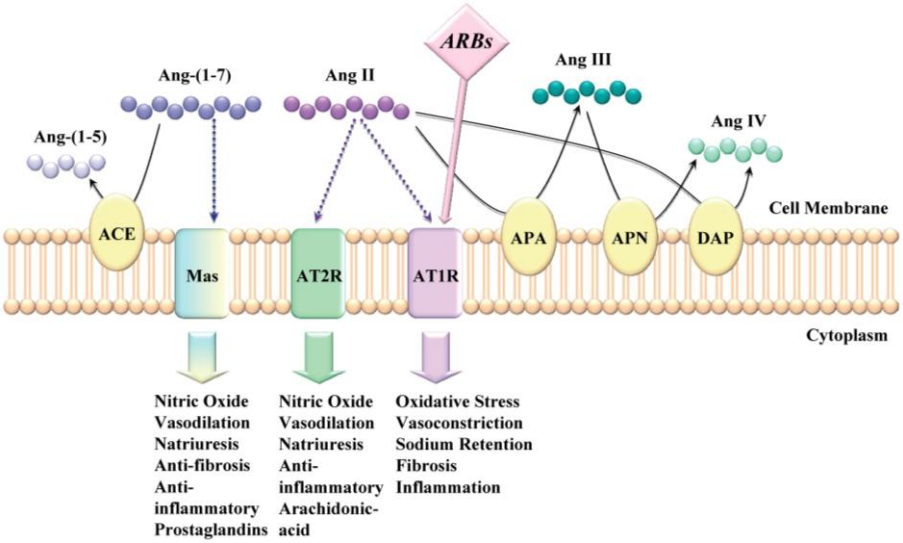

Figure 4. Angiotensin peptides metabolism and main functions. Angiotensin-(1-7) is metabolized by ACE to form Ang-(1-5). Angiotensin II (Ang II) is processed by aminopeptidase A (APA) to form Angiotensin III, which is further hydrolyzed by aminopeptidase N (APN) to form Angiotensin IV. Ang II can be directly cleaved by dipeptidyl aminopeptidase IV (DAP) to Ang IV. Ang-(1-7) binds to Mas receptor to produce anti-inflammatory effects. Ang II can bind to either AT2R or AT1R. By binding to AT2R, eNOS is stimulated to produce nitric oxide (NO) for vasodilation. When Ang II binds to AT1R, it stimulates NADPH oxidase to produce oxygen radicals (ROS) such us superoxide anion (O_2^-). Ang II type 1 receptor blockers (ARBs) inhibit Ang II effects by binding to the AT1R.

Angiotensin II Signal Blockage

The development of renal injury and hypertension is closely related to inappropriate regulation of the RAAS. Hypertensive patients with renal disease are recommended as first-line therapy to use ACE inhibitors (ACEIs) or Angiotensin II type I Receptor Blockers (ARBs) (Fig. 4)[15]. These inhibitors attenuate renal disease in both pre-clinical and clinical studies, and are effective and well tolerated, improving morbidity and mortality associated to cardiovascular diseases[24-25]. They not only exert their effect at a systemic level reducing elevated values of blood pressure but also increase nitric oxide (NO) bioavailability. NO is a potent vasoactive molecule essential for the maintenance of cardiovascular health, which has proved to be affected in spontaneously hypertensive rats (SHR model)[26].

Kidney inflammation and fibrosis

In addition to the cardiovascular system, the kidney is highly affected by the excess of salt. Sodium filters freely through the glomeruli, being reabsorbed 99% of the filtered load along the nephron (mainly in the proximal tubules) by an integrated system of channels, interchangers and ionic transporters. Sodium reabsorption is a determinant factor of the renal oxygen consumption[27-28].

A renal pro-inflammatory response, secondary to the excess of sodium (Fig. 5), favours further sodium retention and thus the development of arterial hypertension[29-30]. The molecular mechanisms of the inflammatory response to salt-sensitive hypertension remain to be completely characterized. The increase in sodium reabsorption in the renal tubules leads to elevated blood flow and thus to glomerular hyper-filtration. This process intensifies the metabolic demand of oxygen, which results in a decrease in tissue oxygen tension (pO_2)[31]. The rise in oxygen consumption leads to relative hypoxia. The latter then triggers a cascade of events, magnifying the production of reactive oxygen species (ROS) that follows a rise of the expression of nuclear pro-inflammatory transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB). NF-κB activates genes involved in the inflammatory and fibrotic responses, resulting in the accumulation of cells responding to inflammation in the kidney as well as the release of adhesion molecules (V-CAM 1, I-CAM 1), chemokines (MCP-1, RANTES) and cytokines (transforming growth factor-beta1, TGF-β1), as well as Ang II[14]. Ang II signalling through AT1R stimulates the enzyme NADPH oxidase which produces more ROS, in turn activating NF-κB, and stimulating the expression of pro-inflammatory genes, closing a positive feedback[32].

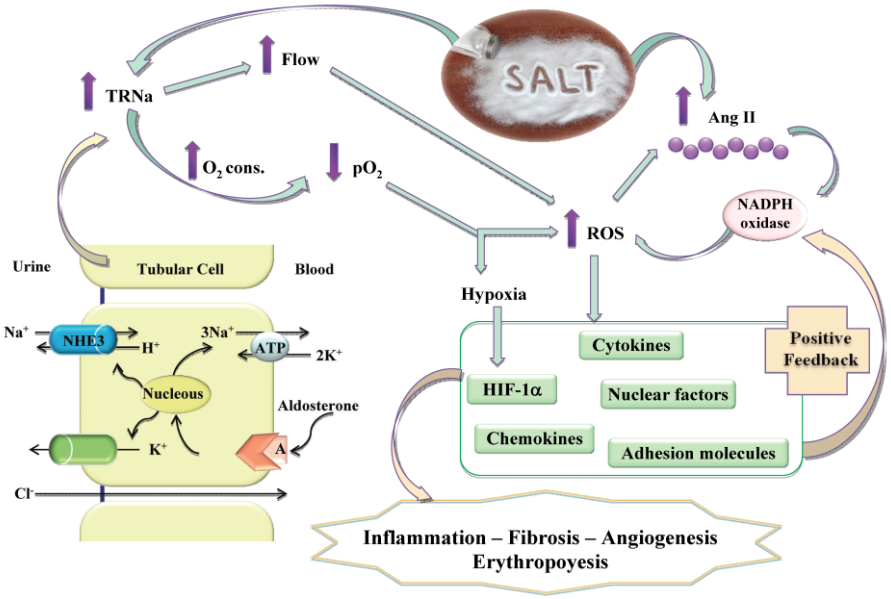

Figure 5. Inflammation markers triggered by excess of salt. High salt increases sodium tubular reabsorption and thus oxygen consumption. Higher oxygen demand leads to reduced O2 availability (hypoxia). Hypoxia, together with an increase of flow due to greater sodium transport, stimulates the formation of oxygen radicals. Angiotensin II production is up-regulated in the kidney and stimulates NADPH oxidase to produce more oxygen radicals (ROS). It follows an activation cascade of adhesion molecules, chemokines, cytokines and nuclear factors, leading to inflammation, fibrosis, angiogenesis, and erythropoyesis, closing a positive feedback.

In previous studies, we have provided *in vivo* evidence that there is early over-expression of the main pro-inflammatory and pro-fibrotic markers produced by acute infusions of hypertonic NaCl in anaesthetised Sprague Dawley rats. The cascade of events observed includes hypoxia, oxidative stress, tubular inflammation, and interstitial fibrosis, evidenced by the increased expression of: TGF-β1, RANTES, HIF-1α, α-SMA, NF-κB, and Ang II[33]. These processes were prevented within the acute period, by the administration of atrial natriuretic peptide[34]. On the contrary, infusion of Ang II maximized the over-expression of the aforementioned markers[35-36].

Damaged cells release ATP under local inflammation and intravascular microthrombus formation. The ATP is converted into adenosine by ectonucleases lining the endothelium of various cell surfaces[37-40]. Examples of this type of integral

membrane enzymes are ecto-nucleotide diphosphohydrolase (E-NTPDase, also Ectoapyrase or CD39) and ecto-5-nucleotidase (Ecto-5'-NTase, also CD73). E-NTPDase converts pro-aggregatory ATP into ADP and less efficiently into anti-aggregatory adenosine (Fig. 6). Ecto-5'-NTase converts ADP into adenosine. These actions limit the extent of intravascular platelet aggregation[41]. E-NTPDase has a role in water handling as well. Over-expression of this enzyme in transgenic mice results in down-regulation of the water channel aquaporin-2, leading to impaired urine concentration[42]. E-NTPDase is highly sensitive for ROS[43-44] leading to diminished expression of this ecto enzyme in ischemic conditions as observed in vascular endothelium of subjects with preeclampsia[45-46], and in the glomerular microvasculature in chronic kidney graft rejection[47]. ROS have been implicated in the signalling cascade induction related to vascular remodelling, cell growth, extracellular matrix protein deposition, inflammation, endothelial dysfunction, and increased vascular tone, all of which are characteristic features of the vascular phenotype in hypertension[48].

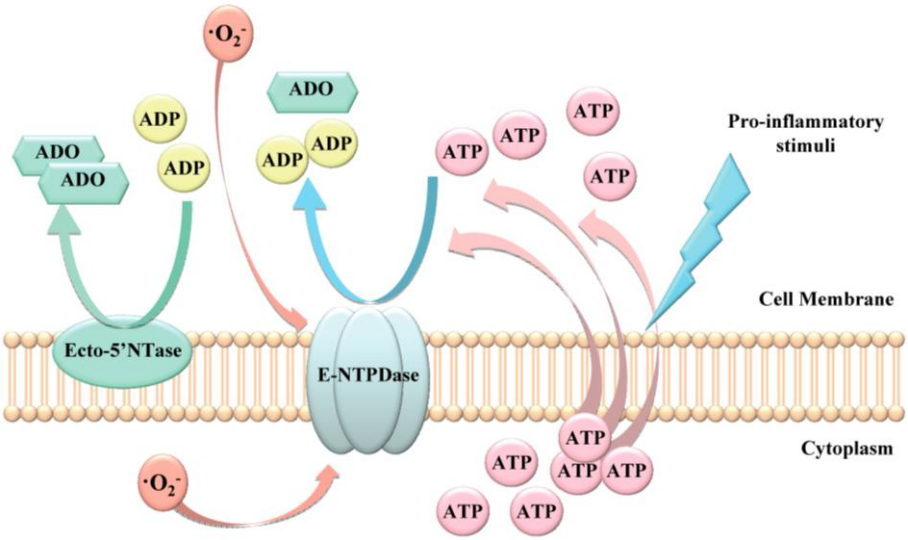

Figure 6. E-NTPDase. In pathological events, such as inflammation, there is accumulation and secretion of ATP from the injured cell, a pro-inflammatory molecule. E-NTPDase transforms these ATP molecules to ADP, and adenosine, although less efficiently. Ecto-5'Ntase converts ADP into adenosine. Adenosine, on the contrary, exerts a potent anti-inflammatory effect. Reactive oxygen species, such as $\cdot O_2^-$, have a direct negative influence on E-NTPDase, by down-regulating its expression.

Inflammation is a crucial event prior to the development of fibrosis, which is the final common pathway to end stage renal disease. It has been demonstrated that TGF-β1 activated by Ang II stimulates collagen and fibronectin synthesis and participates in the development of kidney damage, controlling the deposition of extracellular matrix and its remodelling[16, 24]. Up regulation of TGF-β1 is found in the majority of fibrotic kidney diseases, in patients as well as in animal models. On the contrary, the suppression of TGF-β1 signalling significantly slows the progression of interstitial kidney fibrosis in animals[49]. TGF-β1 is the main stimulant of the phenotypic activation of myofibroblasts. Myofibroblasts are the primary source of extracellular matrix products in active fibrotic sites. In response to tissue damage, these cells undergo a process of activation in order to turn into α-SMA-positive myofibroblasts, an intermediate phenotype between fibroblasts and muscle cells[50]. The interstitial α-SMA-positive myofibroblasts are responsible for the continuous accumulation and deposition of extracellular matrix in the interstitial compartments of damaged kidneys. Therefore these cells are not present in the normal kidney[49-50].

Endothelial dysfunction under salt excess. Role of Nitric Oxide

The vascular endothelium not only serves as a passive barrier to diffusion between the circulating blood and underlying vascular smooth muscle cells, but also regulates vessel diameter, inhibits blood clot formation, and impairs the multiplication of cells involved in plaque formation. Endothelial dysfunction is considered to play an important role in the pathogenesis of vascular disease. Two of the main characteristics are impaired NO availability and increased levels of ROS[51-52].

NO has numerous beneficial effects including the regulation of vascular tone, permeability, inflammation, cellular proliferation, angiogenesis, and vascular remodelling. NO is produced via the oxidative L-arginine pathway catalyzed by a family of three isoforms of nitric oxide synthases: nNOS (neuronal), iNOS (inducible) and eNOS (endothelial)[53-54]. Endothelial cell activation, in response to agonists and mechanical stimuli, leads to NO formation by stimulating eNOS (Fig. 7)[55]. Endothelium-derived NO diffuses to the underlying vascular smooth muscle cells (VSMC) and stimulates soluble guanylyl cyclase to produce cGMP, causing relaxation[56]. Recent data have suggested the involvement of aquaporin-1 (AQP-1) in NO transport out of the endothelium and into VSMC[57-58]. However, more recently, it has been demonstrated that VSMC also express all three NOS isoforms, which may represent an alternative mechanism whereby local NOS expression modulates

vascular functions in an endothelium-independent manner[59-60]. Previous studies have demonstrated that a high salt diet leads to impaired vascular relaxation to acetylcholine and elevated production of superoxide by NADPH oxidase and xantine-xantine oxidase in aorta and mesenteric resistance arteries[61-64]. By incubating the vessels with tempol, apocynin or oxypurinol, the higher superoxide levels are reduced and NO levels are increased, suggesting that the increase of superoxide in animals fed a high salt diet interfere with NO availability and thus, it could play a critical role in reducing endothelium-dependent vascular relaxation, and inducing vascular remodelling, cell growth, and extracellular matrix protein production[65]. However, at the present time, the effect of elevated superoxide levels on eNOS expression in smooth muscle layer from arteries of rats fed high salt diet have not been determined.

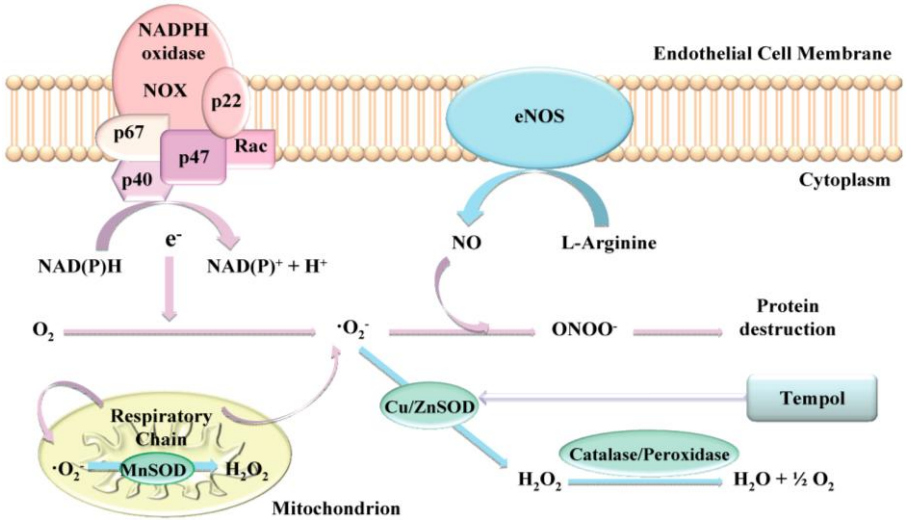

Figure 7. Production and destruction of Reactive Oxygen Species and Nitric Oxide. NADPH oxidase stimulated by mechanical stretch, vasoactive peptides, growth hormones and cytokines, converts NAD(P)H into $NAD(P)^+$ and H^+, releasing an electron that converts the oxygen (O_2) into an oxygen radical, the superoxide anion ($\cdot O_2^-$). The superoxide anion is transformed into H_2O_2 by the Superoxyde Dismutase (SOD) enzyme and into water and oxygen again by Catalase or Peroxidase. The Nitric Oxide Synthase (endothelial, eNOS) produces the vasodilator Nitric Oxide (NO) by metabolizing L-arginine. NO can also bind to $\cdot O_2^-$ and generate peroxinitrite ($ONOO^-$), another oxygen radical that produces protein damage. Tempol is able to mimic SOD actions metabolizing $\cdot O_2^-$.

Oxidative Stress

It has been described that a high salt diet increases oxidative stress in kidneys of salt-sensitive rats, for example, by producing superoxide anion or via lipidic peroxidation[66-67]. ROS are involved in vasoconstriction and anti-natriuresis in the kidneys in vivo[68] and increase sodium transport in vitro[69], suggesting that this anion plays a pathophysiological role in the kidney, contributing to the development of hypertension. Moreover, Ang II can increase the production of ROS through the activity of the enzyme NADPH oxidase (Fig. 7)[70]. On the other hand, superoxide anion activates the transcription of the nuclear factor NF-kB, which regulates the induction of genes involved in the inflammatory and fibrogenic responses through the release of cytokines and accumulation of inflammatory cells in the kidney, as mentioned[14, 71].

Reducing Oxidative Stress

Tempol (4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-N-oxyl) is a small cell-permeable molecule, mimetic of the superoxide dismutase enzyme (SOD), commonly used for the study of oxidative stress in different animal models of salt sensitive-hypertension (Fig. 7). The acute or chronic intravenous administration has also been effective in reducing blood pressure[72]. This effect is accompanied by an increase in sodium excretion and a decrease in oxygen consumption in the kidney. The mechanisms by which tempol improves renal oxygenation are not well established. One possibility is that tempol increases NO bioavailability, which competes with the oxygen in the mitochondrion respiratory chain, thus reducing the use of oxygen by the mitochondria and inhibiting the luminal entrance of sodium[73]. In this regard, Welch *et al.* demonstrated that tempol improves pO_2 in the renal cortex and the relation sodium transport/oxygen consumption, previously reduced by prolonged infusion of Ang II[74]. In addition, the oral administration of tempol to Dahl-sensible rats (a model of salt-sensitive hypertension) fed a diet rich in sodium, prevents the intrarenal increase of angiotensingen[75] and thus, the production of Ang II[15, 75]. However, Silva *et al.* observed that the increase of NaCl in the thick ascending limb of Henle in the kidney medulla is capable by itself to diminish the production of superoxide anion, independently of the effects of Ang II[76].

Aim and scope of the thesis

The aim of this thesis is to deepen the present knowledge of the effects of excess salt on the kidney *in vivo* and on the vascular endothelium *in vitro*, especially in regard to water balance disorders, and inflammatory and fibrotic responses.

In the first part of this thesis, we focused on the regulation of water channels in the kidney and the alterations they undergo when exposed to high sodium. In Chapter 1, we investigated the expression of the main two AQPs in renal tubules of normal rats under a high salt diet. We evaluated the role of tubular Ang II signalling by administering losartan (AT1R blocker), and the participation of oxidative stress and NO by inhibiting ROS production with tempol (mimetic of the superoxide dismutase enzyme). We measured systolic blood pressure and renal function as well as the intrarenal levels of the AQPs together with inflammatory markers. Our next aim was to assess the expression of these AQPs and the inflammatory markers in an acute model of hypernatremia, together with their regulation by the RAAS and oxidative stress (Chapter 2). In this study, we utilized an acute sodium overload treatment in anesthetized as well as in anaesthetized rats. In the second part of the thesis, we studied the effects of salt on inflammation and fibrotic markers in different models *in vivo* and *in vitro*. We first utilized an *in vivo* model of acute sodium overload in normal rats (Chapter 2 and 3), in which we measured mean arterial pressure and renal functional parameters. We evaluated the role of tubular Ang II signalling by administering losartan, and the participation of oxidative stress with tempol. We also studied the regulation of pro-inflammatory cytokines and chemokines and nuclear factors. We then proposed to study the aforementioned processes in a chronic model, as a way to correlate it with salt intake in humans. We used concentrations of salt higher than in regular high salt intake in humans in order to enhance the effects. For this purpose, animals were subjected to a diet with “normal salt” or “high salt” content (Chapters 4, 5 and 6). In Chapter 4, we hypothesized that the oxidative stress induced by chronic salt overload could stimulate inflammatory and fibrogenic signalling pathways in kidneys of normal rats. To prove this hypothesis, we administered tempol in the drinking water of rats fed a high salt diet. In Chapter 5, we investigated a possible adaptive mechanism which attenuates the deleterious effects of oxidative stress and hypoxia that are produced in response to a chronic sodium overload. In Chapter 6, we tested the direct *in vitro* effect of salt on the endothelium and the potential involvement of ROS in the mechanism of damage, by producing an *in vitro* co-culture with a monolayer of endothelial cells and a supernatant with peripheral blood mononuclear cells (PBMC).

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Part I

Salt and Water Balance

Chapter 1

Salt-induced downregulation of renal aquaporins is prevented by losartan

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Regulatory Peptides

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Abstract

The purpose of this study was to investigate the expression of aquaporin-1 (AQP-1) and aquaporin-2 (AQP-2) in the renal tubule of rats fed a high-salt diet and its modulation by the AT1 receptor blocker losartan.

The experiments were performed in four groups of rats fed for 3 weeks with the following diets: regular rat chow (NS), high-salt (8% NaCl) chow (HS), NS plus losartan (NS-L) and HS plus losartan (HS-L). Losartan (40 $mg.kg^{-1}$) was administered in the drinking water. Systolic blood pressure (SBP) and renal function were evaluated. The intrarenal levels of angiotensin II (Ang II), TGF-β1, α-smooth muscle actin (α-SMA), endothelial nitric oxide synthase (eNOS), AQP-1 and AQP-2 were determined by immunohistochemistry. AQP-1 and AQP-2 protein levels were measured by western blot analysis.

A high-sodium diet down-regulated AQP-1 and AQP-2 expression levels in the proximal tubule and collecting duct, respectively. The high-sodium diet also induced Ang II, TGF-β1 and α-SMA over-expression and decreased eNOS expression in the renal cortex and medulla. Losartan increased the diuresis and natriuresis, favouring urinary sodium concentration. Additionally, losartan prevented the pro-fibrogenic response, decreasing Ang II, TGF-β1 and α-SMA levels and normalizing AQP-2 expression in the HS-L group. AQP-1 expression was upregulated by losartan in both the NS-L and HS-L groups.

These results show that increased intrarenal Ang II in rats fed a high-salt diet down-regulates renal AQP-1 and AQP-2 expressions. In addition, although losartan increased diuresis and natriuresis, it prevented the down-regulation of aquaporins, favouring urinary sodium concentration.

1. Introduction

Diverse factors like AQP-1, AQP-2, Ang II and vasopressin can alter the ability of the kidney to recover water from the tubular fluid. AQP-1 mediates the reabsorption of already 80% of the fluid from the glomerular filtrate, being expressed in the luminal and basolateral membranes of the proximal tubules, in epithelial cells of the thin descending limb of Henle's loop and in endothelial cells of the descending vasa recta[1, 2]. In addition, AQP-2 channels reabsorb the remaining 20% of the tubular fluid in the collecting duct. Vasopressin, besides its known properties to stimulate water reabsorption, translocates AQP-2 from the intracellular vesicles to the apical plasma membrane in renal tubular cells and stimulates AQP-2 gene transcription[3].
The renin–angiotensin system is a major hormonal regulator of sodium homeostasis and mediates most of its effects via Ang II[4]. Ang II signals are mediated by stimulation of two receptor subtypes, named Ang II type 1 (AT1) and Ang II type 2 (AT2). AT1 receptors are ubiquitously expressed in the kidney, mediate vasoconstriction of the afferent and efferent arterioles and medullary microvasculature and modulate the glomerular filtration rate, the tubuloglomerular feedback mechanism, and cell growth. In addition, Ang II modulates also the activity of diverse ion transporters in the kidney, such as Na+/H+ interchanger, NKCC2 co-transporter, NCC co-transporter and ENaCs[5]. Altogether, these actions of Ang II contribute in a synergistic manner to increase the capability of the kidneys to conserve sodium and maintain blood pressure within normal levels. AT2 receptors are localized in glomerular epithelial cells, proximal tubules, collecting ducts and in renal vasculature. They are considered as functional antagonists of AT1 receptors, and are associated with vasodilatation, apoptosis, antiproliferation and the increase of natriuresis, by stimulating the NO/cGMP/protein kinase G pathway[6]. It has been recently demonstrated that Ang II also regulates the activity of the main AQPs in the kidney. Wintour *et al.* reported that the in vivo infusion of Ang II increased AQP-1 gene expression in the sheep kidney[7]. Moreover, it has been demonstrated that Ang II may regulate AQP-2 targeting to the plasma membrane in the inner medullar collecting duct cells, through AT1 receptor activation[8], and that the peptide also potentiates vasopressin effects on AQP-2[9] as well. In addition, Bouley *et al.*[10] have shown a biphasic effect of exogenous Ang II administration, since 10^{-8} M to 10^{-9} M of Ang II upregulated and a higher concentration of Ang II (10^{-7} M) downregulated AQP-1 mRNA expression in proximal tubule cells. Furthermore, it was also documented that an autonomous renin–angiotensin system

is present in renal proximal tubules, being Ang II locally produced and secreted, reaching a concentration that is 100-fold higher in the lumen than in plasma[11]. The facts that renin and its mRNA expression were localized in the principal cells of the collecting ducts[12], that angiotensin converting enzyme (ACE) was found in distal nephron segments and that angiotensinogen was found in the urine[13], indicates that the distal part of the nephron is also a place of synthesis of intrarenal Ang II. In this way, locally formed Ang II in the kidney, in pathological conditions, may contribute to renal diseases by inducing sodium retention, inflammation, fibrosis[14] and AQP downregulation[15, 16].

Up to date, there are no reports analyzing the effects of a chronic overload of salt on AQP expression in the kidney. Rats fed a high-sodium diet constitute an experimental model, characterized by low plasma renin activity and circulating Ang II levels but with increased Ang II intrarenal levels[17], which is useful to analyze that relationship. Based on these antecedents, we hypothesized that the increase of renal Ang II observed in rats subjected to sodium overload could downregulate AQP expression through a pro-inflammatory and profibrotic effect, decreasing urinary sodium concentration. Then, the aim of the present study was to examine the immunoexpression of AQP-1 and AQP-2 in rats subjected to a high-salt diet, with or without simultaneous Ang II-AT1 receptor blockade.

2. Materials and methods

2.1. Animal protocol

Male Sprague Dawley rats of 200–230 g body weight were used. Animals were housed in steel cages in a controlled temperature room at 23± 2 °C, exposed to a daily 12-hour light–dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.), and fed for 3 weeks with the diets described below, with free access to tap water. Experiments were conducted in accordance with the institutional guidelines for the care and use of research animals of the University of Buenos Aires, Argentina.

Animals were randomly divided into four groups (n=6 each group): a) NS (control): animals fed a normosodic diet (0.4 g% NaCl); b) HS: animals fed a high-sodium diet (8 g% NaCl); c) NS-L: animals fed a normosodic diet plus losartan (40 $mg.kg^{-1}.day^{-1}$; SigmaAldrich Inc., St. Louis, Missouri, USA) administered together with the drinking water; d) HS-L: animals fed a high-sodium diet plus losartan. At the end of the third week, the systolic blood pressure (SBP) was measured by the tail

cuff method and recorded in a Grass Polygraph 79D, before the sacrifice of the animals by decapitation. The kidneys were quickly removed and processed for histological studies which include the measurement of intrarenal levels of Ang II, TGF-β1, α-smooth muscle actin (α-SMA) and endothelial nitric oxide synthase (eNOS) by immunohistochemistry and AQP-1 and AQP-2 by both immunohistochemistry and Western blot analysis.

To evaluate the renal function, additional four groups were performed. The last day of the third week, the animals were intraperitoneally anesthetized with urethane (1.2 $g.kg^{-1}$). A PE-90 tube (3 cm long) was inserted into the trachea to maintain an open airway and the left femoral vein was catheterized with a Silastic cannula (0.12 mm i.d.) for continuous infusion. The bladder was cannulated for urine collection using a PE-75 cannula. The femoral vein was infused with 0.15 M NaCl isotonic saline solution (ISS) at a rate of 0.04 $mL.min^{-1}$ (Syringe Infusion Pump, Sage™, Orion) for 60 min, to achieve a steady diuresis and allow urine collection (equilibration period). Then, the ISS infusion continued for another 60 min at the same rate (experimental period). A blood sample was collected after 30 min of the experimental period and a urine sample was collected during 60 min of the experimental period, for measurement of urine volume, urinary and plasma sodium and creatinine.

2.2. Urine and blood measurements

Urinary and plasma sodium and creatinine were measured by standard methods using an autoanalyzer. Creatinine clearance was assessed to evaluate the glomerularfiltration rate (GFR). GFR and sodium fractional excretion (FE_{Na}) were calculated according to a standard formula. Urinary flow (UV) is expressed as $\mu L.min^{-1}.kg^{-1}$, sodium urinary excretion (UV_{Na}) as $\mu mol.min^{-1}.kg^{-1}$, urinary sodium concentration (U_{Na}) as $mEq.L^{-1}$, GFR as $mL.min^{-1}$ and FE_{Na} as percentage.

2.3. Kidney processing for histological studies

At the end of the experimental period, both kidneys were rapidly excised, decapsuled, longitudinally cut, fixed in formol-buffer (pH 7.2) and then included in paraffin and cut into 3-μm sections. For immunohistochemistry, sections were deparaffined and rehydrated, and endogenous peroxidase activity was blocked by treatment with 0.5% H_2O_2 in methanol for 20 min. Local expression of AQP-1, AQP-2, Ang II, TGF-β1, α-SMA and eNOS was detected using the following specific antibodies: rabbit anti-AQP-1 (Santa Cruz Biotechnology, Inc., USA; 1:50

dilution), rabbit anti-AQP-2 (Santa Cruz Biotechnology, Inc.; 1:200 dilution), human antiAng II (Península, CA; 1:500 dilution), rabbit anti-TGF-β1 (Santa Cruz Biotechnology, Inc.; 1:200 dilution), rabbit anti-α-SMA (Santa Cruz Biotechnology, Inc.; dilution of 1:200), and rabbit anti-eNOS (Santa Cruz Biotechnology, Inc.; 1:200 dilution). Immunostaining was carried out by means of a commercially modified avidin–biotin–peroxidise complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, CA, USA) and counterstained with hematoxylin. Histological sections were observed in a Nikon E400 light microscope (Nikon Instrument Group, Melville, New York, USA). All measurements were carried out using an image analysis software (Image-Pro Plus ver. 4.5 for Windows, Media Cybernetics, LP, Silver Spring, MD, USA). The results are expressed as percentage of the stained area.

2.4. Kidney processing for western blot analysis of aquaporins

Kidneys were excised and the renal cortex and medulla were immediately dissected. 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, 2 μmol.L^{-1} leupeptin, 1x protease inhibitor cocktail (Roche Diagnostics)). Protein concentration was determined by Lowry technique in the Triton-soluble supernatant. Samples of cortex and inner medulla containing similar amounts of protein (100 μg protein/lane) were separated by electrophoresis in 7.5% SDS polyacrylamide gels (Bio-Rad, Munich, Germany), transferred to a nitrocellulose membrane (Bio-Rad), and then incubated with rabbit polyclonal anti-AQP-1 (Santa Cruz Biotechnology, 1:250 dilution), or rabbit polyclonal anti-AQP-2 (Santa Cruz Biotechnology,1:250 dilution). A secondary immunoreaction with a goat anti-rabbit antibody conjugated with horseradish peroxidase (1:5000 dilution) was then carried out. The samples were revealed by chemiluminescence using ECL reagent for 2–4 min (Amersham Pharmacia Biotech). The density of the respective bands was quantified by densitometric scanning of Western blots using a Hewlett–Packard scanner and a Totallab analyzer software (Biodynamics Corp., Seattle, WA). Protein amounts were calculated by densitometry. Protein levels are expressed as the ratio between AQP-1 or AQP-2 and β-actin bands (Assay Designs Inc) and control optical densities.

2.5. Statistical analysis

Results of urine and blood measurements and SBP are expressed as mean ± SEM (standard error of the mean). Immunostainings are expressed as the percentage of the positively stained area ± SEM. The Gaussian distribution was evaluated by the Kolmogorov and Smirnov method. The groups were compared using ANOVA followed by the Newman–Keuls test. Values of $p > 0.05$ were considered significant.

3. Results

3.1. Systolic arterial pressure and renal function

The SBP increased in the HS group with respect to the NS group (mm Hg, NS: 128± 2, HS: 148±2, $p<0.01$; ΔSBP: 20± 2). Losartan reduced the SBP in the NS-L and HS-L groups, but with a difference in tensional levels that is very similar to that observed between the NS and HS groups (NS-L: 107±2, HS-L: 122±4, $p<0.05$; ΔSBP: 15±2).

Table 1 shows the parameters of renal function. UV increased in the HS group as compared with the NS group. Losartan did not alter UV in the NS-L group, but increased UV further in the HS-L group. UV_{Na} exhibited a very similar behaviour to UV: it was higher in the HS group than in the NS group. Losartan did not modify UV_{Na} in the NS-L group, but increased it further in the HS-L group. U_{Na} increased in the HS group as compared with the NS group; whereas losartan increased U_{Na} in both the NS-L and the HS-L groups, maintaining a significant difference between the NS-L and HS-L groups. GFR was unaltered in the HS group with respect to the NS group. Losartan diminished GFR in the NS-L group, but did not alter it in the HS-L group. FE_{Na} increased in HS with respect to NS; whereas losartan increased FE_{Na} in both the NS-L and the HS-L groups, but with a difference between their FE_{Na} values similar to that observed between the NS and HS groups.

	UV	UV_{Na}	U_{Na}	GFR	FE_{Na}
NS	10.1±2.2	0.4±0.3	19.6±5.2	1.5±0.4	0.06±0.05
HS	16.1±2.1*	2.9±1.6*	164.7±16.7*	1.3±0.3	0.51±0.24*
NS-L	8.6±2.1	0.5±0.1	59.3±9.1†	0.4±0.1†	0.25±0.09†
HS-L	27±1.4*†	8.5±0.5*†	315.0±23.5*†	1.5±0.5*	1.3±0.01*

Table 1. Renal Function Parameters. Abbreviations: UV: Urinary flow ($\mu L.min\text{-}1.kg^{-1}$); UV_{Na}: urinary sodium excretion ($\mu Eq.min^{-1}.kg^{-1}$); U_{Na}: urinary sodium concentration ($mEq.L^{-1}$), GFR: Glomerular Filtration Rate (mL.min-1); FE_{Na}: Sodium Fractional Excretion (%). NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan. Values are expressed as mean ± SEM. *p<0.05 vs. the respective normosodic control (NS and NS-L); † p<0.01 vs. the respective control without losartan (NS and HS).

3.2. Expression of aquaporins in renal sections

Figure 1 shows AQP-1 and AQP-2 abundance determined by Western blot. AQP-1 expression was slightly but significantly diminished in the HS group with respect to the NS group. Losartan administration increased AQP-1 in both the NS-L and HS-L groups. AQP-2 was significantly decreased in the HS group compared with NS rats and was normalized to control levels by losartan (HS-L group).

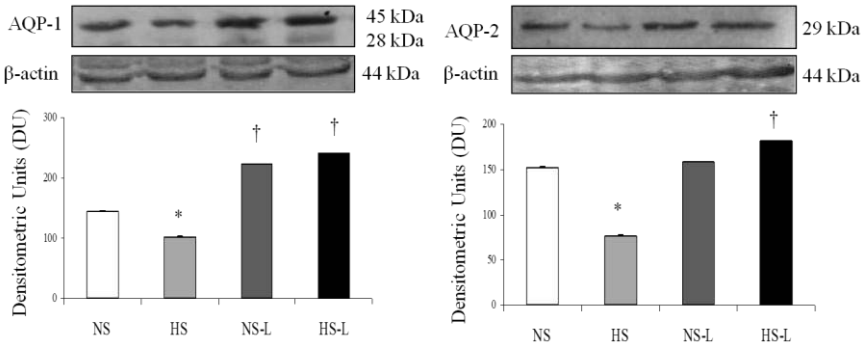
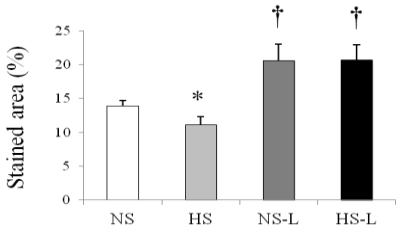


Figure 1. Western blot of AQP-1 and AQP-2. Representative Western blot analysis of AQP-1 abundance in renal cortex and AQP-2 abundance in renal medulla. Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan. Values [means ±

SEM (n=5)] represent the mean of densitometric units (DU) after background subtraction. All experiments were performed in triplicate. Each blot was normalized to ß-actin expression band in the same gel. * p<0.01 vs. the respective normosodic control (NS and NS-L); † p<0.01 vs. the respective control without losartan (NS and HS).

Figure 2 shows the immunostaining of AQP-1 in renal tissue. AQP-1 immunoexpression was decreased in proximal tubules of HS animals, and increased by the administration of losartan in both the NS-L and HS-L groups.

Panel A



Panel B

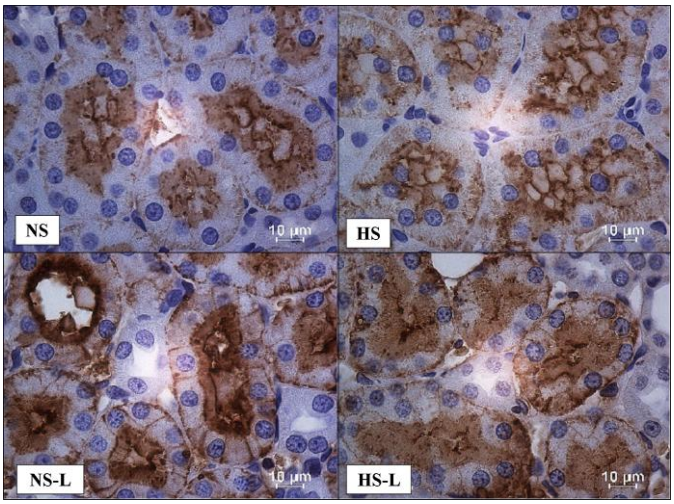
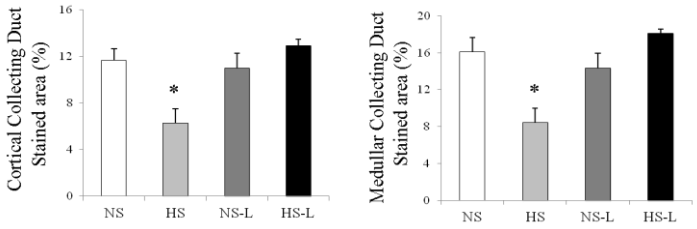


Figure 2. AQP-1 immunoexpression in renal tissues. Panel A. Histograms illustrate the values of AQP-1 expression in renal tissue. Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan. Values are expressed as percentage (%) of positive stained area ± SEM; n=5; *p <0.01 vs NS group; †p< 0.01 vs the respective control without losartan. Panel B. Representative image of positive staining corresponding to AQP-1 expression in renal cortex (400x).

Figure 3 shows the immunostaining of AQP-2 in renal tissue. AQP-2 immunoexpression decreased in the cortical and medullar collecting ducts of the HS group. This decrease was prevented by losartan administration.

Panel A



Panel B

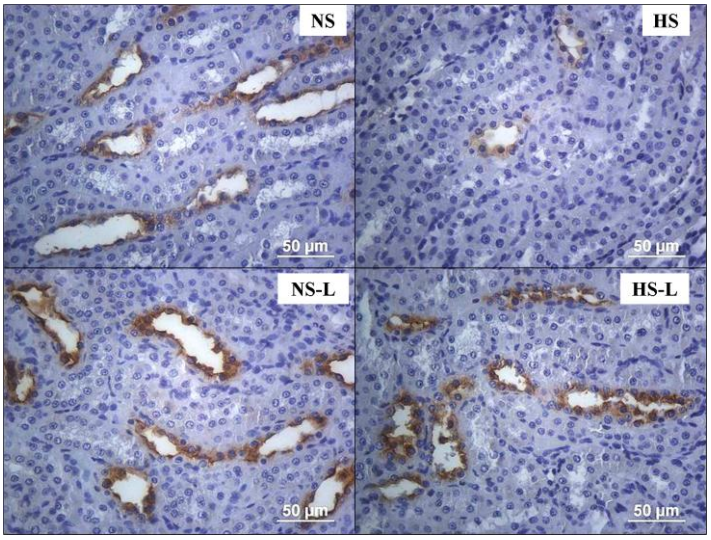


Figure 3. AQP-2 immunoexpression in renal tissues. Panel A. Histograms illustrate the values of AQP-2 expression in renal tissue. Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan. Values are expressed as percentage (%) of positive stained area ± SEM; n=5; *p <0.01 vs. NS group. Panel B. Representative image of positive staining corresponding to AQP-2 expression in renal medulla (400x).

3.3. Immunohistochemical staining of Ang II and profibrotic markers in renal sections

Table 2 and Figures 4 to 7 show the immunostaining of Ang II, TGF-β1, α-SMA and eNOS in renal tissues, respectively. Immunoexpression of Ang II (Table 2 and Fig. 4) and TGF-β1 (Table 2 and Fig. 5) increased in renal tubular cells of the HS group, while that of α-SMA (Table 2 and Fig. 6) increased in the interstitium of the renal cortex and medulla. Losartan (HS-L group) prevented the increase in Ang II, TGF-β1 and α-SMA expression elicited by the sodium load, in renal cortex and medulla. On the other hand, losartan treatment did not alter Ang II and TGF-β1 staining in the NS-L group, although it slightly increased α-SMA staining in renal cortex and medulla of the mentioned group.

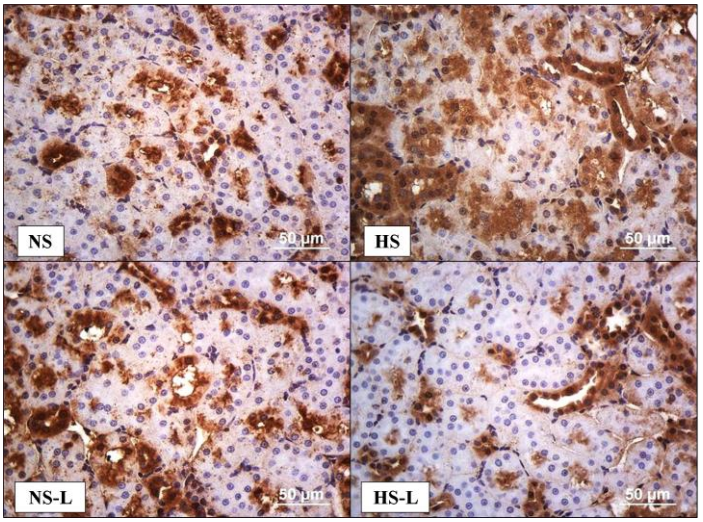


Figure 4. Ang II immunoexpression. Representative image of positive staining corresponding to Ang II expression (400x). Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan.

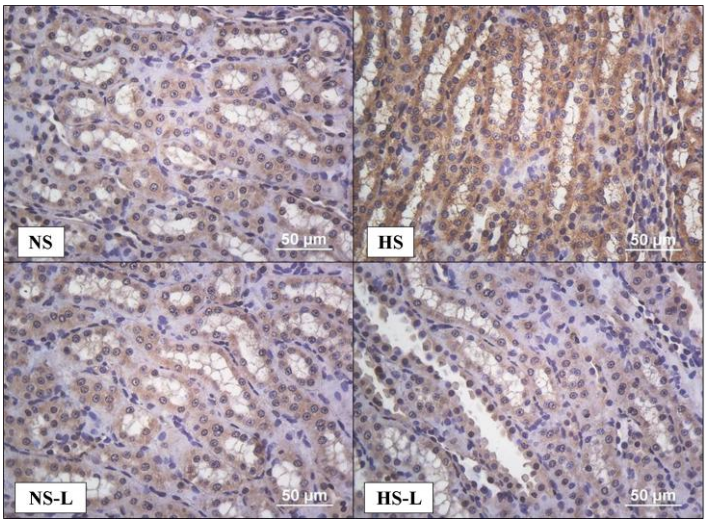


Figure 5. TGF-β1 immunoexpression. Representative image of positive staining corresponding to TGF-β1 expression (400x). Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan.

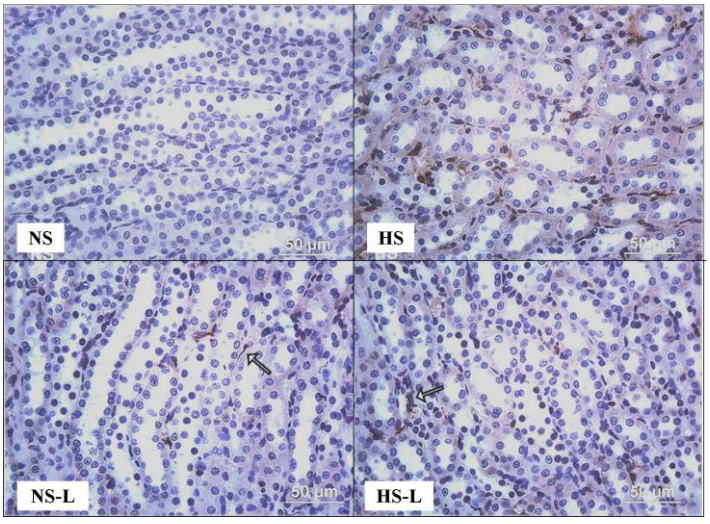


Figure 6. α-SMA immunoexpression. Representative image of positive staining corresponding to α-SMA expression (400x). Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan.

Conversely, the intrarenal expression of eNOS (Table 2 and Fig. 7) was markedly decreased in tubular cells of the renal cortex and the medulla in the HS group with respect to NS rats. Losartan, which showed no effects in NS rats, prevented the decrease in eNOS staining in the HS-L group, showing a similar stain to that observed in the NS group.

		Ang II (%)	eNOS (%)	TGF-β1 (%)	α-SMA (Nº mf)
CORTEX	NS	16.6±1.8	23.4±2.1	26.9±0.6	4.2±0.8
	HS	44.7±0.8*	11.2±2.2*	48.7±1.1*	33.2±1.3*
	NS-L	20.9±0,9	20.7±3.3	29.1±1.1	16.8±2.4*
	HS-L	17.0±1.1†	28.6±1.5†	24.2±0.8†	18.7±1.9*†
MEDULLA	NS	15.5±1,6	24.3±1,2	19.3±0.5	10.5±0.9
	HS	35.7±1.1*	10.4±2.3*	36.8±0.8*	127.4±1.9*
	NS-L	13.8±0.8	18.3±3.0	16.5±0.8	22.9±2.7*
	HS-L	17.1±1.1†	24.9±1.3†	17.3±0.7†	19.7±1.4*†

Table 2. Expression of angiotensin II (Ang II), endothelial nitric oxide synthase (eNOS), transforming growth factor-β1 (TGF-β1) and alpha-smooth muscle actin (α-SMA) in renal cortex and medulla. Abbreviations: %: percentage of stained area; Nºmf: number of miofibroblasts per area; NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan. *p< 0.01 vs NS; †p< 0.01 vs respective control without losartan.

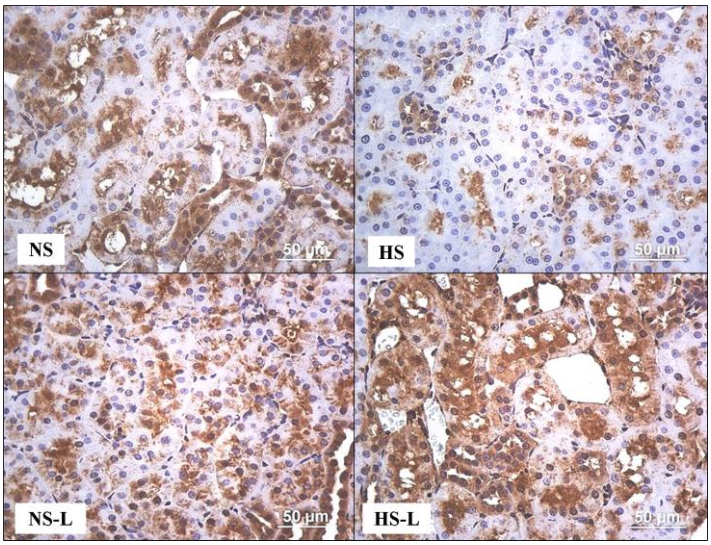


Figure 7. eNOS immunoexpression. Representative image of positive staining corresponding to α-SMA expression (400x). Abbreviations: NS: normosodic diet; HS: high-sodium diet; NS-L: normosodic diet plus losartan; HS-L: high-sodium diet plus losartan.

4. Discussion

The present study constitutes the first report demonstrating that an excess in dietary salt downregulates AQP-1 and AQP-2 levels in proximal tubules and collecting ducts respectively, and that this downregulation is prevented by Ang II-AT1 blockade by losartan. In addition, losartan increased AQP-1 expression in proximal tubules independently of sodium load, promoting the concentration of sodium in the urine. Furthermore, we showed that an excess of salt in the diet increases renal Ang II expression and develops a profibrogenic response in the kidney, as evidenced by Ang II, TGF-β1 and α-SMA overexpression in renal tissues. All these alterations were prevented by the administration of the AT1 receptor antagonist, losartan.

In the present study, we observed that Ang II immunoexpression increased in the renal cortex and medulla. This increase could be a result from a greater uptake of circulating Ang II by stimulation of AT1 receptor and/or from *de novo* generation of intrarenal Ang II, derived from local intrarenal angiotensinogen, produced and secreted by proximal tubular cells[4]. Data in literature are contradictory. Clinical and

experimental studies have demonstrated that the excessive salt intake increases oxidative stress, AT1 receptor expression as well as the renal Ang II levels[18–21], whereas AT2 receptors are down-regulated[22]. However, Fox et al. reported that, in normal conditions, an increase in salt intake inhibits both, circulating Ang II and the tissue renin–Ang II system[23]. In addition, Lara *et al*. described that Sprague–Dawley rats subjected to a high salt diet for 2 weeks did not show changes in urinary angiotensinogen excretion although they exhibited enhanced deposition of collagen and increased NADPH oxidase activity in the kidneys[24]. However, it must be pointed out that, the oxidative stress is able to induce conformational molecular changes in the angiotensinogen molecule which facilitates a more rapid generation of Ang I, when it is exposed to renin[25]. In this order, we have previously demonstrated an increase of NADPH oxidase activity and a profibrogenic response in the kidneys of normal rats fed a high salt diet, being all these changes prevented by the administration of tempol, a superoxide dismutase mimetic[26].

Since a dietary salt supplement increased blood pressure, we cannot exclude that the lower expression of AQPs found in rats fed a high-sodium diet, could be a consequence of hypertension. However, Mc Donough *et al*. reported that acute variations in tensional levels may cause rapid and reversible changes of sodium pump activity, as well as a relocation of the apical sodium transporters[27]. Therefore, we cannot exclude that chronic changes in blood pressure could be implicated in the downregulation of AQP expression in rats fed a high-salt diet. In order to investigate the effects of renal Ang II, through AT1 receptor stimulation, on AQP regulation in rats fed a high-salt diet, we analyzed the response caused by losartan. In the HS group, renal Ang II levels were increased and AQP levels were decreased, being these effects reversed by losartan administration. These results could be related to the inhibition of intrarenal rather than circulating Ang II, considering that in our experimental model, circulating Ang II would be decreased by the inhibition of renin secretion by sodium overload. In this order, it has been recently recognized that local renal Ang II is a powerful pro-inflammatory cytokine and growth factor, implicated in the pathogenesis of progressive renal diseases and the development of tubular–interstitial fibrosis through AT1 receptor binding[28], whereas stimulation of AT2 receptor reduces inflammation and fibrosis in the ischemic kidney through nitric oxide and cGMP production[29]. It is generally admitted as a functional negative crosstalk between AT1 and AT2 receptors in several pathophysiological conditions including hypertension[30]. In addition, AT2 receptor activation may directly antagonize AT1 receptor mediated actions by forming heterodimers with AT1 receptors[31]. Therefore, further studies are needed to elucidate the role of AT1 and

AT2 receptors in losartan effects on renal AQP expression. In addition, it has been also suggested that the chronic interstitial fibrosis caused by Ang II could be the cause of AQP downregulation and that this fact would be clinically associated with the inability of the kidney to concentrate the urine in different pathological conditions. In this order, Cao *et al.*[15] have demonstrated that during acute lung injury, Ang II levels are negatively correlated with AQP-1 mRNA in lung tissues. Similarly, Jensen *et al.* showed that the treatment with the AT1-receptor inhibitor candersartan prevented the decrease in AQP-2 expression in response to the bilateral ureteral obstruction and attenuates the post-obstructive polyuria as well as the renal sodium loss[16]. Moreover, Hasler *et al*. showed that the activation of NF-kB transcriptional factor by pro-inflammatory factors such as Ang II reduces AQP-2 gene transcription[32]. In the present study, we observed that in the HS group, Ang II and TGF-β1 immunostaining increased in tubular cells of the renal cortex and medulla, and α-SMA staining increased in the renal interstitium, whereas eNOS was decreased in the renal cortex and medulla. Therefore, since losartan caused a marked decrease of the expression of profibrogenic markers, and prevented changes in eNOS expression, it is possible to suggest that the enhancement of intrarenal Ang II expression in the HS group may be responsible for the downregulation of AQPs in the kidney, as a consequence of a pro-inflammatory or a pro-fibrogenic action. Moreover, the protective action of losartan could not only be due to AT1 blockade, but also to Ang II stimulation on the available AT2 receptors and the response through eNOS–nitric oxide–cGMP signalling. The activation of the AT2 signalling pathway could prevent the pro-inflammatory response and up-regulate AQPs in renal tissues. In addition to preventing AQP-1 downregulation, losartan upregulated AQP-1 expression in the proximal tubule from both NS-L and HS-L groups, suggesting that this effect was independent of the sodium overload. Further studies are required to elucidate the significance of these observations.

In the present study, a high-salt intake caused a meaningful increase in UV_{Na} and FE_{Na}, without changing the GFR. The simultaneous administration of losartan increased even more UV_{Na} and FE_{Na}, but did not alter the GFR. Conversely, in the control group fed with normosodic diet, losartan caused a decrease in SBP and GFR, but did not modify sodium excretion, thus increasing the FE_{Na}. The control of sodium excretion and blood pressure by Ang II is exerted through multiple intrarenal as well as extrarenal mechanisms. The most important intrarenal effects of Ang II include efferent arteriolar constriction through AT1 receptor stimulation, as well as direct effects on sodium transport. The constrictor effect on efferent arterioles is also important in preventing reductions of GFR in circumstances associated with

impaired renal perfusion. Therefore, the fall in blood pressure and the direct effect on efferent arterioles produced by losartan in normal rats could cause reductions in the GFR, as we observed in the present work. Our studies revealed that losartan exerted a natriuretic effect at the tubular level, independently of its hypotensive effect and of GFR changes. Moreover, Ang II over-expression in rats fed a high-salt diet was accompanied by a marked decrease in eNOS expression in the renal cortex and medulla, being this decrease normalized by losartan treatment. It is well documented that renal AT1 receptors, via activation of renal sodium transporters, produce anti-natriuretic effects[27], whereas AT2 receptors activate the nitric oxide/cGMP pathway leading to an increase in urinary sodium excretion[33, 34], thus influencing renal sodium/fluid homeostasis. Therefore, the natriuretic action of losartan could not only be due to AT1 blockade, but also to Ang II stimulation on the available AT2 receptors. Finally, we observed that losartan increased urinary sodium concentration. In this order, it has been described that AQP-2 expression in the kidney is related to changes in nitric oxide synthase activity[35]. Additionally, it has been shown that the activation of a cGMP-dependent pathway results in AQP-2 insertion to the cell membrane, a fact that can be mediated by different cGMP pathway activators, such as the atrial natriuretic peptide, L-arginine and nitric oxide[36]. Therefore, we can suggest that the decrease of eNOS expression, elicited by increased intrarenal Ang II levels, could be responsible for AQP-2 down-regulation in the renal tubules of rats subjected to HS diet, and that losartan administration could exert an antidiuretic effect through Ang II–AT2–nitric oxide–cGMP signalling.

As it is illustrated in Figure 8, a high salt diet may contribute to increase Ang II expression in renal tubular cells. In addition, intracellular Ang II, via AT1 receptor binding may stimulate cytokine production causing inflammation, fibrosis and downregulating of AQP expression. The AT1 blocker losartan, prevented intrarenal Ang II overexpression and the fibrogenic response, and increased AQP immunoexpression. In contrast, the signalling cascade AT2–eNOS–NO could be suppressed by a high salt diet. These effects were prevented by losartan administration, suggesting that intrarenal Ang II also has a deleterious profibrogenic effect and decreases urinary concentration ability. The fact that losartan also normalized eNOS expression, suggests that its antifibrotic, natriuretic and antidiuretic effects could be partly attributed to unmasked AT2 receptor stimulation by Ang II.

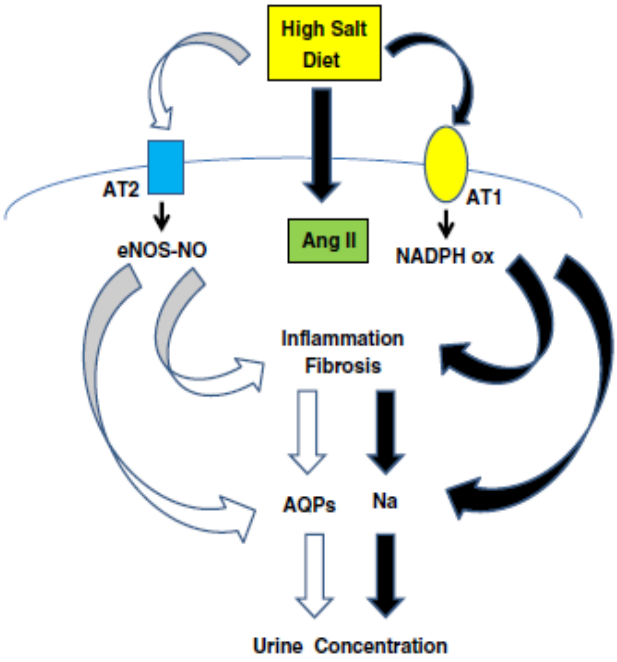


Figure 8. Schematic figure summarizing our findings. White arrows indicate inhibition, black arrows indicate stimulation. A high salt diet may contribute to increase Ang II expression in renal tubular cells. Intrarenal Ang II, via AT1 receptor binding may stimulate cytokine production causing inflammation and fibrosis, NADPH oxide activation, AQP downregulation and sodium retention, enhancing urine concentration. On the other hand, the signalling cascade AT2–eNOS–NO which normally counterbalances AT1 receptor stimulatory actions could be suppressed by a high salt diet, reinforcing the effects of AT1 stimulation. These effects were prevented by AT1 receptor blockade by losartan administration, suggesting that intrarenal Ang II also has a deleterious profibrogenic effect and decreases urinary concentration ability in the kidney. The fact that losartan also normalized eNOS expression, suggests that its antifibrotic, natriuretic and antidiuretic effects could be partly attributed to unmasked AT2 receptor stimulation by Ang II.

In summary, our findings support the idea that renal Ang II is closely involved in AQP downregulation when an excess of sodium is included in the diet. In addition, AT1 receptor blockade by losartan administration during a dietary salt supplementation could exert beneficial effects, enhancing the ability of the kidney to concentrate the urine.

5. Acknowledgments

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Chapter 2

Role of Angiotensin II and Oxidative Stress on Renal Aquaporins Expression in Hypernatremic Rats

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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, 1M NaCl), either alone or with losartan ($10mg.kg^{-1}$) or tempol ($0.5mg.min^{-1}.kg^{-1}$) during two hours. 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.

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. 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; §$p<0.05$) 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, prevents AQP-2 over-expression.

1. Introduction

Under normal conditions, plasma sodium levels are regulated within a physiological 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[1]. 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[2], 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[3-6]. 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[7, 8]. The vasopressin hormone (AVP), which is released from the pituitary gland into the bloodstream in cases of hypernatremia, enhances collecting duct water permeability due to AQP-2 accumulation at the cell surface and increase AQP-2 protein abundance[9]. However, it has been shown that AQP-2 expression levels decreased shortly (>3 h) following hypertonic challenge implying another regulation mechanism AVP-non dependent[10]. 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 demosntrated 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)[12-14]. 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[15, 16]. In this regard, 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[17, 18]. 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[19]. 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 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.

2. Methods

2.1. 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-hour 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).

2.2. Experimental Protocols

2.2.1. 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 minutes 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-minute stabilization period, the animals were infused with isotonic saline solution (0.15M 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 minutes (experimental period). Urine was collected every 30 minutes 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 minutes (experimental period).
2) C-Los group (n=5): Injected with losartan (10mg. 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 minutes.
3) C-Temp group (n=5): Infused with isotonic saline solution plus tempol (0.5 $mg.min^{-1}.kg^{-1}$) during the 120 minutes of the experimental period.
4) Na group (n=5): Infused with of hypertonic saline solution (NaCl 1.0M) for 120 minutes.
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 1.0M) for 120 minutes.
6) Na-Temp group (n=5): Infused with hypertonic saline solution (NaCl 1.0M) plus tempol (0.5 $mg.min^{-1}.kg^{-1}$) during the 120 minutes of the experimental period.

2.2.2.Unanesthetized Rats

In order to confirm that the results observed in Na group were caused by the hypertonicity of the plasma and not by the sodium overload *per se*, 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 48hs 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 ($2mL.100g^{-1}$ of body weight, i.p.): control group: C group (0.15 $mol.L^{-1}$ NaCl) and experimental group: Na group (0.8 $mol.L^{-1}$ 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 1ml 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-1, AQP-2, Ang II, NF-kB and eNOS protein expression, the kidneys were isolated, rapidly

excised, decapsulated, longitudinally cut and harvested for immunohistochemistry and Western blot as described below.

2.3. Urine and Blood Measurements

Urine flow rate (UV), urine and plasma sodium and plasma potassium (respectively U_{Na}, PL_{Na}, 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 $mEq.L^{-1}$, Urinary osmolarity (U_{Osm}) as $mOsmol.Kg^{-1}$, FE_{Na} as the percentage (%) of filtrated sodium. UV is expressed as $\mu L.min^{-1}$, U_{Na}, PL_{Na} and PL_K as $mEq.L^{-1}$.

2.4. 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 $\mu mol.L^{-1}$ pepstatin, 2 $\mu mol.L^{-1}$ leupeptin, 1x protease inhibitor cocktail (Roche Diagnostics). Protein concentration in the Triton-soluble supernatant was determined by the Lowry technique[20].

2.5. 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 (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 1:5000). 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.

2.6. 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 minutes. Immunohistochemical assays were conducted as reported previously[18]. 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).

2.7. Quantitative Imaging and Morphological Analysis

Histological sections of five animals were studied in each group. All tissue samples were evaluated blindly and separately by two researchers. All measurements were carried out on 20 fields per tissue section, using image analyzer Image-Pro Plus, version 4.5 for Windows (Media Cybernetics, LP, Silver Spring). Data were averaged and the results of positive staining for AQP-1, AQP-2, Ang II, eNOS and NF-kB were expressed as a percentage of positive stained area in renal tubules ± SEM. Identification of the different tubule segments was based on main cell characteristics observed in the histological photos.

2.8. Statistical Analysis

All results are expressed as mean ± SEM. Gaussian distribution was evaluated by the Kolmogorov and Smirnov method and comparisons between groups were made using ANOVA followed by the Bonferroni test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Studies in Anesthetized rats

3.1.2. Mean Arterial Pressure (MAP)

As reported previously[19], MAP did not increase after 2 hours 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).

	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	1764 ± 49
C-Los	65 ± 3*	139 ± 1	3.5 ± 0.2	0.36 ± 0.09	1630 ± 35
C-Temp	87 ± 1	140 ± 1	3.6 ± 0.1	0.45 ± 0.09	1835 ± 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*§	1012 ± 15*§
Na-Temp	94 ± 4	154 ± 1*	3.6 ± 0.1	13.74 ± 1.37*§	1070 ± 22*§

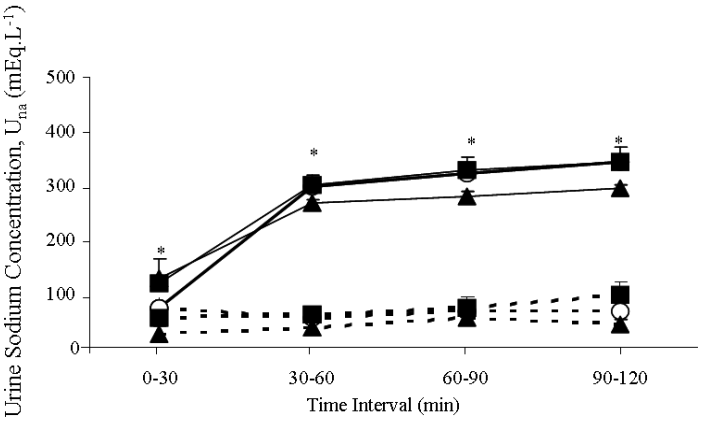
Table 1. Mean arterial pressure, electrolytes and urinary osmolality in anesthetized rats. All values are mean ± SEM (n= 5-6 per group), * $p< 0.05$ vs C group, §$p<0.05$ vs. Na group. Abbreviations: MAP: mean arterial pressure, PL_{Na}: sodium plasmatic concentration, PL_K: potassium plasmatic concentration, FE_{Na}: fractional excretion sodium, U_{Osm}: urinary osmolality, C: control group infused with isotonic saline solution; C-Los: control group infused with isotonic saline solution plus losartan; C-Temp: control group 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.

3.1.3. 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 shown significant changes in both parameters.

Figure 1 shows results of UV (panel A) and U_{Na} (panel B). As reported previously[19], 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.

Panel A



Panel B

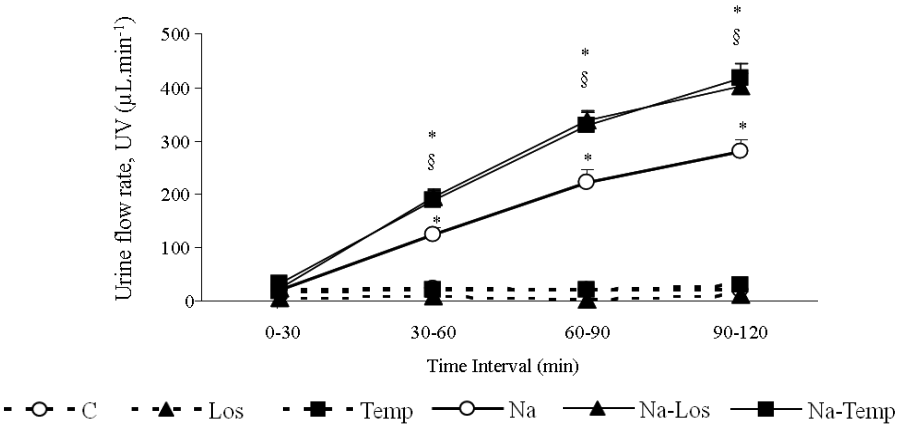


Figure 1. Urinary flow (UV, $\mu L.min^{-1}.kg^{-1}$) (panel A) and Urinary sodium concentration (U_{Na}, $mEq.L^{-1}$) (panel B). C: Control group infused with isotonic saline solution, Na: sodium overload, Los: isotonic saline solution plus losartan, Temp: isotonic saline solution plus tempol, Na-Los: sodium overload plus losartan, Na-Temp: sodium overload plus tempol. 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.

3.1.4. Immunohistochemistry and Western Blot 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.

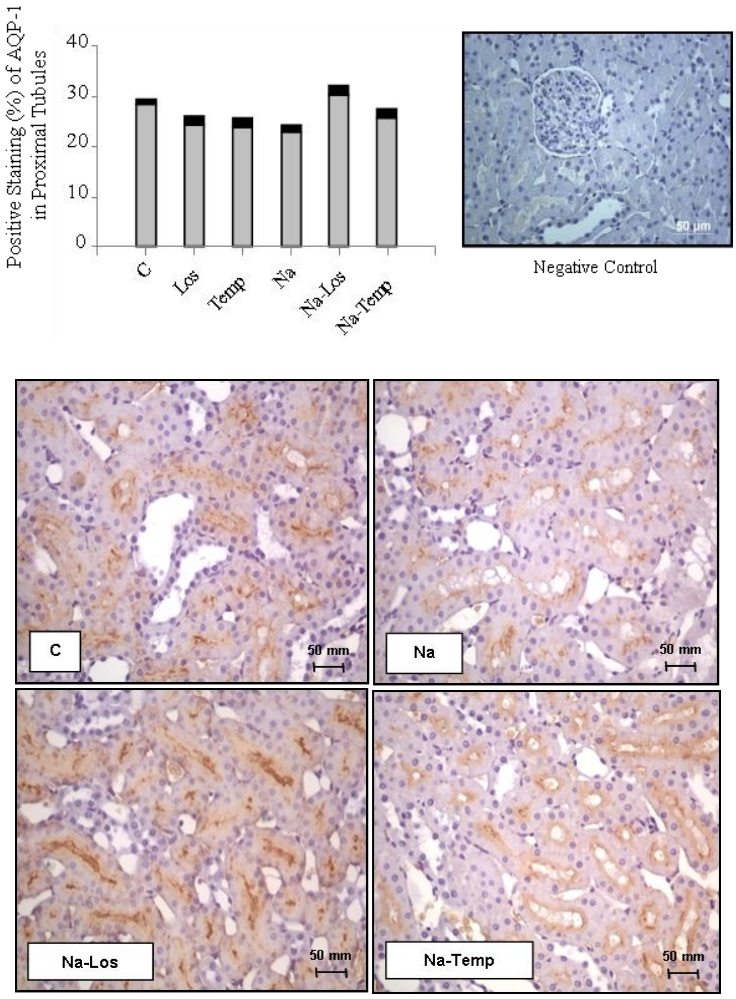


Figure 2, Panel A. Histograms illustrate the values of AQP-1 expression (top) in proximal tubules of renal cortex. Values are expressed as a percentage (%) of positive stained area ± SEM; n=5; §p<0.01 vs. Na group. Representative immunohistochemical images of negative staining control, positive staining of AQP-1 in renal cortex (bottom) 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 x 400.

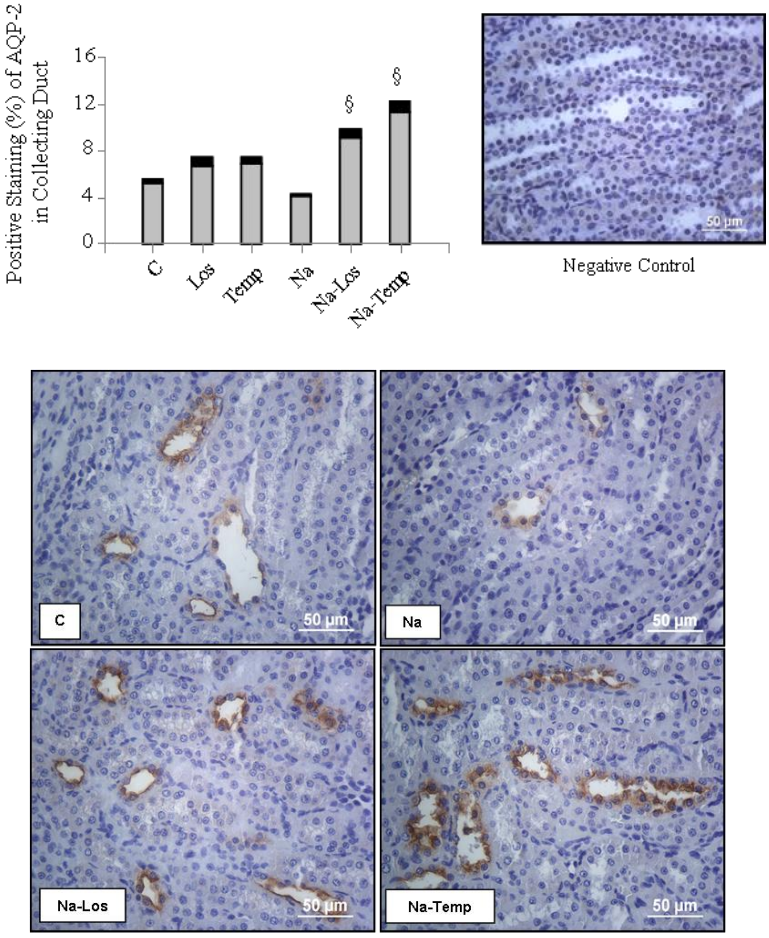


Figure 2. Panel B. Histograms illustrate the values of AQP-2 expression (top) in medullar collecting duct. Values are expressed as a percentage (%) of positive stained area ± SEM; n=5; §p<0.01 vs. Na group. Representative immunohistochemical images of negative staining control, positive staining of AQP-2 in renal medulla (bottom) 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 x 400.

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 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.

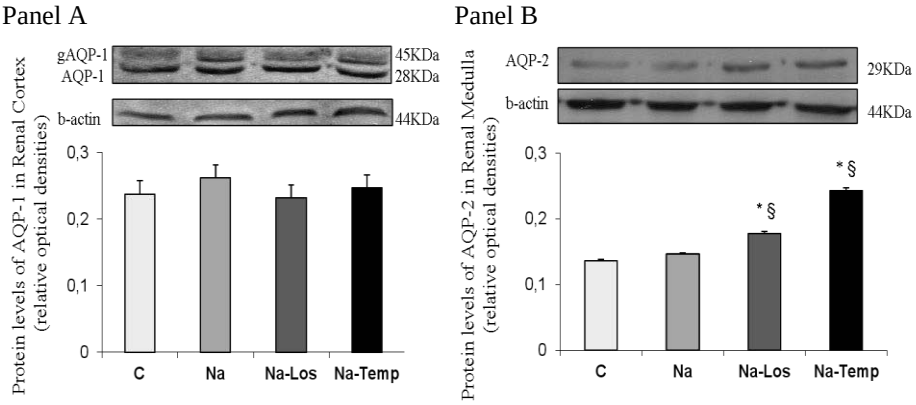


Figure 3. Representative Western blot analysis of AQP-1 in renal cortex (panel A) and AQP-2 in renal medulla (panel B) of control group infused with isotonic saline solution (C) and experimental groups infused with sodium overload (Na), sodium overload plus losartan (Na-Los), and sodium overload plus 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.

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, figure 4 shows immunostaining images of NF-kB nuclear factor.

	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*

Table 2. Ang II, eNOS and NF-kB immunostaining in renal medulla of anesthetized rats. 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. All values are mean ± SEM; n= 5-6 per group; * p< 0.05 vs respective control group.

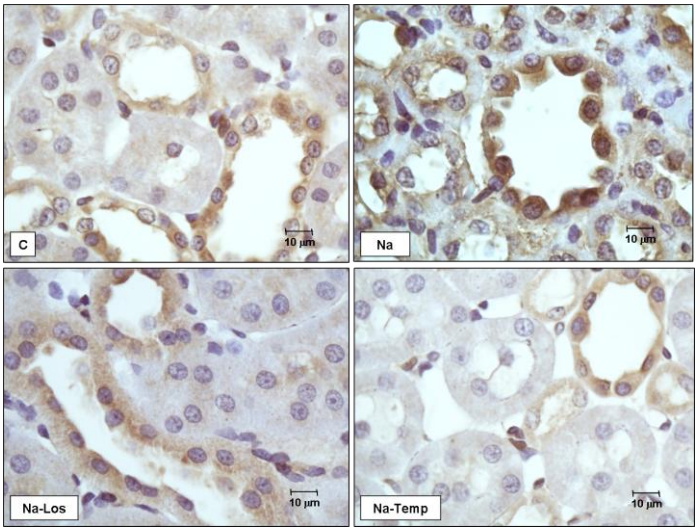


Figure 4. Representative immunohistochemical images of positive staining of NF-kB in renal medulla of control group infused with isotonic saline solution (C) and experimental groups infused with sodium overload (Na), sodium overload plus losartan (Na-Los), and sodium overload plus tempol (Na-Temp). Original magnification x 400.

3.2. Studies in Unanesthetized Rats.

Table 3 shows the results of diuresis, 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.

	PL_{Na} ($mEq.L^{-1}$)	PL_K ($mEq.L^{-1}$)	UV ($\mu L.min^{-1}.kg^{-1}$)	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*	-----

Table 3. Electrolytes, diuresis and water intake in unanesthetized rats. Abbreviations: PL_{Na}: sodium plasmatic concentration, PL_K: potasium 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. All values are mean ±SEM (n= 5-6 per group), *p< 0.05 vs. respective control group; §p<0.05 vs. respective group with access to drinking water.

Figure 5 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 (panel A) and medulla (panel B). 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).

Panel A

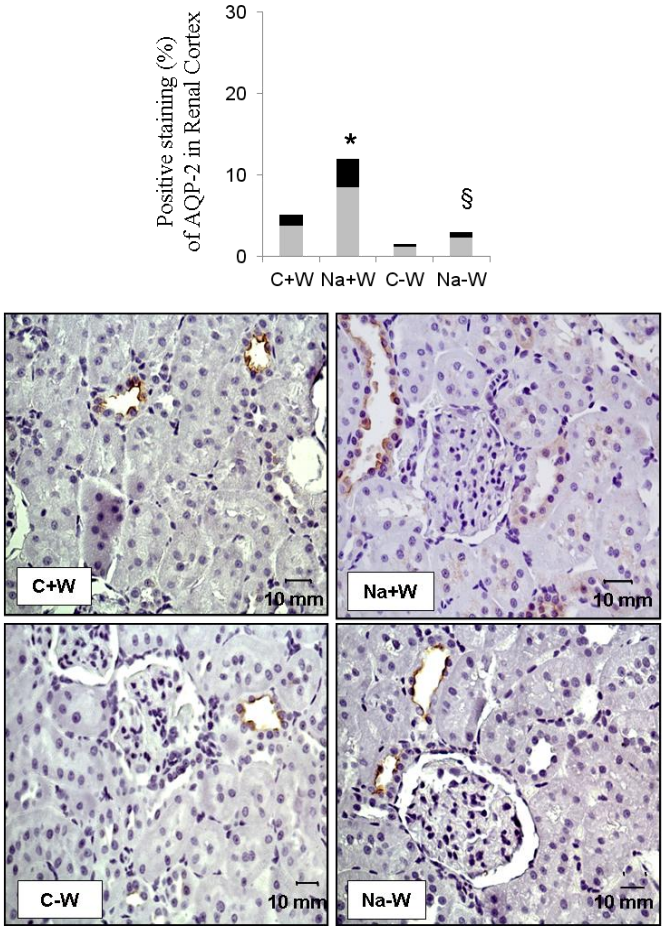


Figure 5. Panel A. Histograms illustrate the values of AQP-2 expression in renal cortex (top) of unanesthetized rats intraperitoneally injected with saline solution in two concentrations: C group (control, 0.15 mol.L^{-1} NaCl) and experimental group: Na group (0.8 mol.L^{-1} NaCl), with (+W) or without (-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). Original magnification x 400.

Panel B

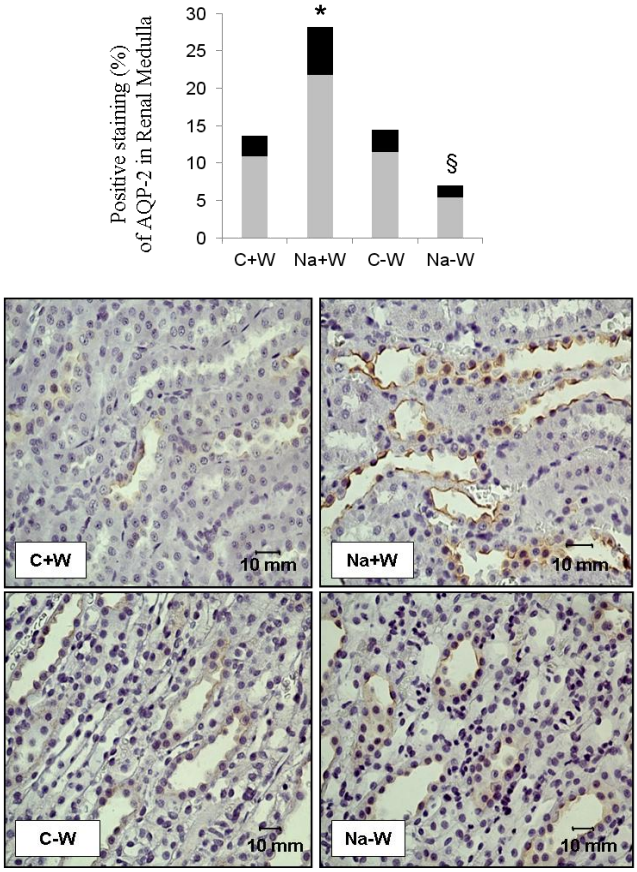
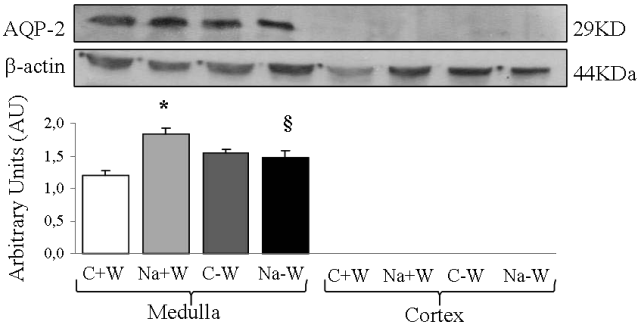


Figure 5. Panel B. Histograms illustrate the values of AQP-2 expression in renal medulla (top) of unanesthetized rats intraperitoneally injected with saline solution in two concentrations: C group (control, 0.15 mol.L^{-1} NaCl) and experimental group: Na group (0.8 mol.L^{-1} NaCl), with (+W) or without (-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 medulla (bottom). Original magnification x 400.

Figure 6 illustrates renal protein expression of AQP-2 (panel A) in renal medulla and eNOS expression (panel B) in cortex and medulla from unanesthetized rats as measured by 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.

Panel A



Panel B

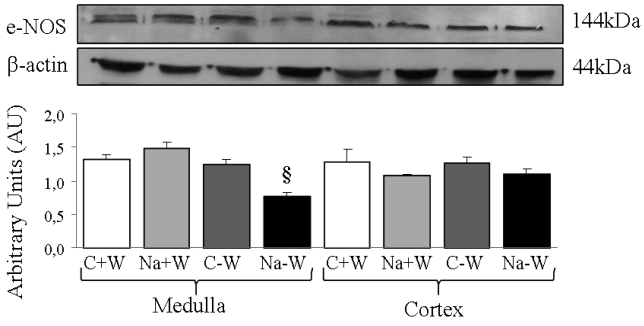


Figure 6. Representative Western blot analysis of AQP-2 (panel A) and eNOS (panel B) in renal cortex and medulla of unanesthetized rats injected intraperitoneally with saline solution at two concentrations: C group (control, 0.15 mol.L^{-1} NaCl) and experimental group: Na group (0.8 mol.L^{-1} NaCl), 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; *$p<0.01$ vs. C group, §$p<0.01$ vs. Na group.

4. Discussion

The present results indicate that increased endogenous Ang II and oxidative stress in response to hypernatremia produced by acute sodium overload could early prevent AQP-2 over-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, favouring greater flow and sodium transport, which produces oxidative stress[19]. 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. The increased AQP-2 expression is reflected in changes of urinary osmolality but not of U_{Na}. 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. 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[19]. Considering that plasmatic hypertonicity independently of AVP increase 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.

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 did not show enhancement of AQP-2 and eNOS 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, in which should increase AQP-2 expression, we observed decreased or no changes, 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 over-expression 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.

It is known that NF-kB downregulates AQP-2 expression by binding to its promoter, which contains two highly conserved kB elements[21]. 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[16]. Our group has previously reported the presence of an imbalance between Ang II and eNOS expression in sodium overloaded rats[22]. This imbalance was now confirmed, as shown in the 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[23, 24]. 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 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[21]. 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[15]. 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[24]. 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 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 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[25]. 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; 2) NO may increase AQP-2 expression and inhibit tubular water reabsorption, being this effect masked by AQP-1 inhibition.

In conclusion, 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 an anti-apoptotic role, it is therefore reasonable to consider that the appropriate down-regulation 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.

6. Acknowledgements

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Part II

Salt and Inflammation

Chapter 3

Different Protective Actions of Losartan and Tempol on the Renal Inflammatory Response to Acute Sodium Overload

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Abstract

The aim of this work was to study the role of local intrarenal angiotensin II (Ang II) and the oxidative stress in the up-regulation of pro-inflammatory cytokines expression observed in rats submitted to an acute sodium overload.

Sprague–Dawley rats were infused for 2 h with isotonic saline solution (Control group) and with hypertonic saline solution alone (Na group), plus the AT1 receptor antagonist losartan (10 $mg.kg^{-1}$ in bolus) (Na–Los group), or plus the superoxide dismutase mimetic tempol (0.5 $mg.min^{-1}.kg^{-1}$) (Na–Temp group).

Mean arterial pressure, glomerular filtration rate, and fractional sodium excretion (FE_{Na}) were measured. We evaluated by immunohistochemistry, the expression of Ang II, nuclear factor kappa-B (NF-κB), hypoxia inducible factor-1alpha (HIF-1α), transforming growth factor-beta1 (TGF-β1), smooth muscle actin (α-SMA), endothelial nitric oxide synthase (eNOS), and RANTES. Ang II, NF-κB, TGF-β1 and RANTES early inflammatory markers were over-expressed in Na group, accompanied by enhanced HIF-1α immunostaining, lower eNOS expression, and unmodified α-SMA. Losartan and tempol increased FENa in sodium overload group. Although losartan reduced Ang II and NF-κB staining and increased eNOS expression, it did not restore HIF-1α expression and did not prevent inflammation. Conversely, tempol increased eNOS and natriuresis, restored HIF-1α expression, and prevented inflammation. Early inflammatory markers observed in rats with acute sodium overload is associated with the imbalance between HIF-1α and eNOS expression. While both losartan and tempol increased natriuresis and eNOS expression, only tempol was effective in restoring HIF-1α expression and down-regulating TGF-β1 and RANTES expression.

The protective role of tempol, but not of losartan, in the inflammatory response may be associated with its greater antioxidant effects.

1. Introduction

In normal physiological conditions, the antioxidative function of nitric oxide (NO) and superoxide dismutase (SOD) activity in the tissues, counterbalances superoxide anion ($\cdot O_2^-$) oxidative effects, maintaining its production at a minimum level. However, this balance is altered when tissular NO production by the endothelial nitric oxide synthase (eNOS) or the adequate removal of NO by $\cdot O_2^-$ is impaired, allowing $\cdot O_2^-$ accumulation in the tissues[1]. Consequently, $\cdot O_2^-$ activates transcription nuclear factor NF-κB, which in turn modulates the induction of genes involved in renal inflammatory and fibrogenic responses through release of cytokines and accumulation of inflammatory cells in the kidney[2]. Several studies have demonstrated that a disbalance between NO and $\cdot O_2^-$ in the kidney determines the initial condition of oxidative stress, which alters renal hemodynamia and the excretory function, leading to the development of sodium retention, hypertension, and renal injury[1]. It is well known that a high salt intake leads to the development of hypertension in Dahl salt-sensitive rats, which is associated with an increase in oxidative stress and a decrease in the antioxidant capacity[3, 4]. NO deficiency is also known to impair kidney function, contributing to the development of salt-sensitive hypertension[5, 6]. Moreover, an antioxidant treatment increases NO bioavailability, contributing to reduce renal damage in salt-sensitive rats[7].

It is known that $\cdot O_2^-$ might increase PKC-α activity in thick ascending limb and trigger the activation of the NHE-3 exchanger and Na^+-K^+-$2Cl^-$ co-transporter, which in turn stimulate tubular sodium reabsorption[8]. Furthermore, studies carried out *in vitro* in macula densa have shown that the transport of luminal NaCl to the inside of tubular cells induces the depolarization of the tubular cell membrane, which appears to be the signal for NADPH oxidase activation and $\cdot O_2^-$ production[9, 10]. In addition, mechanic factors like the cellular stretch, tubular flow[11, 12], and/or fluid hypertonicity[13, 14] are able by themselves to increase $\cdot O_2^-$ production. This cascade of events leads to the development of a vicious circle in which a small increase in sodium transport begets an increase in $\cdot O^-_2$ production.

Intrarenal angiotensin II (Ang II) is also linked to oxidative stress and the inflammation observed in salt-sensitive models[15]. Besides its actions on hydrosaline balance regulation, the attention has been recently focused on the possibility that intrarenally formed Ang II behaves like a true cytokine, modulating the induction of genes involved in renal inflammatory and fibrogenic responses[16]. In this way, Ang II increases $\cdot O_2^-$ production through the activation of NADPH oxidase, which is a

major source of $\cdot O_2^-$ in the kidney[17]. Ang II activates transforming growth factor-beta1 (TGF-β1) expression, stimulates collagen and fibronectin synthesis, and participates in renal failure development, controlling the deposition and remodelling of extracellular matrix[18]. Then, the increase in intrarenal Ang II production or sodium tubular transport as well as the mechanic effect mediated by the increase in the tubular flow or the stretch can generate oxidative stress and tissular inflammation in the kidney.

Studies carried out in vivo in our laboratory have shown that an acute sodium overload up-regulated intrarenal Ang II and the expression of diverse pro-inflammatory and pro-fibrogenic markers in renal tissues[19-21]. However, up to date it remains unclear which are the pathophysiological mechanisms involved in the early expression of inflammatory markers after an acute sodium overload.

Based on these antecedents, the aim of our study was to evaluate the role of tubular Ang II signalling through the AT1 receptor (AT1R) and the participation of the oxidative stress and NO in the development of renal pro-inflammatory response observed in rats submitted to an acute hypertonic sodium overload. The AT1R antagonist losartan or the SOD mimetic tempol was administered to sodium overloaded rats in order to block renal Ang II effects and to inhibit oxidative stress production, respectively.

2. Methods

2.1. Animal Preparation

Male Sprague–Dawley rats (10–12 weeks old; 270–350 g body weight) were used. Animals were housed at controlled temperature (23±28ºC) 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 (Isidro Casanova, Buenos Aires, Argentina). Experiments were conducted in accordance with the Institutional University of Buenos Aires guidelines for the care and use of research animals.

The rats were intraperitoneally anesthetized with urethane (1,2 $g.kg^{-1}$). After then, a tracheotomy was 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 continuous mean arterial pressure (MAP) measurement, by means of a Statham GOULD P23ID transducer

coupled to a Grass Polygraph 79D. The bladder was cannulated for urine collection using a PE-75 cannula. A45-min infusion with isotonic saline solution, 0.15M NaCl (ISS) allowed reaching a steady diuresis and permitted urine collection in all groups. Then, all the animals were infused for 120 min, at the same rate of 0.04 $mL.min^{-1}$ (Syringe Infusion Pump, SageTM, Orion, Mass, USA). The following groups were studied: C (control): infused with SSI (NaCl 0.15 M), and three experimental groups subjected to an acute sodium overload: Na: infused with hypertonic saline solution (NaCl 1.0 M); Los: infused with NaCl 0.15M + losartan (Merck, Buenos Aires, Argentina) (10 $mg.kg^{-1}$ in bolus); Temp: infused with NaCl 0.15M + tempol (Sigma-Aldrich Inc., Saint Louis, Missouri, USA) (0.5 $mg.min^{-1}.kg^{-1}$); Na–Los: infused with NaCl 1.0M + losartan (10 $mg.kg^{-1}$ in bolus) and Na–Temp: infused with NaCl 1.0M + tempol (0.5 $mg.min^{-1}.kg^{-1}$). Two blood samples were collected at 60 and 120 min and urine was collected along 30 min periods (from 0 to 120 min) for sodium, potassium, and creatinine measurements. MAP was continuously monitored during all the procedures.

2.2. Urine and blood measurements

Urinary and plasmatic sodium, potassium, and creatinine were measured by standard methods using an autoanalyzer. Creatinine clearance was assessed in order to evaluate the glomerular filtration rate (GFR). GFR and sodium fractional excretion (FE_{Na}) were calculated according to a standard formula. Urinary flow (UV) is expressed as $ml.min^{-1}$; plasmatic sodium and potassium as $mEq.L^{-1}$, sodium as $mmol.min^{-1}$, GFR as $mL.min^{-1}$, and FE_{Na} as percentage.

2.3. Kidney processing for histological examination

At the end of the infusion period, the left kidney was perfused with ISS through the abdominal aorta until the blood was washed out and the parenchyma showed a pale appearance. The kidney was rapidly excised, decapsulated, longitudinally cut, and harvested for immunohistochemical studies.

Tissues were fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. For immunohistochemistry, sections were deparaffined and rehydrated, and endogenous peroxidise activity was blocked by treatment with 0.5% H_2O_2 in methanol for 20 min. Samples were sectioned to 3mm thickness, stained with Masson's trichromic, and were examined by light microscopy for tubular injury and interstitial fibrosis. Immunostaining of Ang II, hypoxia inducible factor-1alpha (HIF-1α), NF-kB, TGF-β1, endothelial nitric oxide synthase (eNOS), smooth

muscle actin (α-SMA), and RANTES were detected using the following specific monoclonal antibodies: human anti-Ang II (Peninsula, CA; dilution of 1:500), rabbit anti-HIF-1α (Novus Biologicals, Inc., Littleton, CO; dilution: 1:1,000), rabbit anti-NF-κB p65 (Santa Cruz Biotechnology, Inc, California, USA), rabbit anti-TGF-β1 (Santa Cruz Biotechnology, Inc, California, USA), rabbit anti-eNOS (Santa Cruz Biotechnology, Inc, California, USA), mouse anti-α-SMA (Santa Cruz Biotechnology, Inc, California, USA), and goat anti-RANTES (Santa Cruz Biotechnology, Inc, California, USA; dilution: 1:200), respectively. Immunostaining was carried out by means of a commercial modified avidin–biotin–peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, Burlingame, California, USA) and counterstained with hematoxylin.

Histological sections were observed in a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY). All measurements were carried out using image analysis software (Image-Pro Plus ver. 4.5 for Windows, Media Cybernetics, LP, Silver Spring, MD). The identification of different nephron segments was based on main cell characteristics, which were observed in the histological pictures[22]. Immunoreactivities for Ang II, HIF-1α, NF-κB, TGF-β1, RANTES, α-SMA, and eNOS are expressed as percentage of positive stained area ± SEM.

2.4. Statistical analysis

Results from urine and blood measurements and MAP levels are expressed as mean ± SEM. Gaussian distribution was evaluated by the Kolmogorov and Smirnov method, and comparisons among groups were carried out using ANOVA followed by the Newman–Keuls test. P values <0.05 were considered significant.

3. Results

3.1 Mean arterial pressure and plasma measurements

As we have previously reported[11], the acute 2-h salt overload (Na group) did not increase MAP (mmHg) compared with basal levels (basal 95±2 vs. after salt overload 91±2). When losartan was infused alone (Los group), it decreased MAP values compared with its own basal levels (basal: 96±4 vs. after Los: 63±4, $p<0.01$). In addition, when losartan was simultaneously administered with sodium overload (Na–Los group), no change was observed compared with its own basal period levels (basal: 94±4 vs. after Na–Los: 84±6), but it decreased MAP with respect to C group after 120 min of ISS infusion (C: 98±4 vs. after Na–Los: 84±6, $p<0.05$). On the other hand, tempol administration did not reduce MAP levels, neither when it was administered alone (basal: 92±2 vs. after tempol 87±2), nor when it was co-administered with sodium overload (Na–Temp, basal: 96±5 vs. after tempol 96±3). As we have previously reported[19], plasmatic Na concentration ($mEq.L^{-1}$) increased after sodium overload, compared with the control group (C: 137.6±0.4, Na: 154.0±1.4, $p<0.01$). Neither losartan nor tempol administration elicited significant changes in plasmatic Na concentrations in Na overloaded rats (Na–Los: 153.2±1.6; Na–Temp: 154.1±1.0). Plasmatic potassium levels ($mEq.L^{-1}$) were altered neither by sodium overload nor by losartan or tempol, when compared to control animals (C: 2.8±0.1, Na: 3.0±0.1, Na–Los: 3.0±0.1, and Na–Temp: 2.8±0.1).

3.2. Urinary measurements

Figure 1 shows urine flow, urinary sodium excretion, and urinary sodium and potassium concentrations. We have previously reported that an acute sodium overload increased urine flow and sodium excretion[19]. Losartan administration caused an increase in urine flow in acute sodium overloaded rats in a greater proportion than that of urinary sodium excretion, when compared with Na group; thus, urine sodium concentration diminished. In contrast, tempol administration increased both urinary sodium excretion and urinary flow in a similar proportion. Therefore, tempol did not decrease urinary sodium concentration as losartan did. Neither losartan nor tempol administration modified urinary potassium excretion, which was increased by the acute sodium overload.

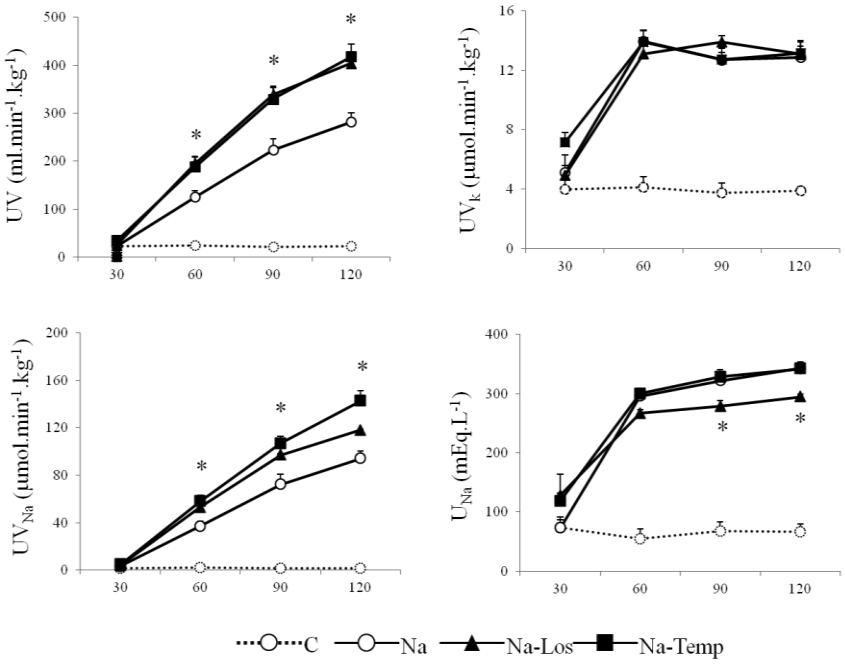


Figure 1. Renal Function. Urinary flow (UV, $\mu L.min^{-1}.kg^{-1}$) (top left); potassium urinary excretion (UV^K, $\mu mol.min^{-1}.kg^{-1}$) (top right); sodium urinary excretion (UV_{Na}, $\mu mol.min^{-1}.kg^{-1}$) (bottom left) and sodium urinary concentration (U_{Na}, $mEq.L^{-1}$) (bottom right). Abbreviations: C: control group infused with isotonic saline solution (dash line and open circle), Na: sodium overload (full line and open circle), Na-Los: sodium overload plus losartan (closed triangle), and Na-Temp: sodium overload plus tempol (closed square). Values are expressed as mean ± SEM; n=5-8; *p<0.05 vs. Na group.

3.2.1. Glomerular filtration rate and fractional sodium excretion

We have shown[19] that an acute sodium overload increased the GFR, inducing glomerular hyperfiltration. Losartan prevented that increase in Na group after the first 60 min infusion period. On the other hand, tempol did not modify the GFR in the Na group at any time (Fig. 2, top). As shown in Figure 2 (bottom), the acute sodium overload increased FE_{Na}. When losartan or tempol were co-administered, a further increase in FE_{Na} was observed.

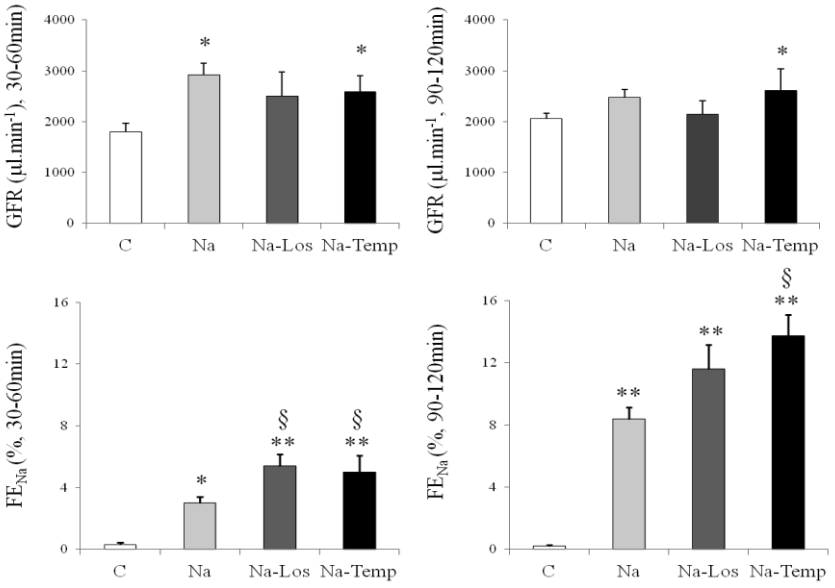
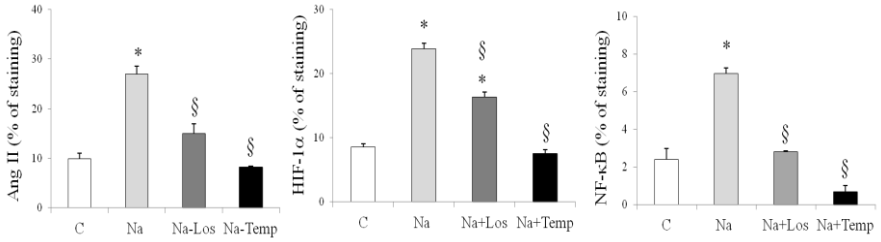


Figure 2. Glomerular Filtration Rate (GFR) and Sodium Fractional Excretion (FE_{Na}). GFR ($\mu L.min^{-1}$, top) and FE_{Na} (%, bottom) along 60min (left) and 120min (right). Abbreviations: C: control group infused with isotonic saline solution, Na: sodium overload, Na-Los: sodium overload plus losartan, and Na-Temp: sodium overload plus tempol. Values are expressed as mean ±SEM; n=5-8. *p<0.05 and **p<0.01 vs. C group; §p<0.05 vs. Na group.

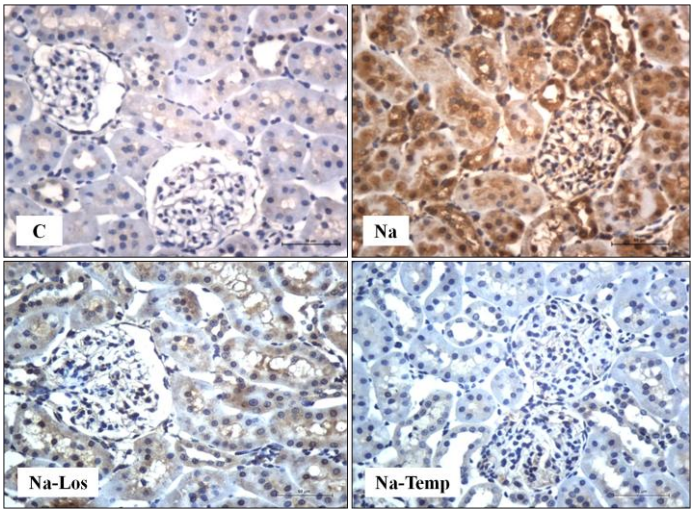
3.3. Immunohistochemical expression in renal sections

The analysis of renal sections obtained from rats subjected to sodium overload revealed increased Ang II, HIF-1α, NF-κB (Fig. 3), and TGF-β1 (Fig. 4) staining, which was mainly localized in proximal convolute tubules (PCT) and cortical collecting ducts (CCDs) (Fig. 5) and medullar collecting ducts (MCDs) (Fig. 6), with respect to control group. RANTES expression only increased in CCD (Fig. 5) and MCD (Fig. 6). Acute sodium overload reduced eNOS expression, compared to control group, in renal cortex (C: 5.49±0.24%, Na: 4.30±0.18%; $p<0.05$) and medulla (C: 4.08±0.21%, Na: 2.87±0.13%; $p<0.05$). Neither the staining with Trichromic-Maisson, nor the α-SMA immunoexpression revealed histopathological signs of fibrosis in Na group (data not shown).

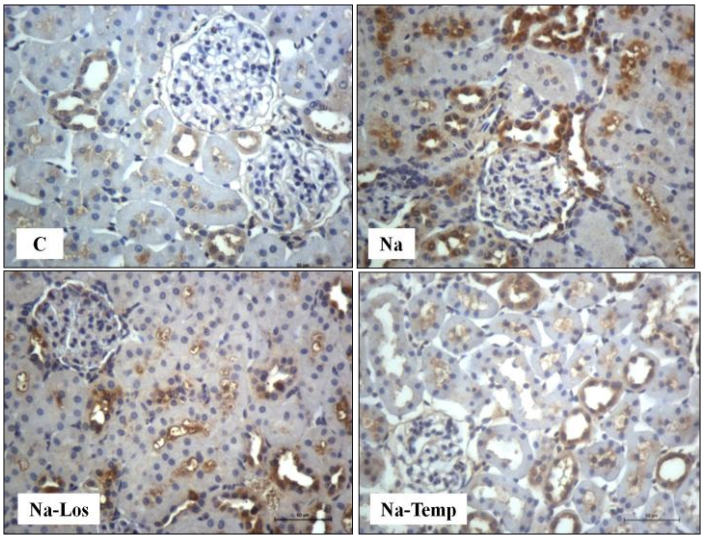
Panel A



Panel B



Panel C



Panel D

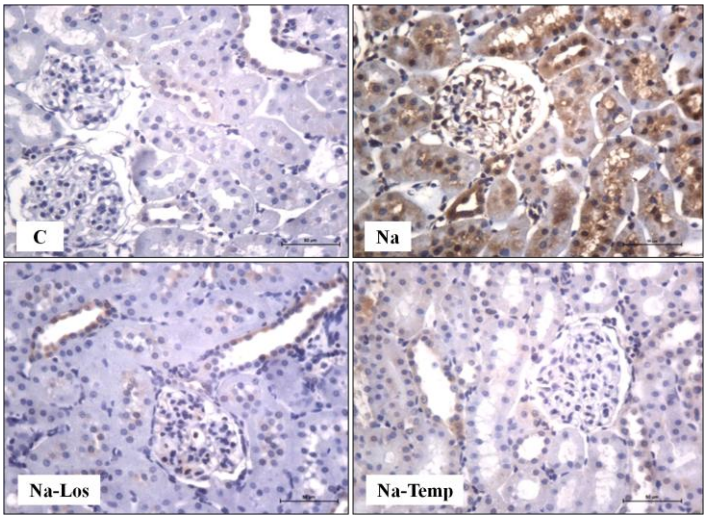
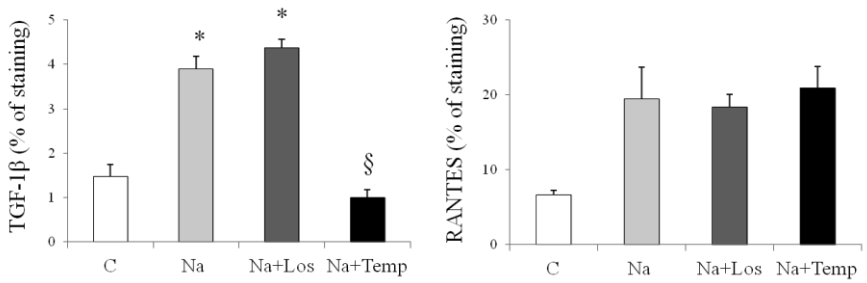


Figure 3. Panel A. Immunohistochemical expression of Ang II, HIF-1α, and NK-κB in proximal convoluted tubules (PCT). Abbreviations: C: control group infused with isotonic saline solution, Na: sodium overload, Na-Los: sodium overload plus losartan (closed triangle), and Na-Temp: sodium overload plus tempol. Values are expressed as mean ± SEM; n=5-8. *p<0.05 and **p<0.01 vs. C group; §p<0.05 vs. Na group. Representative images of positive staining (original magnification 400x) of Ang II (Panel B), HIF-1α (Panel C), and NF-κB (Panel D) in renal cortex.

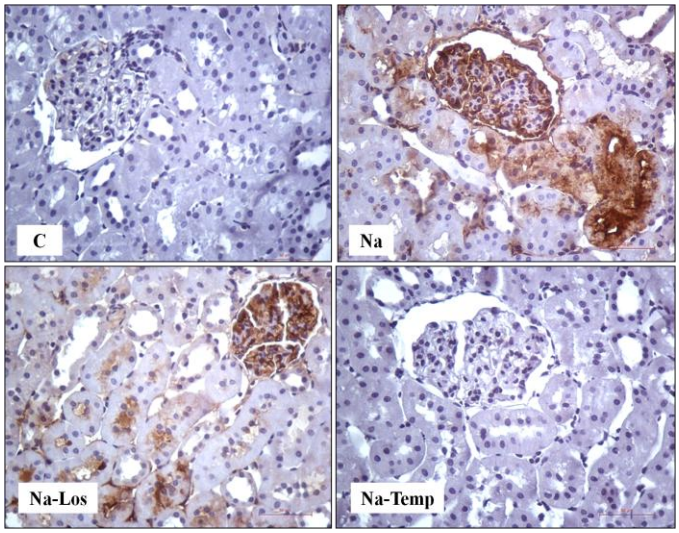
3.3.1. Effects of losartan and tempol on renal proximal tubules

The renal staining of none of the inflammatory markers studied in rats infused with ISS was modified by losartan or tempol administration, in any of the areas examined (data not shown). Increased Ang II, HIF-1α, and NF-κB staining in Na overloaded rats was reduced in proximal tubules when losartan was administered (Fig. 3) but losartan failed to restore HIF-1α and to decrease TGF-β1 staining (Fig. 4). On the other hand, tempol caused a further inhibition of Ang II, HIF-1α, and NF-κB expression (Fig. 3) and restored TGF-β1 staining in Na overloaded rats, reaching similar levels to those observed in C group (Fig. 4). RANTES staining was neither modified by sodium overload nor by losartan or tempol co-administration (Fig. 4). In addition, losartan increased eNOS expression in renal cortex, compared with Na group (7.09±0.35% vs. 4.30±0.18%; $p<0.001$) and medulla (6.18±0.20% vs. 2.87±0.13%; $p<0.001$). Moreover, the increase in eNOS expression elicited by tempol was higher than that stimulated by losartan in renal cortex (10.44±0.59% vs. 7.09±0.35%; $p<0.001$) and medulla (7.49±0.49% vs. 6.18±0.20%; $p<0.05$).

Panel A



Panel B



Panel C

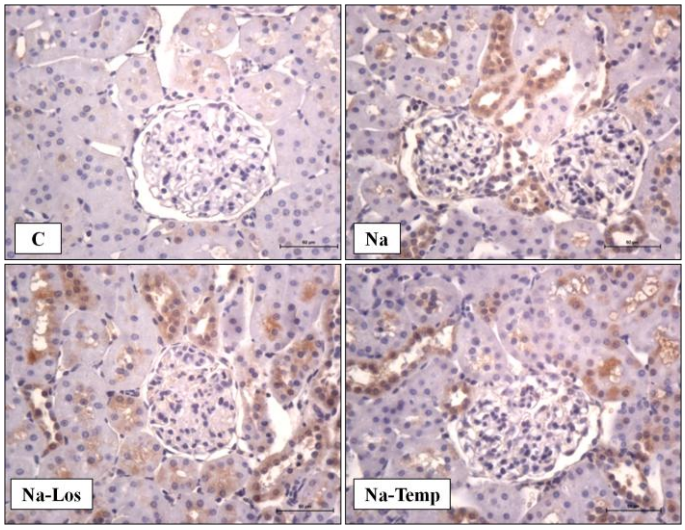


Figure 4. Panel A. Immunohistochemical expression of TGF-β1 and RANTES in proximal convoluted tubules (PCT). Abbreviations: C: control group infused with isotonic saline solution, Na: sodium overload, Na-Los: sodium overload plus losartan (closed triangle), and Na-Temp: sodium overload plus tempol. Values are expressed as mean ± SEM; n=5-8; *p<0.05 and **p<0.01 vs. C group; §p<0.05 vs. Na group. Representative images of positive staining (original magnification 400x) of TGF-β1 (Panel B), and RANTES (Panel C) in renal cortex.

3.3.2. Effects of losartan and tempol on cortical collecting ducts

Losartan reduced Ang II, HIF-1α, and NF-κB staining in CCDs of sodium overloaded rats, but it did not attenuate TGF-β1 and RANTES staining. Whereas tempol reduced Ang II, HIF-1α, NF-κB, and TGF-β1 staining in CCDs of sodium overloaded rats, it lacked any effect on RANTES staining, like losartan (Fig. 5).

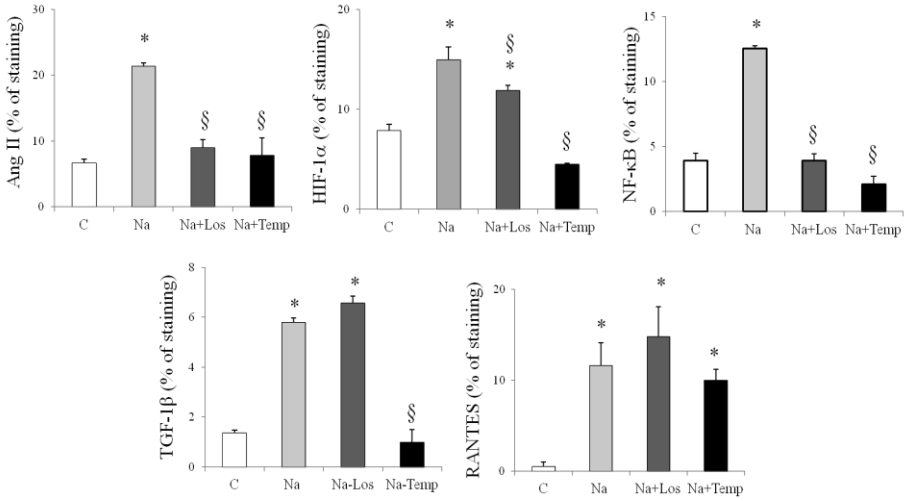


Figure 5. Immunohistochemical expression of Ang II, HIF-1α, NK-κB, TGF-β1 and RANTES in cortical collecting ducts (CCD). Abbreviations: C: control group infused with isotonic saline solution, Na: sodium overload, Na-Los: sodium overload plus losartan (closed triangle), and Na-Temp: sodium overload plus tempol. Values are expressed as mean ± SEM; n=5-8; *p<0.05 vs. C group; §p<0.05 vs. Na group.

3.3.3. Effects of losartan and tempol on medullar collecting ducts

Losartan and tempol markedly reduced Ang II, HIF-1α, and NF-κB expression in MCDs in acute sodium overloaded rats. While losartan did not attenuate TGF-β1 and RANTES staining, already overstained in these rats, tempol diminished the over-expression of both markers (Fig. 6).

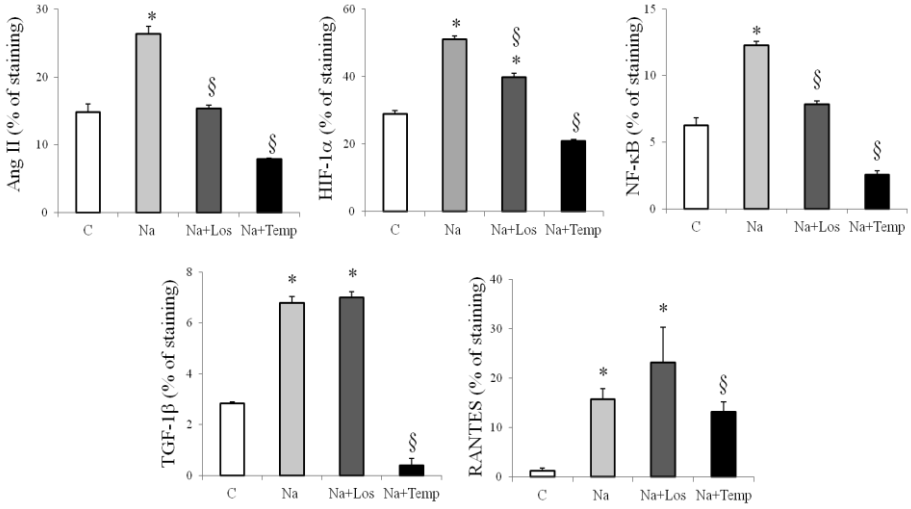


Figure 6. Immunohistochemical expression of Ang II, HIF-1α, NK-κB, TGF-β1 and RANTES in medullar collecting ducts (MCD). Abbreviations: C: control group infused with isotonic saline solution, Na: sodium overload, Na-Los: sodium overload plus losartan (closed triangle), and Na-Temp: sodium overload plus tempol. Values are expressed as mean ± SEM; n=5-8; *p<0.05 vs. C group; §p<0.05 vs. Na group.

4. Discussion

In the present study, we demonstrate that the early expression of inflammatory markers observed in the kidney of rats submitted to sodium overload was associated with the simultaneous decrease in eNOS expression and increase in HIF-1α expression, which suggests greater oxidative stress. Both losartan and tempol had a natriuretic effect and inhibited Ang II up-regulation. However, only tempol showed an anti-inflammatory effect accompanied by an antioxidant action, since it increased eNOS expression and lowered HIF-1α expression to control level. On the contrary, AT1 receptor inhibitor losartan, though it increased eNOS expression, did not restore HIF-1α expression and did not prevent the over-expression of inflammatory markers in renal tubules. These results demonstrate an anti-inflammatory effect of tempol associated with an antioxidant action, independently of renal Ang II expression.

In earlier studies from our laboratory we have observed that rats submitted to sodium overload showed hyperfiltration, an increased Na tubular reabsorption, accompanied by enhanced renal Ang II and HIF-1α expression and inflammatory markers in renal tubules[19]. In the present study, besides we observed lower eNOS expression. These considerations suggest that the oxidative stress produced by an imbalance between the production of NO and $\cdot O_2^-$ in the kidney could play a role in the pathogenesis of the inflammation. It is well known that several factors, like the hyperfiltration as well as the peripheral sympathetic nervous system or the oxidative stress, regulate angiotensinogen expression and consequently, Ang II production in proximal tubules[23, 24]. Furthermore, Ang II induces, through AT1 receptor stimulation, angiotensinogen gene expression, and in consequence, Ang II expression is enhanced by a positive feedback mechanism[25, 26]. It is also known that renal tubular Ang II regulates NADPH oxidase-dependent $\cdot O_2^-$ production, and consequently the HIF-1α stabilization[27]. On the other hand, Ang II regulates tubular sodium transport, stimulates Na^+–K^+–ATPase activity and ATP consumption, decreases ATP concentration, leading to a diminished eNOS expression[28]. In order to evaluate the participation of intrarenal Ang II in the inflammatory response, we studied losartan effects, as a specific antagonist of AT1 receptors. Losartan normalized Ang II and NF-κB expression in Na group reaching similar levels to controls animals, but it failed to restore GFR and HIF-1α immunoexpression, which was partially decreased. Moreover, losartan did not modify RANTES and TGF-β1 expression, although it simultaneously enhanced eNOS expression. In addition, losartan exerted a natriuretic effect without changing the MAP. While losartan inhibited partially the hyperfiltration, it normalized the Ang II expression to control levels. We assume that another cause independent from the hyperfiltration could be involved in Ang II expression, that is, sympathic nervous activity or oxidative stress produced by a mechanical effect[12, 23]. However, Ang II, besides its known binding to AT1 or AT2 plasma membrane receptors, it may be internalized and translocated to the nucleus, where it would directly interact with the receptor located near the nuclear membrane[26]. In this way, specific AT1 receptors have been shown at the nuclear membrane, where they induce the transcription of renin and angiotensinogen mRNA modulating their synthesis[25]. Then, we cannot exclude that losartan modulates the signalling pathway of Ang II independently of hyperfiltration. These facts could explain why, while GRF inhibition was a partial effect, on the other hand, losartan normalized Ang II expression to control levels. Independent of the cause that produces upregulation of Ang II expression, losartan enhanced eNOS

expression but could neither restore HIF-1α expression nor prevent the inflammatory response. Our results suggest that another pathway, independent of the up-regulation of renal Ang II, may be responsible of HIF-1α increase in sodium overloaded rats. Besides, since losartan increased natriuresis and FE_{Na}, we suggest that increased renal Ang II is more involved in Na tubular transport.

On the other hand, tempol, a SOD mimetic, which scavenges $\cdot O_2^-$ production and enhances NO bioavailability[29], normalized Ang II, NF-κB, and TGF-β1 immunoexpression in PCT and CCD and RANTES in MCD to control levels, and augmented natriuresis without affecting MAP or hyperfiltration. These effects were simultaneously accompanied by HIF-1α normalization and eNOS expression, suggesting inhibition of oxidative stress. The oxidative stress produced by the increase of GFR or the tubular stretch may be the signalling for angiotensinogen–renin–Ang II cascade activation. Tempol inhibited Ang II staining restoring the levels observed in Na group and simultaneously maintained the hyperfiltration observed salt overloaded animals. These results suggest that hyperfiltration would not be the cause of Ang II upregulation. However, we cannot exclude that the hyperfiltration is the cause of oxidative stress which may activate the angiotensinogen–Ang II cascade. Another possibility to justify tempol effects on renal Ang II would be based on its action over the renal sympathetic system. Renal sympathetic system stimulates Ang II production[23], and tempol by inhibiting this system, could be reducing Ang II synthesis[30].

The natriuretic action of tempol agrees with the literature. It has been shown that tempol produces natriuresis regulating Na^+–K^+–ATPase, Na^+/H^+ interchanger, Na^+–K^+–$2Cl^-$ co-transporter and ENaC activities and increasing medullar renal blood flow[29, 31]. Furthermore, tempol prevents dopamine D1 receptors down-regulation in proximal tubules of rats submitted to oxidative stress, leading to a reduction in sodium reabsorption by proximal tubules[32]. In addition, tempol administration to salt-sensitive rats fed a high salt diet prevented the enhancement of intrarenal angiotensinogen expression, decreasing in this way anti-natriuretic Ang II actions[33]. However, considering that both tempol and losartan inhibited sodium transport and increased natriuresis, but only tempol decreased the over-expression of inflammatory markers, while losartan lacked these effects, we suggest that the beneficial effect of tempol was more related to its antioxidant role, than to its natriuretic effects.

Our results are in agreement with previous reports, which have shown that the acute response to tempol administration in anesthetized rats was an antioxidant effect, besides its diuretic and natriuretic actions[31].

Then, it is possible that another source of $\cdot O_2^-$, independent of Ang II–AT1 receptor signalling, may be responsible for the inflammatory marker over-expression in sodium overloaded rats, which were not modified by losartan. As known, some mechanical factors as hyperfiltration, tubular epithelial stretch and/or the epithelial sodium transport can be able to generate oxidative stress, inducing in turn cytokines and chemokines over-expression[11, 12]. It has been demonstrated that TGF-β1 was up-regulated by the oxidative stress and inhibited by NO[34, 35]. Therefore, $\cdot O_2^-$ up-regulation as well as lower NO availability, may contribute, at least in part, to stimulate TGF-β1 production. We have observed that tempol, but not losartan, prevented the increase in TGF-β1 expression in rats submitted to sodium overload. Therefore, it is probable that TGF-β1 over-expression in sodium overloaded group was a consequence of the oxidative stress independently of Ang II.

RANTES over-expression was observed in CD but not in PCT. Renal interstitial hypertonicity is one of the main causes of oxidative stress by production of reactive oxygen species of mitochondrial origin[36, 37] and inflammation. Then, a higher interstitial hypertonicity in CD than in PCT may be related to different RANTES staining in those nephron segments. In addition, tempol favours renal blood flow and inhibits sodium transport, increasing this way natriuresis and diminishing medullar interstitial tonicity. Then, we suggest that the different response to tempol in PTC and MCD regarding RANTES staining may be related to tempol actions tending to diminish medullar tonicity and reduce the oxidative stress. Moreover, RANTES over-expression observed in CD of sodium overloaded rats was unchanged by losartan, but it was diminished by tempol only in renal medulla. High extracellular sodium concentration constitutes a challenge to the normal cell function and stimulates the inflammatory response in intestinal, bronchial, and renal epithelial cells[38]. A hyperosmotic stress results in activation of osmosensitive NF-κB, MAPK signalling pathways and expression of IL-8 and RANTES[39]. In addition to NF-κB, the gene expression of a variety of other transcription factors (AP-1, CREB) has been shown to be affected by salt-induced stress. Present results do not rule out the possibility that increased RANTES could be a consequence of Ang II stimulation of AT2 receptors[40].

In conclusion, Ang II and the oxidative stress are involved in the activation of transcriptional factors in rats submitted to acute sodium overload. However, only

tempol was effective in down-regulating early inflammatory markers TGF-β1 and RANTES expression. These effects can be associated with an enhanced eNOS and simultaneously decreased HIF-1α expression. Further studies are required to characterize and delineate these interactions at the level of the production of $\cdot O_2^-$ and NO in the kidney.

5. Acknowledgements

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET PIP 6161), PICT 05-13775 ANPCYT, and Universidad de Buenos Aires (UBACYT B014 and B018). We would like to thank Mrs. Cecilia Medici for the excellent technical support and assistance.

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Chapter 4

High-sodium diet promotes a profibrogenic reaction in normal rat kidneys: effects of Tempol administration

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Abstract

Studies carried out *in vitro* have recently shown that salt loading induces an increasing mechanical stretch and a flow-induced superoxide production in the thick ascending limb of Henle's loop. In this regard, we hypothesized that the oxidative stress induced by salt overload could stimulate inflammatory and fibrogenic signalling pathways in normal rats.

Sprague Dawley rats were fed with an 8% NaCl high- (HS) or 0.4% NaCl normal-salt (NS) diet for 3 weeks, with or without Tempol (T) administration (1 mM, administered in drinking water). Mean arterial pressure (MAP), glomerular filtration rate (GFR) and urinary sodium excretion (UV_{Na}) were measured. NAD(P)H oxidase p47phox, angiotensin II (Ang II), transforming growth factor-beta1 (TGF-β1), alpha-smooth muscle actin (α-SMA) and nuclear factor-kappa B (NF-κB) expression were evaluated in renal tissues by immunohistochemistry.

A high NaCl diet produced a slight but significant increase in MAP and enhanced UVNa and oxidative stress. Administration of a high NaCl diet induced the over-expression of TGF-β1, α-SMA and NF-κB in cortex and medulla, while Ang II increased in proximal convoluted tubules, and decreased in cortical collecting ducts. Tempol administration prevented these changes and simultaneously normalized MAP accompanied by an enhancement in GFR and UV_{Na}.

The results showed that a high NaCl diet is able to produce a renal profibrotic response also in normal rats, which could be associated with oxidative stress rather than intrarenal Ang II expression.

1. Introduction

It is well known that a high NaCl intake enhances superoxide production, lipid peroxidation and oxidative stress in salt sensitive rat kidneys[1, 2]. In addition, it has been shown that the superoxide anion exerts direct renal vasoconstrictor and anti-natriuretic effects *in vivo*[3] and increases sodium transport *in vitro*[4], suggesting that the superoxide anion plays an important pathophysiological role in the kidney, contributing to the development of hypertension. An increase in intrarenal angiotensin II (Ang II) expression in salt-sensitive rats can induce renal injury development, despite the suppression of the systemic renin-angiotensin system and even before a rise in arterial pressure takes place[5-7]. It is well known that Ang II may increase the production of reactive oxygen species through reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity[8]. Superoxide anion activates transcription nuclear factor-kappa B (NF-κB), which modulates the induction of genes involved in the inflammatory and fibrogenic response through the release of cytokines and accumulation of inflammatory cells in the kidney[9]. Inflammation is a crucial event, previous to the subsequent development of fibrosis, which is the final contributing factor to kidney failure. In this order, it has been demonstrated that the Ang II-activated transforming growth factor-beta1 (TGF-β1) expression stimulates collagen and fibronectin synthesis and participates in renal failure development, controlling the extracellular matrix deposition and remodelling[10, 11]. The peritubular accumulation of myofibroblasts that express alpha-smooth muscle actin (α-SMA) and contribute to abnormal extracellular matrix production constitutes an early event in the fibrotic process.

However, there is no strong evidence whether high salt intake in normal rats can promote renal damage. In this regard, a recent study has provided further evidence that the current dietary intake of salt in Western societies is an important factor for the genesis of essential hypertension and may even partly cause blood pressure–independent target organ damage, including the kidney[12]. The issue of whether the reduction of dietary salt intake should be recommended, even to normotensive individuals, is currently debated in the political field, and there are examples to show that salt intake reduction can be a successful recommendation in the general population. Considering that assertion, we investigated for the first time whether a high-salt diet administered to normal rats is able to induce the early expression of tubulointerstitial pro-fibrogenic and pro-inflammatory markers. Additionally, we studied the participation of oxidative stress and tubular Ang II in the genesis of those

markers' expression. With that purpose, Sprague Dawley rats were fed a high-salt diet for 3 weeks in the presence or absence of Tempol administration to observe oxidative stress blockade effects on high sodium overload–derived inflammation and fibrosis. Blood pressure changes, renal excretory function, oxidative stress through NADPH oxidase p47phox, as well as expression of intrarenal pro-inflammatory factors Ang II, TGF-β1, α-SMA and NF-κB were determined.

2. Methods

2.1. Animal protocol

Male Sprague Dawley rats, 5-6 weeks old (180-200g body weight), were used in the experiments. The animals were housed in steel cages in a controlled temperature animal room at 23±2ºC, exposed to a daily 12-hour light-dark cycle (light on from 07:00 am to 07:00 pm), fed for 3 weeks with the diets described below and given tap water *ad libitum*. Experiments were conducted in accordance with the institutional University of Buenos Aires guidelines for the care and use of research animals. The experiments were performed in animals randomly divided into 4 groups (n=6, each group): (a) NS (control): animals fed a normal diet (0.4% NaCl); (b) HS: fed a high NaCl diet (8% NaCl); (c) NS-T: fed a normal diet (0.4% NaCl), plus Tempol (Sigma-Aldrich Inc, St. Louis, MO, USA) administered in drinking water (1 mM); and (d) HS-T: fed a high NaCl diet (8% NaCl), plus Tempol administered in drinking water (1 mM). After a 3-week diet, the rats were intraperitoneally anesthetized with urethane (1.2 $g.kg^{-1}$). A PE-90 tubing (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 for continuous mean arterial pressure (MAP) recording; by means of a Statham GOULD P23ID transducer coupled to a Grass Polygraph 79D during all the procedures. The bladder was cannulated for urine collection using a PE-75 cannula. A femoral vein infusion with isotonic saline solution (ISS, 0.15 M NaCl) was performed at a 0.04 $mL.min^{-1}$ rate (Syringe Infusion Pump, Sage, Orion) for 60 minutes, to allow the reaching of a steady diuresis and permitting urine collection in all groups. Then, ISS infusion continued for another 60 minutes at the same rate as an experimental period. Blood samples were collected at 30 minutes, and urine samples were collected during the 60-minute ISS infusion, for sodium, potassium and creatinine measurement.

2.2. Urine and blood measurements

Urinary and plasma sodium, potassium and creatinine were measured by standard methods using an autoanalyzer. Creatinine clearance was assessed to evaluate glomerular filtration rate (GFR). GFR and sodium fractional excretion (FE_{Na}) were calculated according to a standard formula. Urinary flow (UV) is expressed in $\mu L.min^{-1}$, plasmatic and urinary sodium (PL_{Na}, U_{Na}) and potassium (PL_K, U_K) concentration in $mEq.L^{-1}$, sodium and potassium urinary excretion (UV_{Na}, UV_K) in $\mu mol.min^{-1}$, GFR as ml/min and FE_{Na} as a percentage (%).

2.3. Kidney processing for histological examination

At the end of the infusion period, the left kidney was perfused with ISS through the abdominal aorta until the blood was washed out and the parenchyma showed a pale appearance. The kidney was rapidly excised, decapsuled, longitudinally cut and harvested for immunohistochemical studies. Tissues were fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. Samples were sectioned to 3-µm thickness and stained with Masson's trichrome. Examined by light microscopy, the samples did not reveal histopathological signs for tubular injury or interstitial fibrosis in the HS group (data not shown).

For immunohistochemistry, renal sections were deparaffined and rehydrated, and endogenous peroxidase activity was blocked by treatment with 0.5% H_2O_2 in methanol for 20 minutes. Local Ang II, TGF-β1, α-SMA and NF-κB were detected using the following specific monoclonal antibodies: human anti-Ang II (dilution of 1:500; Peninsula, CA, USA), rabbit anti-TGF-β1 (dilution: 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-α-SMA (dilution: 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-NF-κB p65 (dilution: 1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. Immunostaining was carried out by means of a commercial modified avidin-biotin-peroxidase complex technique (Vectastain ABC kit, Universal Elite; Vector Laboratories, CA, USA) and counterstained with hematoxylin. Histological sections were observed under a Nikon E400 light microscope (Nikon, Melville, NY, USA). All measurements were carried out with image analysis software (Image-Pro Plus, ver. 4.5 for Windows; Media Cybernetics, LP, Silver Spring, MD, USA). Immunoreactivities for Ang II, TGF-β1 and RANTES are expressed as percentage of positive stained area ± SEM in proximal convoluted tubules (PCT), distal convoluted tubules (DCT), thick ascending limb (THAL) of Henle's loop, cortical

collecting duct (CCD) and medullar collecting duct (MCD). The identification of different nephron segments was based on main cell characteristics, which were observed in the histological pictures[13]. For immunofluorescence staining slices were incubated with monoclonal antibodies for NAD(P)H oxidase-p47phox (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were next incubated with conjugated secondary antibodies. Tubular cells were documented by cell staining of hematoxylin, and nuclear integrity was confirmed using 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) staining. For analysis, slides were viewed using a fluorescence microscope (Eclipse 600, Nikon, Melville, NY, USA), and images were digitally captured by a digital camera.

2.4. Statistical analysis

Results from urine, blood measurements and MAP are expressed as means ± SEM. Gaussian distribution was evaluated by the Kolmogorov-Smirnov method, and comparisons between groups were made using ANOVA followed by the Newman-Keuls test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Body weight and MAP

Body weight and MAP values are shown in Table 1. There were no significant differences in body weight between the experimental and control groups. MAP increased in HS-fed animals compared with NS-fed rats. Tempol administration reduced MAP in the HS-T group, reaching very similar levels to those of the NS group. In addition, Tempol did not modify MAP levels in NS-fed animals.

3.2. Plasma electrolytes, GFR and excretory function

Table 1 shows HS diet and Tempol effects on glomerular hemodynamics and renal excretory function in the NS and HS groups. PL_{Na} was not significantly modified in any experimental group. HS diet raised U_{Na} and FE_{Na}% compared with the NS group. On the other hand, PL_K, U_K and GFR were not altered by an HS diet. Tempol administration increased PL_K and U_{Na} in both NS-T and HS-T groups, and also raised GFR in HS-T animals. Figure 1 shows diuresis (UV), UV_{Na} and UV_K values. UV did not differ between NS and HS groups, but Tempol increased it in the HS-T group. UV_{Na} exhibited a very similar pattern to U_{Na}: HS diet led to a marked

increase in UV_{Na}, which was further increased in HS-T. In addition, UV_K was not different between NS and HS, but Tempol markedly increased potassium excretion in HS-T animals, compared with the other groups.

	NS	HS	NS-T	HS-T
BW (g)	330±9	320±6	314±13	320±7
MAP (mmHg)	96±2	107±3 *	100±2	95±3
PL_{Na} ($mEq.L^{-1}$)	141±1	140±3	142±1	143±2
PL_K ($mEq.L^{-1}$)	3.1±0.1	2.9±0.2	3,5±0.1 #	3.6±0.1#
U_{Na} ($mEq.L^{-1}$)	15.7±2.7	198.0±17.6*	54.5±20.8#	332.0±38.5*#
U_K ($mEq.L^{-1}$)	122.8±17.4	111.0±11.1	180.5±29.8	79.1±13.0*
GFR ($mL.min^{-1}$)	1.62±0.12	1.48±0.09	1.22±0.24	3.09±0.69*#
FE_{Na} (%)	0.1±0.1	0.5±0.1*	0.1±0.1	1.2±0.4*

Table 1. BW: body weight; MAP: mean arterial pressure; PL_{Na}: plasmatic sodium; PL_K: plasmatic potassium; U_{Na}: urinary sodium concentration; U_K: urinary potassium concentration; GFR: glomerular filtration rate; FE_{Na}: fractional excretion; NS: normosodic diet group, HS: hypersodic diet group, NS-T: normosodic diet plus tempol group; HS-T: hypersodic diet plus tempol group. Values are expressed as mean ± SEM; *p<0.05 vs. respective control NS, #p<0.05 vs. respective control without tempol.

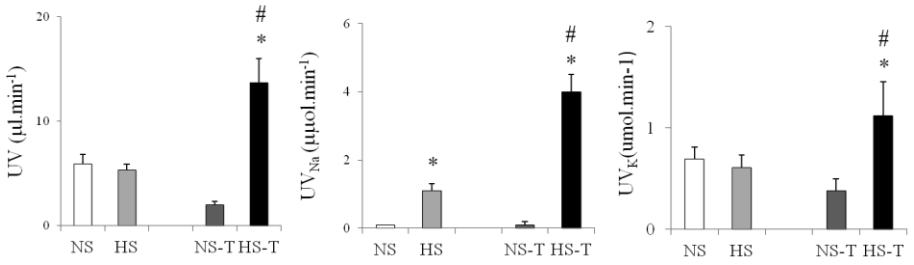


Figure 1. Renal Function. Abbreviations: Diuresis: urinary flow, UV ($\mu L.min^{-1}$) (left); Natriuresis: urinary sodium excretion, UV_{Na} ($\mu mol.min^{-1}$) (middle); and Kaliuresis: urinary potassium excretion, UV_K ($\mu mol.min^{-1}$) (right); NS: normal salt diet, HS: high salt diet, NS-T: normal salt diet plus Tempol, HS-T: high salt diet plus Tempol. Values are expressed as mean ± SEM; n=5-8; *p<0.05 vs. NS; #p<0.05 vs. respective control without tempol.

3.3. Intrarenal NADPH oxidase p47phox

NF-κB staining was increased in Na group (Figure 2), in each tubular segment except in THAL (%, PCT, NS: 4.2±0.3, HS: 11.9±0.8*; DCT, NS: 3.7±0.3, HS: 5.9±0.2*; CCD, NS: 2.7±0.4, HS: 5.1±0.5*; MCD, NS: 3.3±0.3, HS: 6.5±0.6*, p<0.001; THAL, NS: 2.3±0.3, HS: 2.0±0.2, *p< 0.001 vs NS). Tempol prevented increased staining in all tubular segments from HS group without changes in NS group (%, PCT, NS-T: 3.9±0.3, HS-T: 3.7±0.2; DCT, NS-T: 3.2±0.3, HS-T: 3.3±0.4; CCD, NS-T: 2.0±0.4, HS-T: 2.4±0.4; MCD, NS-T: 3.7±0.3, HS-T: 3.4±0.4; THAL, NS-T:2.6±0.4, HS-T:2.0±0.3).

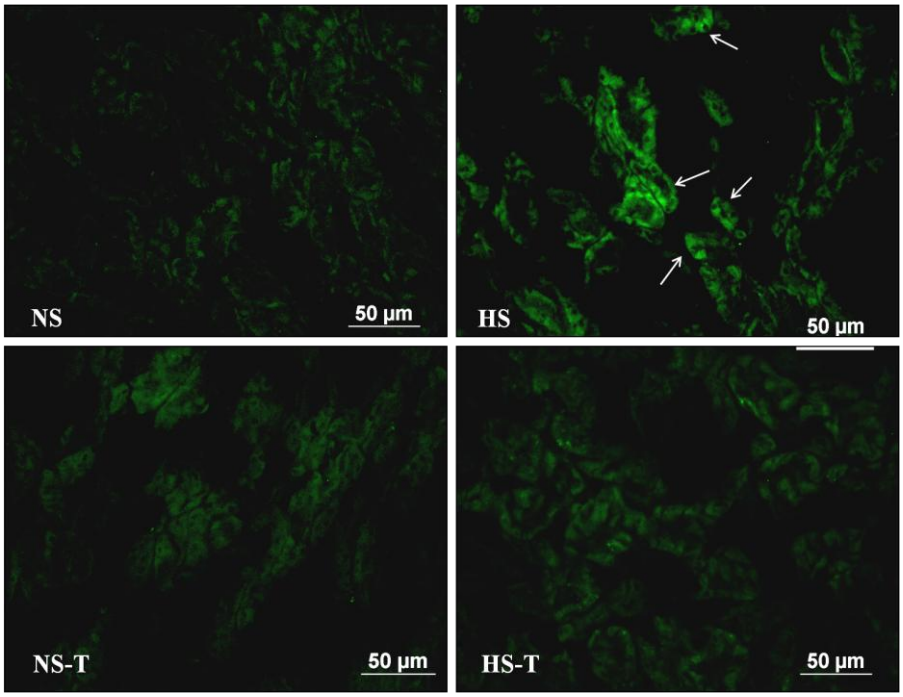
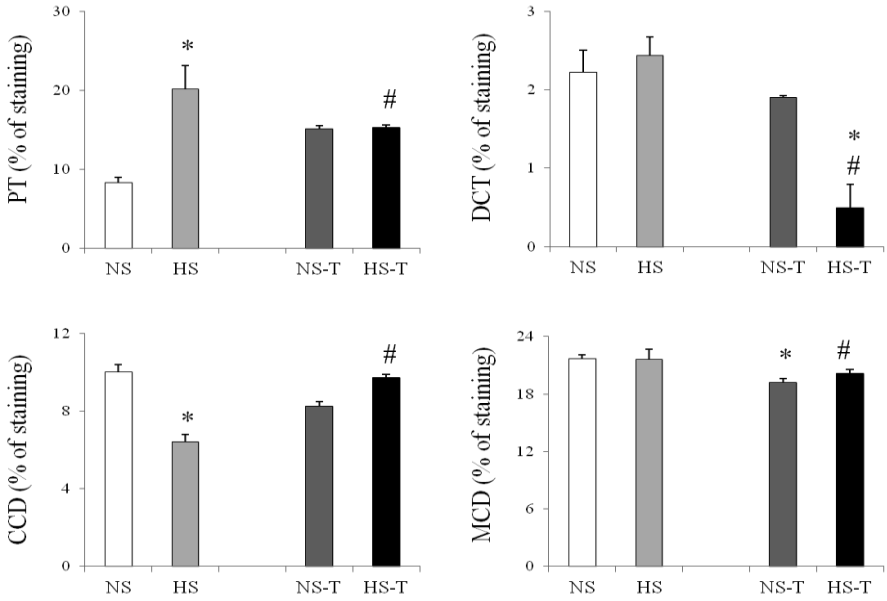


Figure 2. Immunofluorescence images of NADPH oxidase p47phox. Arrows point strong expression of the p47phox, cytosolic component in cortical tubular cells in HS group. Abbreviations: NS, normal salt diet; HS, high salt diet; NS-T, normal salt diet plus Tempol; HS-T, high salt diet plus Tempol.

3.4. Intrarenal Ang II expression

Figure 3 shows Ang II immunoexpression in renal tissues. Immunohistochemical analysis of renal sections obtained from rats subjected to high salt intake revealed positive staining for Ang II in all tubular sections examined, although Ang II staining was very low in THAL (data not shown). HS diet increased Ang II expression in PCT but decreased it in CCD compared with those in the NS group, without altering it in the rest of the examined tubular segments. It is worth noting that immunohistochemical staining in PCT showed greater staining of Ang II in the nucleus than in the cytoplasm. Tempol administration in NS rats only reduced Ang II staining in MCD, without changing its staining in other tubules. In addition, Tempol administration in HS diet-fed rats, substantially decreased Ang II staining in PCT, DCT and MCD and increased Ang II levels in CCD, restoring the staining observed in the NS group. No changes elicited by Tempol were observed in THAL (data not shown).

Panel A



Panel B

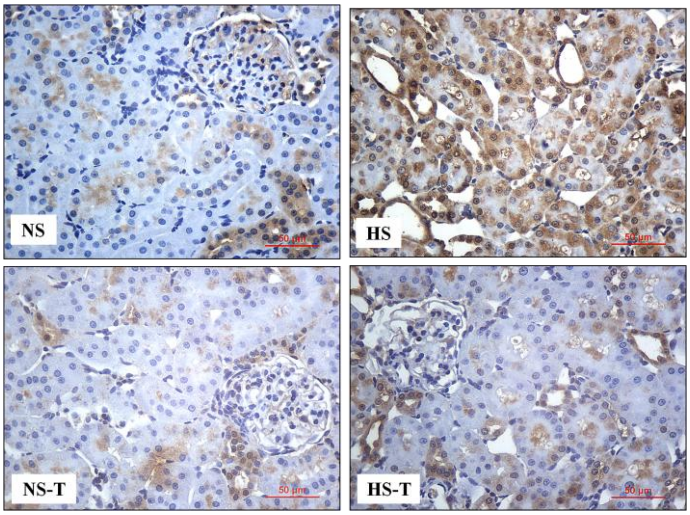
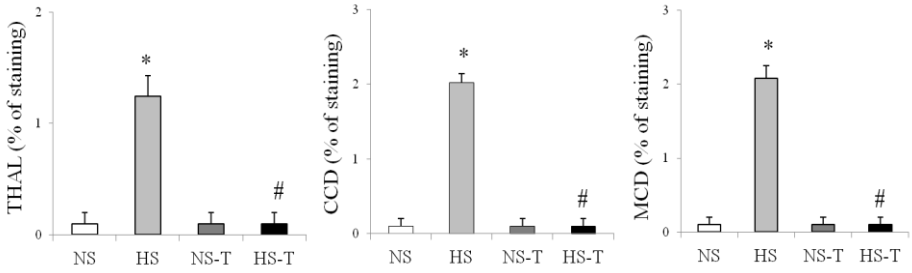


Figure 3. Panel A. Intrarenal Angiotensin II Immunostaining (Ang II). Abbreviations: PT: proximal tubule; DCT: distal convolute tubule; CCD: cortical collecting duct; MCD: medullar collecting duct. NS: normosodic diet group, HS: hypersodic diet group, NS-T: normosodic diet plus tempol group; HS-T: hypersodic diet plus tempol group. Quantitative representation of positive staining/µ2; expressed as mean percentage ± SEM; *p<0.05 vs. respective control NS, #p<0.05 vs. respective control without tempol. Panel B. Intrarenal Angiotensin II Immunostaining. Representative images of positive staining (Original magnification x 400).

3.5. Intrarenal TGF-β1 expression

TGF-β1 inmmunoexpression was markedly increased in THAL, CCD and MCD kidney segments of the HS group. TGF-β1 expression was normalized in the HS-T group, reaching a similar staining to that in the NS group. No changes in TGF-β1 staining were observed in renal tubular segments obtained from rats fed with normal diet, with or without Tempol administration (Fig. 4).

Panel A



Panel B

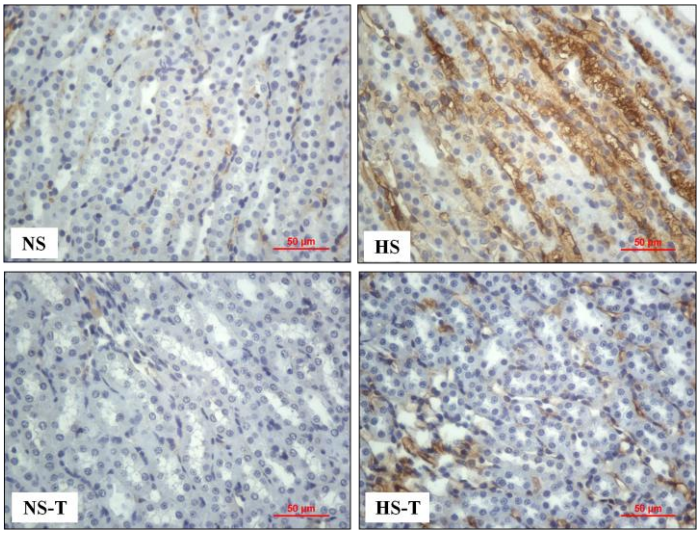


Figure 4. Panel A. Intrarenal Transforming Growth Factor-β1 immunostaining (TGF-β1). Abbreviations: THAL: thick Henle ascending loop; CCD: cortical collecting duct; MCD: medullar collecting duct; NS: normosodic diet group, HS: hypersodic diet group, NS-T: normosodic diet plus tempol group; HS-T: hypersodic diet plus tempol group. Quantitative representation of positive staining/µ2; expressed as percentage ± SEM. Values are expressed as mean ± SEM.* $p<0.05$ vs. respective control NS, #p vs. respective control without tempol. Figure 4. Panel B. Representative images of positive staining (Original magnification x 400) in renal medulla.

3.6. Intrarenal α-SMA expression

α-SMA immunolabelling was markedly increased in interstitium from renal cortex (% staining: NS: 3.09±0.12; HS: 18.11±0.61*; NS-T: 4.03±0.17 and HS-T: 4.98±0.16, *p<0.001 vs NS) and medulla (% staining: NS: 5.37±0.13; HS: 24.98±0.68*; NS-T: 5.91±0.16 and HS-T: 7.19±0.17, *p<0.001 vs NS) obtained from rats subjected to HS diet, showing α-SMA peritubular interstitial localization. Tempol normalized α-SMA expression in the HS-T treated group.

3.7. Intrarenal NF-κB expression

NF-κB staining was increased in the HS group, in each tubular segment except in THAL (%, PCT: NS: 4.2% ± 0.3%, HS: 11.9% ± 0.8% [**p<0.001 vs. NS]; DCT: NS: 3.7% ± 0.3%, HS: 5.9% ± 0.2% [**p<0.001 vs. NS]; CCD: NS: 2.7% ± 0.4%, HS: 5.1% ± 0.5% [**p<0.001 vs. NS]; MCD: NS: 3.3% ± 0.3%, HS: 6.5% ± 0.6% [**p<0.001 vs. NS]; THAL: NS: 2.3% ± 0.3%, HS: 2.0% ± 0.2% [**p<0.001 vs. NS]). Tempol prevented increased staining in all tubular segment from the HS group without changes in the NS group (%, PCT: NS-T: 3.9% ± 0.3%, HS-T: 3.7% ± 0.2%; DCT: NS-T: 3.2% ± 0.3%, HS-T: 3.3% ± 0.4%; CCD: NS-T: 2.0% ± 0.4%, HS-T: 2.4% ± 0.4%; MCD: NS-T: 3.7% ± 0.3%, HS-T: 3.4% ± 0.4%; THAL: NS-T: 2.6% ± 0.4%, HS-T: 2.0% ± 0.3%).

4. Discussion

In the present study, we analyzed the profibrogenic and proinflammatory response in kidneys of normal rats subjected to high salt intake, in the presence and absence of the superoxide anion scavenger, Tempol. We observed that the HS group exhibited a slight increase in MAP, and an enhancement of oxidative stress, profibrotic markers such as NF-κB in cortical and medullar tubules, expression of TGF-β1 in the distal nephron and α-SMA in the interstitium of renal cortex and medulla. In addition, Ang II staining increased in PCT, while it decreased in CCD. Tempol administration prevented all of these changes observed in the HS group, a fact that was accompanied by a potent natriuretic and diuretic effect. Since the profibrogenic response bears no association with Ang II localization, the present results suggest that a high-salt diet administered to normal rats was able to induce an early

profibrogenic response, independently of any Ang II inflammatory signalling, but associated to the oxidative stress.

In our experiments, a chronic salt overload (8% NaCl diet during 3 weeks) raised the UV_{Na}, and produced a slight but significant increment in MAP. In addition, we observed that Tempol administration normalized MAP elevated levels observed in the HS group, and it also enhanced GFR, FE_{Na} and UV_{Na}. These results are in agreement with previous studies, where treatment with Tempol improved renal hemodynamics and electrolyte excretory function in salt-sensitive hypertension models, while it abolished salt-sensitive hypertension as well as systemic, vascular and renal oxidative stress[14, 15]. Superoxide anion is a free radical known to stimulate sodium reabsorption by the thick ascending limb via activation of Na^+-K^+-$2Cl^-$ cotransport[4], Na^+-H^+ exchange[16] and Na^+-K^+-ATPase stimulation[14], processes which are prevented by Tempol. Moreover, Tempol can raise the blood flow to the renal medulla more than to the renal cortex in different experimental models, contributing to increased natriuresis and the reduction of MAP levels[17]. It is well known that Tempol moderates the tubuloglomerular feedback response, thus reducing macula dense stimulation and afferent arteriole vasoconstrictor responses, consequently inducing renal vasodilation and a fall in MAP levels. Therefore, our findings confirmed that the glomerular hyperfiltration and the natriuretic effect elicited by Tempol could contribute to preventing enhanced blood pressure.

The present results showed an increased Ang II immunoexpression in PCT, but simultaneously decreased Ang II expression in CCD in rats fed with high NaCl diet. It is well known that the PCT synthesizes and secretes Ang II into the lumen[18]. In addition, we have indicated that Ang II levels are higher in the nucleus than in the cytoplasm of tubular cells in the HS group. These findings are in agreement with Li *et al*, who showed that Ang II may directly stimulate nuclear AT1R to induce transcriptional responses, which may be associated with stimulation of tubular epithelial sodium transport, cell growth and proinflammatory cytokine expression[19]. However, our experiments did not show increments in TGF-β1 levels in PCTs of kidneys from rats fed with a high NaCl diet. Therefore, we suggest that the elevated levels of Ang II observed in PCT of animals fed with a high NaCl diet could regulate sodium transport rather than activate proinflammatory cytokines. Moreover, in high NaCl diet–nourished rats, an unexpected Ang II effect on PCT increasing sodium transport has recently been shown[20]. In this way, Ang II could stabilize nephron functions, avoiding a sodium overload to distal segments rather than contributing to salt homeostasis. It is known that a high-sodium diet produces oxidative stress, which up-regulates AT1R in the PCT, enhancing Ang II levels by

internalization from the luminal fluid. In addition, it has been described that oxidative stress leads to dopamine D1 receptor dysfunction in PCT. Tempol, which reduces the oxidative stress, restores AT1 and D1 receptor functions[21]. Furthermore, the fact that Tempol may reduce AT1R up-regulation, supports the hypotheses that this inhibition would permit a lesser protective effect of Ang II on distal nephrons. This may cause distal sodium overload, which in turn may increase the tubuloglomerular feedback thus decreasing the GFR. Nevertheless, Tempol might reduce sodium signaling in the macula densa as well as afferent arteriole responses, which would contribute to moderate the tubuloglomerular feedback response and to increase the GFR, as we have observed in our experiments. On the other hand, decreased Ang II levels, as we found in the CCD, could be well correlated with the increase in UV_{Na} and in the FE_{Na} that we observed in the HS-fed group. It has been reported that Ang II stimulates H^+ secretion to the lumen in CCD tubules[22]. In turn, proton secretion forces pendrin-mediated Cl^- absorption by the apical Cl^-/HCO_3^- exchanger and generates a more favourable electrochemical gradient for sodium reabsorption, mediated by the epithelial sodium channel (ENaC)[22]. Regarding Tempol effects in the HS-fed group, our results showed that the increased Ang II expression in PCT and decreased expression in CCD was normalized by Tempol treatment. Tempol also diminished Ang II expression in the DCT of the HS-fed group, more than in the NS-fed group. Under normal sodium load conditions, the Na^+/Cl^- cotransporter (NCC) mediates the electroneutral NaCl reabsorption in the DCTs. In this regard, Ang II stimulates the acute trafficking of NCC to the apical membrane[23]. Some evidence has emerged that the NCC may participate in natriuresis and diuresis modulation. In this way, a fall in tubular Ang II levels can induce the retraction of NCC from the apical plasma membrane, with the NCC internalized in the cell. Therefore, Tempol could facilitate sodium excretion, by inhibiting Ang II expression in the DCT. Altogether, our findings support the idea that the changes observed in Ang II expression along the tubular segments of the nephron may represent an adaptive mechanism to sodium overload.

After 3 weeks of high salt load we found an enhanced tubulointerstitial TGF-β1 expression in distal nephrons and α-SMA immunolabeling in renal cortex and medulla. Both over-expressions were prevented by Tempol treatment. It has been demonstrated that TGF-β1 is up-regulated by Ang II and oxidative stress, and inhibited by nitric oxide. Recently published data have shown that, despite the positive-feedback loop between TGF-β1 and nitric oxide under physiological conditions, the inhibitory effect of nitric oxide on TGF-β1 production was reduced after salt intake in Dahl salt-sensitive rats[24, 25]. The authors showed that

administration of the antioxidant kallistatin restored nitric oxide levels and prevented salt-induced TGF-β1 overexpression. Since we have not observed any correlation between Ang II and TGF-β1 immunolabeling, the profibrogenic response (showed by α-actin and TGF-β1 enhanced staining) observed in our experiments could be related to the balance of oxidative stress and nitric oxide, rather than to renal Ang II expression.

Our results confirmed that Tempol, a superoxide anion scavenger, exerts a beneficial action in high-salt diet–fed rats since it ameliorated the high arterial pressure, an effect that may be associated with its antioxidant and natriuretic and diuretic effects. During salt loading, intrarenal Ang II levels showed an opposite behaviour according to the different nephron segments studied. Surprisingly, Ang II levels increased in PCT, but decreased in CCD, while TGF-β1 expression was enhanced in distal nephrons, and α-SMA staining increased in renal cortex and medulla. The chronic administration of Tempol prevented these changes and decreased Ang II levels in DCT. These results provide evidence that intrarenal Ang II expression is not correlated with proinflammatory and profibrogenic marker expression in the kidney of HS diet–fed animals. Moreover, the changes in TGF-β1 expression, observed in the HS diet–fed group, would not result from Ang II-initiated inflammation signalling[26]. While in salt-sensitive rats fed with a high-salt diet, the development of hypertension and renal disease are associated with intrarenal Ang II expression, our results suggest that in normal rats, the early fibrogenic response of the kidney to a sodium overload would be directly associated with the oxidative stress.

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Chapter 5

Renal Over-expression of Atrial Natriuretic Peptide and Hypoxia Inducible Factor-1α as Adaptive Response to a High Salt Diet

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Submitted

Abstract

In the kidney, a high salt intake favours oxidative stress and hypoxia, and causes the development of fibrosis. Both atrial natriuretic peptide (ANP) and hypoxia inducible factor-1 alpha (HIF-1α) exert cytoprotective effects. We tested the hypothesis that renal expression of ANP and HIF-1α is involved in a mechanism responding to the oxidative stress produced in the kidneys of rats chronically fed a high sodium diet.

Sprague-Dawley rats were fed a normal-salt (0.4% NaCl) (NS) or a high-salt (8% NaCl) (HS) diet for 3 weeks, with or without the administration of tempol (T), an inhibitor of oxidative stress, in the drinking water. We measured the mean arterial pressure (MAP), glomerular filtration rate (GFR), and urinary sodium excretion (UV_{Na}). We evaluated the expression of ANP, HIF-1α, and transforming growth factor-beta1 (TGF-ß1) in renal tissues by immunohistochemistry. The animals fed high salt diet showed increased MAP and UV_{Na} levels, and enhanced renal immunostaining of ANP, HIF-1α, and TGF-ß1. The administration of tempol together with the sodium overload increased the natriuresis further, prevented the elevation of blood pressure, and the increased immunostaining of ANP, TGF-ß1, and HIF-1α expression compared to their control.

These findings suggest that HIF-1α and ANP, synthesized by the kidney, are involved in an adaptive mechanism in response to a sodium overload to prevent or attenuate the deleterious effects of the oxidative stress and the hypoxia on the development of fibrosis.

1. Introduction

Reactive oxygen species (ROS) have been demonstrated to play an important pathophysiological role in the kidney[1-4]. ROS can activate the mitochondrial uncoupling protein 2 (UCP-2), leading to inefficient renal O_2 usage and contributing to renal hypoxia[5]. Changes in cellular oxygen concentrations induce tightly regulated response pathways that attempt to restore oxygen supply to cells and modulate cell function in hypoxic conditions. Most of these responses occur through the induction of the transcription factor hypoxia-inducible factor-1alpha (HIF-1α) which coordinates the expression of diverse adaptive genes against the hypoxic injury[6, 7]. HIF-1α transcriptionally upregulates the expression of metabolic proteins (GLUT-1), adhesion proteins (integrins), soluble growth factors (TGF-β1 and VEGF), and extracellular matrix components (type I collagen and fibronectin), which enhance the repair process. For these reasons, HIF-1α is viewed as a positive regulator of organ repair and tissue fibrosis[8, 9].

In the kidney, a predominant enzyme involved in oxidative stress development is NADPH-oxidase, which is up-regulated by increased sodium tubular transport, luminal flow or cytokines release[10, 11]. In turn, the superoxide anion increases tubular NaCl transport, further enhancing oxidative stress[12]. It is well known that a high salt intake increases the oxidative stress in the kidneys of normal and salt-sensitive rats[1-4]. In this regard, we have reported that a high salt diet in Sprague-Dawley rats is able to increase the oxidative stress. We also showed that the administration of tempol (4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-N-oxyl), which is a permeate superoxide dismutase mimetic commonly used to inhibit the oxidative stress, prevented those changes and produced a potent natriuretic and diuretic effect. Thus, we concluded that the increase of the oxidative stress induced by sodium overload could account for anti-natriureris[1]. It has also been described that in models of experimental salt-sensitive hypertension, tempol improved the renal hemodynamic response and electrolyte excretory function, while abolishing salt-sensitive hypertension and renal oxidative stress[13].

A growing number of mammalian genes have been identified to play a key role in the cellular adaptive response to counter-regulate renal hypoxia and the consequent process of fibrosis, among which is atrial natriuretic peptide (ANP). ANP is a member of a natriuretic peptide family which, besides its role in the regulation of volume homeostasis, has been noted to exert protective effects in several cell types in response to oxidative stress[14] and fibrosis[15] and on the adaptation to hypoxia[16].

Based on these data, we hypothesized that endogenous renal ANP and HIF-1α could constitute endogenous adaptive mechanisms in response to the oxidative stress produced in the kidney of rats chronically fed with a high sodium diet. Therefore, in the current study we determined the effect of a high-salt diet on the regulation of renal expression of HIF-1α, ANP, and TGF-β1 in Sprague-Dawley rats. In addition, we evaluated whether tempol administration, by inhibiting oxidative stress, prevents the increase of these molecules. Understanding these endogenous mechanisms can lead to finding a better therapeutic approach in salt-sensitive hypertension.

2. Methods

2.1. Animal protocol and experimental measurements

Experiments were conducted in accordance to the care and use of research animals of international guiding principles and local regulations concerning the care and use of laboratory animals for biomedical research (ANMAT, 6344/96; Institute of Laboratory Animal Resources, 1996)[17], as well as the "International Ethical Guiding Principles for Biomedical Research on Animals" established by the CIOMS (Council for International Organizations of Medical Sciences)[18]. These protocols were approved by Universidad de Buenos Aires (UBACYT B113) and the National Scientific and Technical Research Council (CONICET, PIP 1337/09).

Male Sprague Dawley rats, 5-6 weeks old (180-200g body weight), were used in the experiments. The animals were housed in steel cages in a controlled temperature animal room at 23±2ºC, exposed to a daily 12-hour light-dark cycle (light on from 07:00 a.m. to 07:00 p.m.), fed the diets described below for three weeks, and were given free access to tap water. The experiments were performed in rats randomly divided into four groups (n=6 for each group): a) NS (control): fed a normal salt diet (0.4 g% NaCl); b) HS: fed a high salt diet (8 g% NaCl); c) NS-T: fed a normal salt diet (0.4 g% NaCl) plus 1 mM tempol (Sigma-Aldrich Inc, St. Louis, Missouri, USA), administered in the drinking water; d) HS-T: fed a high salt diet (8 g% NaCl) plus 1 mM tempol administered in the drinking water. After 3 weeks, the rats were anaesthetized intraperitoneally with urethane (1.2 $g.kg^{-1}$) 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 catheterized with a T4 tube for blood sampling and for continuous mean arterial pressure recording (MAP) by means of a Statham GOULD

P23ID transducer coupled to a Grass Polygraph 79D during all the procedures. The bladder was cannulated for urine collection using a PE-75 cannula. A femoral vein infusion with isotonic saline solution (ISS, 0.15M NaCl) was performed at a rate of 0.04 $mL.min^{-1}$ (Syringe Infusion Pump, SageTM, Orion) for 60 minutes to allow for a steady diuresis and to permit urine collection in all groups. Then, ISS infusion continued for another 60 min at the same rate during the experimental period. From each animal, a blood sample was collected at 30 minutes and a urine sample was collected from 0 to 60 minutes for sodium, potassium and creatinine measurements. At the end of the experimental period, other blood samples were obtained from the abdominal cava vein, immediately placed into plastic tubes containing 15% EDTA, and kept on ice for ANP dosage. The kidney was rapidly excised, decapsulated, longitudinally cut and harvested for immunohistochemical studies.

2.2. ANP radioimmunoassay

The plasma ANP extraction procedure was followed as described by Cavallero *et al*. [19]. The radioimmunoassay (RIA) was performed using an ANP-rat RIA commercial kit (Phoenix Pharmaceuticals, Burlingame, CA)[20].

2.3. Urine and blood measurements

Urinary and plasma sodium and creatinine were measured by standard methods using an autoanalyzer. Creatinine clearance was assessed in order to evaluate the glomerular filtration rate (GFR). GFR and sodium fractional excretion (FE_{Na}) were calculated according to a standard formula. Urinary flow (UV) is expressed as $\mu L.min^{-1}$; plasmatic sodium (PL_{Na}) concentration as $mEq.L^{-1}$, sodium urinary excretion (UV_{Na}) as $\mu mol.min^{-1}$, GFR as $mL.min^{-1}$ and FE_{Na} as percentage.

2.4. Kidney processing for histological examination

Renal tissues were fixed in phosphate-buffered 10% formaldehyde (pH 7.20) and embedded in paraffin. For immunohistochemistry, sections were deparaffinated and rehydrated, and endogenous peroxidase activity was blocked by treatment with 0.5% H_2O_2 in methanol for 20 minutes. Local ANP, HIF-1α and TGF-β1 were detected using the following specific antibodies: rabbit anti-ANP (Phoenix Pharmaceutical; dilution 1:500), rabbit anti-HIF-1α (Novus Biologicals, Inc., Littleton, CO; dilution 1:1000), and rabbit anti TGF-β1 (Santa Cruz Biotechnology; dilution 1:200). Immunostaining was performed with a commercial modified avidin-biotin-peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector

Laboratories, CA) and with counterstaining with haematoxylin. Histological sections were observed in a Nikon E400 light microscope (Nikon Instrument Group, Melville, New York, USA). All measurements were carried out using image analysis software (Image-Pro Plus ver. 4.5 for Windows, Media Cybernetics, LP. Silver Spring, MD, USA). Immunoreactivities for ANP, HIF-1α and TGF-β1 are expressed as percentage of positive stained area ± standard error of the mean (SEM) in proximal convoluted tubules (PT), distal tubules (DT), thick ascending limb of the loop of Henle (THAL), cortical collecting ducts (CCD), and medullary collecting ducts (MCD).

2.5. Statistical analysis

Results from urine and blood measurements and MAP are expressed as mean ± SEM. Gaussian distribution was evaluated by the Kolmogorov and Smirnov method and the comparison among groups was carried out using ANOVA followed by the Bonferroni test. P values < 0.05 were considered significant.

3. Results

3.1. Body Weight and Mean Arterial Pressure

There were no significant differences in body weight between control and experimental groups (grams; NS: 330±9; HS: 320±6; NS-T: 314±13; HS-T: 320±7). The MAP was increased in the HS-fed group compared to the NS-fed group (mmHg; NS: 94±3 vs HS: 107±3*). Tempol administration did not modify MAP levels in NS animals, but normalized MAP in the HS-T group, reaching MAP levels very similar to those observed in NS rats (mmHg; NS-T: 97±2 vs HS-T: 95±3†). *$p< 0.05$ vs. respective NS group, †$p< 0.05$ vs. respective group without tempol.

3.2. Plasma Sodium and Urinary Sodium Excretion (Table 1)

High salt diet and tempol administration did not alter plasma sodium in any experimental group. The administration of a high salt diet did not alter the GFR, which was increased by tempol in HS-T group with respect to the HS group. Tempol administration also increased GFR in the NS-T group as compared with the NS group.

The administration of a high salt diet led to a greater UV_{Na}, which was further increased in HS-T group with respect to the NS-T and HS groups. Moreover, tempol increased UV_{Na} in NS-fed rats. The administration of a high salt diet raised FE_{Na} in HS group with respect to the NS group. Tempol administration increased the FE_{Na} further in the HS-T group as compared with NS-T and to the HS group. FE_{Na} was not altered in the NS-T group.

	NS	HS	NS-T	HS-T
PL_{Na} ($mEq.L^{-1}$)	142±1	145±2	143±1	143±2
GFR ($mL.min^{-1}$)	1.51±0.1	1.48±0.1	3.4±0.8†	3.1±0.7†
UV_{Na} ($\mu mol.min^{-1}.kg^{-1}$)	0.23±0.1	3.50±0.64*	2.00±0.92†	13.0±1.5*†
FE_{Na} (%)	0.04±0.02	0.53±0.08*	0.12±0.06	1.20±0.20*†

Table 1. Renal Function Parameters. Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group, PL_{Na}: Plasmatic sodium concentration, GFR: glomerular filtration rate, UV_{Na}: Urinary sodium excretion, FE_{Na}: fractional sodium excretion. All values are mean ±SEM (n= 5-6 per group), *p< 0.05 vs. respective NS group, †p< 0.05 vs. respective group without tempol.

3.3. Plasmatic ANP

Plasma ANP concentration ($pg.mL^{-1}$) did not differ significantly between the NS (325.75 ± 66.69) and HS (260.00±7.38) groups, as measured by RIA.

3.4. Intrarenal ANP Expression

Figure 1 shows quantified levels of ANP immunoexpression in renal tissues. Representative images of ANP positive staining are shown in Figure 2 panel A (renal cortex) and B (renal medulla). The analysis of renal sections obtained from the HS group revealed increased positive staining for ANP in glomeruli, THAL, and CCD with respect to the NS group. Tempol prevented the elevation of ANP staining in HS-T group in glomeruli, THAL, and CCD, and decreased ANP staining in PT and MCD of both NS-T and HS-T groups.

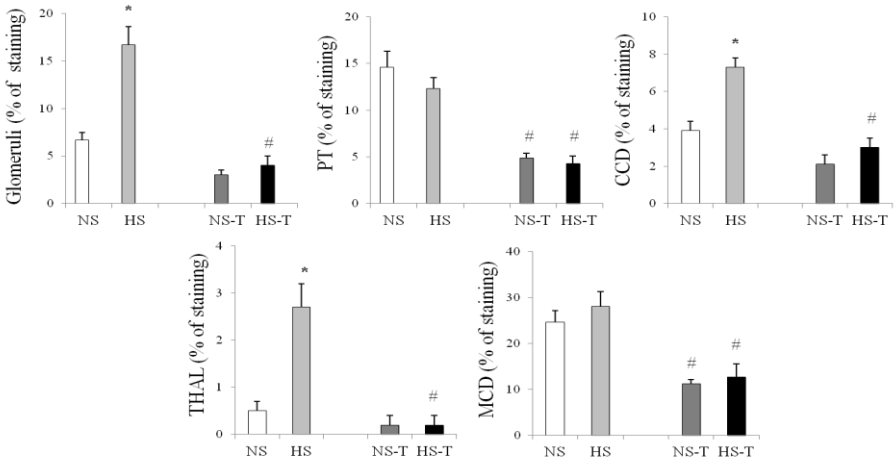
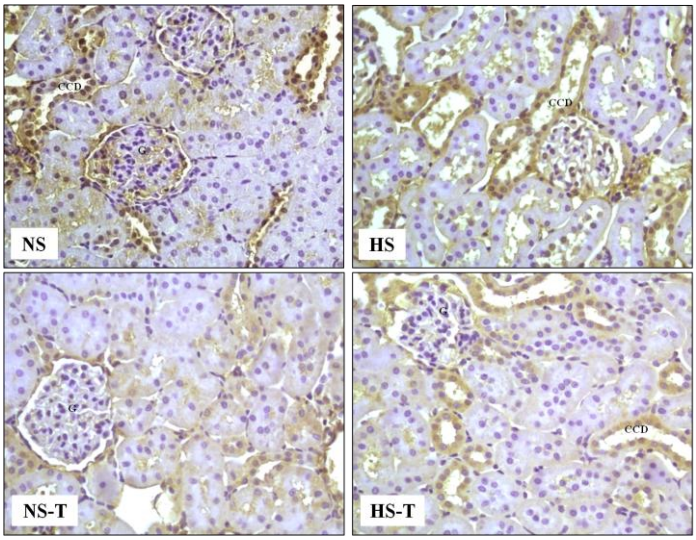


Figure 1. Quantitative representation of positive intrarenal atrial natriuretic peptide (ANP). Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group. PT: proximal tubule, CCD: cortical collecting duct, THAL: thick ascending limb of the loop of Henle and MCD: medullar collecting duct. Values are expressed as percentage ± SEM. *p<0.05 vs respective NS control; #p<0.05 vs. respective control without tempol.

Panel A



Panel B

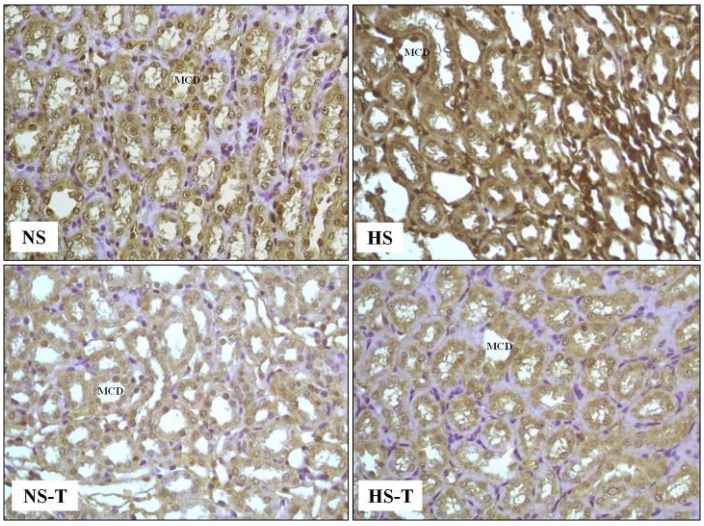


Figure 2. Panel A: Representative images of positive staining of atrial natriuretic peptide (ANP) in renal cortex. Panel B: Representative images of positive staining of ANP in renal medulla. Original magnification 400x. Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group. G: glomerulus, CCD: cortical collecting duct, MCD: medullar collecting duct.

3.5. Intrarenal HIF-1α Expression

Figure 3 shows quantified levels of HIF-1α immunoexpression in renal tissues. Representative images of HIF-1α positive staining are shown in figure 4 panel A (renal cortex) and B (renal medulla). Immunostaining analysis of renal sections obtained from HS group revealed that HIF-1α staining was increased in all examined tubular segments (PT, DT, CCD, THAL, and MCD) with respect to the NS group. The administration of tempol did not alter HIF-1α staining in the NS-T group, except in THAL where it was augmented. Whereas in HS-T group, HIF-1α staining decreased in all tubular segments except in THAL compared with HS group.

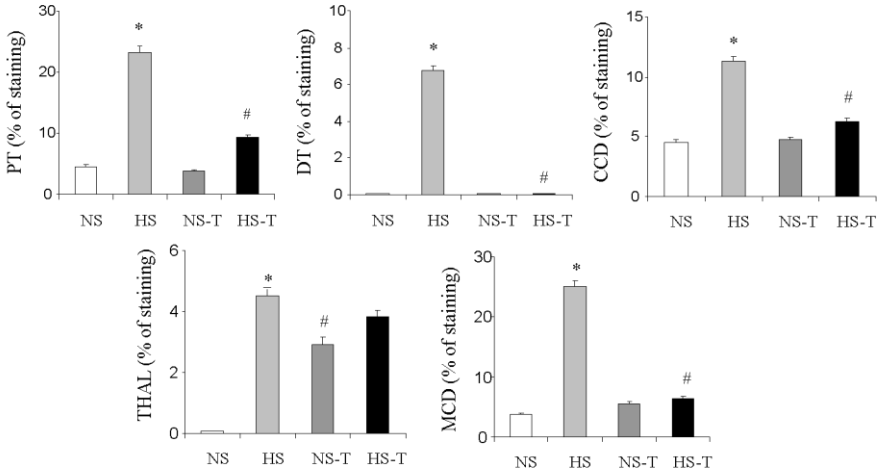
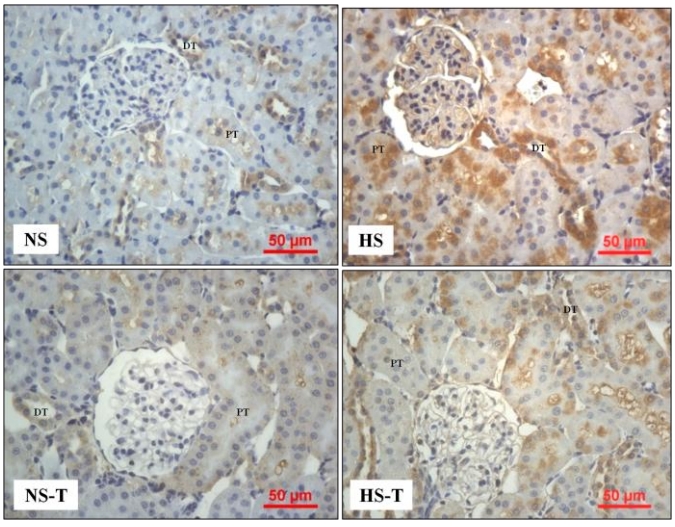


Figure 3. Quantitative representation of positive intrarenal hypoxia-inducible factor-1 (HIF-1α). Values are expressed as percentage ± SEM. *p<0.05 vs respective NS control; #p<0.05 vs. respective control without tempol. Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group. PT: proximal tubule, DT: distal tubule, CCD: cortical collecting duct, THAL: thick ascending limb of the loop of Henle and MCD: medullar collecting duct.

Panel A



Panel B

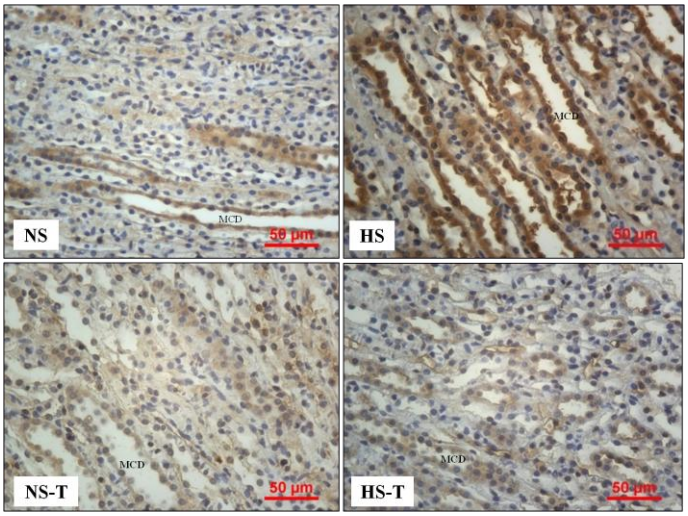


Figure 4. Panel A: Representative images of positive staining of hypoxia inducible factor-1 (HIF-1α) in renal cortex. Panel B: Representative images of positive staining of HIF-1α in renal medulla. Original magnification 400x. Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group. PCT: proximal convoluted tubule, DT: distal tubule, CCD: cortical collecting duct, MCD: medullar collecting duct.

3.6. Intrarenal TGF-β1 Expression

Figure 5 shows quantified levels of TGF-β1 immunoexpression in renal tissues. Representative images of TGF-β1 positive staining are shown in figure 6 in panel A (renal cortex) and B (renal medulla). Immunostaining analysis of renal sections revealed increased positive staining for TGF-β1 in glomeruli, THAL, CCD, and the MCD in HS group, with respect to the NS group. Tempol did not show additional effects in the NS-T group as compared with its control, and prevented the increase of TGF-β1 staining in HS-T group.

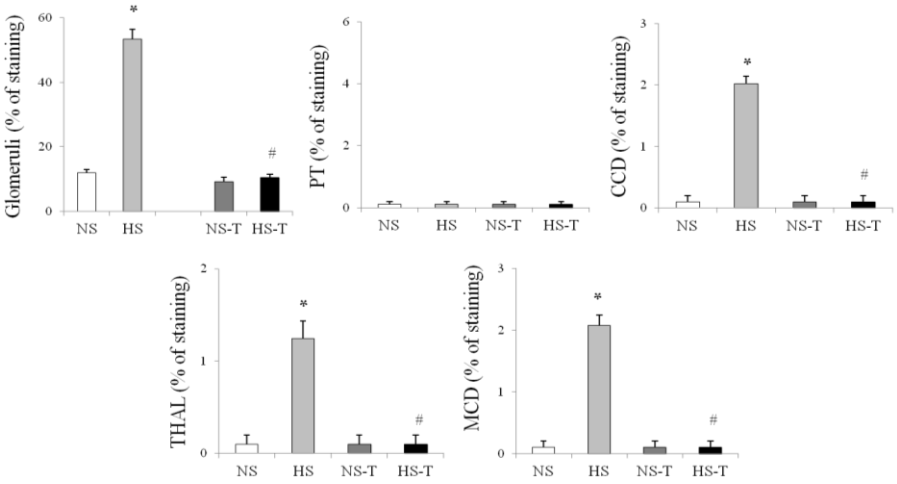
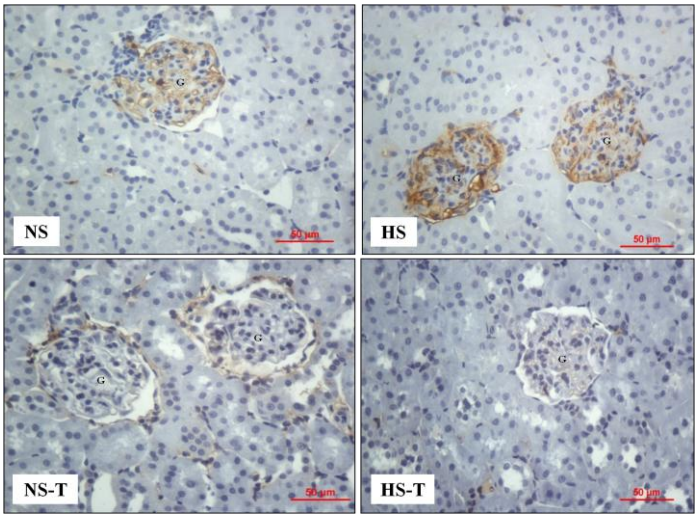


Figure 5. Quantitative representation of positive intrarenal transforming growth factor (TGF-β1). Values are expressed as percentage ± SEM. *p<0.05 vs respective control NS; #p<0.05 vs. respective control without tempol. Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group. PT: proximal tubule, CCD: cortical collecting duct, THAL: thick ascending limb of the loop of Henle and MCD: medullar collecting duct.

Panel A



Panel B

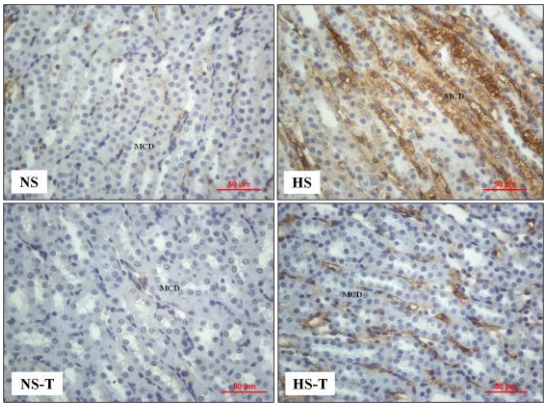


Figure 6. Panel A: Representative images of positive staining of TGF-β1 in renal cortex. Panel B: Representative images of positive staining of TGF-β1 in renal medulla. Original magnification 400x. Abbreviations: NS: normal salt diet group, HS: high salt diet group, NS-T: normal salt diet plus tempol group, HS-T: high salt diet plus tempol group. G: glomerulus, MCD: medullar collecting duct.

4. Discussion

Our results showed that a high salt diet in normal rats resulted in a greater ANP expression in glomeruli, THAL and CCD, where the pro-fibrotic marker TGF-β1 was also increased. In addition, enhanced HIF-1α expression was observed, not only in the renal medulla as it has been previously described[21], but also in renal cortex. We found that tempol administration, a superoxide dismutase mimetic, favoured urinary sodium excretion, prevented the increase of ANP and TGF-β1 expression, and normalized HIF-1α expression, the latter except in THAL. These results suggest that increased ANP and HIF-1α expression in renal cortex and medulla could be involved in an adaptive response to the oxidative stress resulting from a high salt diet.

As we have previously reported, a chronic salt overload (8% NaCl diet during 3 weeks) does not alter body weight or plasma sodium, but causes higher MAP levels as well as urinary sodium excretion[1]. Even though the animals used in this study have their blood pressure taken under anesthesia, we have recently reported the same effect of a high salt diet on the MAP in conscious animals[22], allowing us to conclude

that the anesthesia does not affect the blood pressure when the different diet groups are compared. On the other hand, the administration of tempol normalized MAP levels observed in the HS group, and it also increased further urinary sodium excretion. These results are in agreement with previous studies, where treatment with tempol improved renal hemodynamic and electrolyte excretory function in salt-sensitive hypertension models[1, 23]. The effects of tempol, promoting natriuresis and preventing endogenous increase of ANP, could suggest that by inhibiting oxidative stress, tubular sodium transport decreases and thus prevents the endogenous ANP expression as natriuretic hormone. However, we observed a clear raise in ANP expression also in glomeruli from rats fed a high salt diet, in which sodium transport does not occur. Therefore, in addition to a natriuretic effect, endogenous ANP could be exerting other effects in glomeruli and tubules. Furthermore, the increase of renal ANP levels coincided with TGF-β1 over-expression in glomeruli, THAL and CCD. In this regard, it is known that TGF-β1 up-regulates the transcription of the serum and glucocorticoid-dependent kinase hSGK1, involved in the regulation of two important factors for cell volume regulation, i.e. the renal epithelial Na+ channel ENaC and the thick ascending limb Na^+-K^+-$2Cl^-$ cotransporter NKCC[24]. The increase of cell volume stimulates protein synthesis and inhibits protein degradation, contributing to enhance the net formation and deposition of matrix proteins. In addition, TGF-β1 transduces intracellular signals through type 1 (TGF-βR1) and type 2 (TGF-βR2) receptors, via the nuclear translocation of Smad3 proteins, thus contributing to a fibrotic response[25]. It has been demonstrated that the activation of ANP/cGMP/PKG signaling phosphorilates Smad3 and disrupts TGF-β1-induced nuclear translocation of pSmad3 and later downstream events, including myofibroblast transformation and the proliferation and expression of extracellular matrix molecules[26, 27]. Moreover, we have previously demonstrated that an increase in TGF-β1 expression produced by an acute saline overload was prevented and reversed by the administration of low and non-hypotensive doses of ANP[28]. Taking this into account, the present results suggest that the raise of ANP expression in rats fed a high salt diet could constitute a counter-regulatory mechanism against anti-natriuretic and/or pro-fibrotic TGF-β1 actions.

Meanwhile, the plasmatic concentration of ANP was not affected by dietary salt, suggesting that these animals did not change cardiac ANP secretion[19]. Several reports provide evidence that plasmatic ANP increases after a chronic salt loading given by drinking 1–18% NaCl solutions[29, 30] or by a rat chow with high salt content[31]. However, the literature also shows conflicting results reporting that ANP mRNA expression and circulating levels of ANP remained unaltered after the

ingestion of high sodium diets[32]. The present results show that renal ANP expression levels are independent of circulating ANP levels and are subjected to a different regulation. These findings are in accordance with Sun et al, who have previously demonstrated that a dietary salt supplementation may selectively increase ANP levels in the kidney by down-regulating its clearance receptor (NPR-C)[33].

Our study also shows that a high salt intake increased HIF-1α expression not only in the renal medulla, as it has been described before[20], but also in renal cortex. Considering that a high salt intake increases oxidative stress, renal tubular transport and hypoxia, it may constitute one of the possible factors involved in the up-regulation of HIF-1α expression in renal tissues[6, 34]. Additionally, it has been reported that a high salt intake inhibits PHD-2 expression, the predominant isoform in renal medulla of prolyl-hydroxylase enzyme, which promotes the degradation of HIF-1α, increasing its expression level[35]. In this way, recent studies have shown that HIF-1α may regulate the encoding genes of some enzymes in the THAL, as those of nitric oxide synthase (NOS), cyclooxygenase-2 (COX-2) and hemeoxygenase-1 (HO-1), which are highly expressed in renal medulla[36, 37]. However, the tempol administration, which prevents oxidative stress and enhanced urinary sodium excretion, normalized HIF-1α expression in the HS group, except in THAL from both groups fed a normal or a high salt diet. These data suggest that tempol could prevent the hypoxia produced by the oxidative stress as it has been described[5, 22], but not the hypoxia produced by greater sodium transport in THAL, where the enhancement of sodium reabsorption in this tubular segment may be compensating the inhibition of sodium transport in PT, elicited by tempol.

In summary, the present findings suggest that HIF-1α and ANP could represent a main adaptive mechanism in normal rats, in response to a salt overload, counter-regulating the hypoxia and fibrosis produced by oxidative stress, and playing a crucial role in the maintenance of sodium balance. The administration of tempol, as a scavenger of superoxide anion, prevents over-expression of HIF-1α and ANP. The disruption of this salt adaptive pathway in salt-sensitive rats could be the cause of sodium retention, oxidative stress, inflammation and fibrosis in these animals. The pharmacological potentiation of endogenous ANP may be a therapeutic approach for the management of oxidative stress in salt-sensitive hypertension.

5. Acknowledgements

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Chapter 6

A Slight Increase of Salt Induces Down-regulation of CD39 and Type-1 Angiotensin II Receptor in Endothelial Cells in Vitro

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In preparation

Abstract

Small increases of plasma sodium are thought to affect vascular tone. Recent data suggest that endothelial alterations may play a major role in this phenomenon. The objective of the present study was to investigate the possible endothelial loss of ecto-apyrase (CD39) and the angiotensin II type 1 receptor (AT1R) expression that may occur as a result of minor increase of salt content of the culture mediums in vitro and the potential involvement of reactive oxygen species in the mechanism of damage.

The expression of CD39 and AT1R was tested by flow cytometry, western blot and immunostaining of cytospins *in vitro* after co-culturing with peripheral blood mononuclear cells (PBMC) using medium supplemented with low concentrations of salt. In addition, production of reactive oxygen species (ROS) was measured in PBMC of these cultures.

Endothelial cells showed reduced CD39 expression after increase of 1.0%- 3.0% of salt ($p<0.01$) and reduced AT1R expression after increase of 0.5%-2.0% salt into the medium ($p<0.01$) as compared with standard medium. Significant raise of ROS production by PBMC occurred after enhancement of salt up to 1.0% ($p<0.05$).

Down regulation of endothelial CD39 and AT1R can be achieved by relatively low amounts of salt *in vitro*. Concomitant increase of ROS in PBMC after addition of salt suggests that the mechanism of this type of endothelial injury in vitro is mediated by oxidative stress, although potential additional mechanisms remain to be investigated.

Introduction

High dietary salt intake is associated with hypertension, cardiovascular and renal risk. Recently, it has been suggested that plasma sodium plays an important role in the control of blood pressure, as a small increase in plasma sodium was observed in subjects with hypertension[1, 2]. Although little is known about the complete mechanism by which salt may increase blood pressure, evidence is accumulating that vascular endothelium could participate in a sodium-mediated blood vessel function[3-5]. For instance, endothelial cell elasticity, determined by the sodium concentration has been proposed as an important parameter in the control of blood pressure[6].

High salt may also decrease the production of a powerful vasodilator, nitric oxide (NO), and enhance the production of superoxide anion ($\cdot O_2^-$), both which can lead to the formation of peroxynitrite. Besides its local effect on tissue, the latter oxidant may also result in reduced availability of NO[7].

CD 39 is an ecto-phosphatase lining the vascular endothelium and present on various other cell surfaces. This ecto-enzyme, also known as ecto-apyrase or Ecto-Nucleoside Triphosphate Diphosphohydrolase 1 (ENTPD1), is able to hydrolyze extracellular ATP and ADP[8, 9]. Regulation of these nucleotides by CD39 reflects an essential control mechanism for local inflammation and intravascular microthrombus formation[10, 11]. CD39 is highly sensitive for reactive oxygen species (ROS)[12, 13] leading to diminished expression of this ecto-enzyme in ischemic conditions as observed in vascular endothelium of subjects with preeclampsia[14, 15], and in the glomerular microvasculature in chronic kidney graft rejection[16].

Another important molecule found on the endothelium is Angiotensin II type-1 receptor (AT1R), which mediates most of the physiological and pathophysiological actions of Ang II. However, still little is understood about the importance of this receptor on endothelial cell signaling[17].

Recent evidence suggests that AT1R activation is critical in the pathogenesis of endothelial dysfunction by increasing the production of oxidants[18]. Preliminary data in our laboratory suggested that increased salt intake may affect expression of CD39 and AT1R on monocytes and endothelial cells in vitro. The question emerged whether salt intake increments as low as approximately 1%, which is the rise of plasma sodium after high- versus low-salt diets in healthy volunteers[19], affects CD39 and AT1R expression on endothelial cells. We tested the hypothesis that endothelial loss of CD39 and AT1R expression occurs as a result of minor increase of salt

content of the culture medium. In addition, we investigated whether this putative affect is dependent on ROS production in the co-culture system used since high salt has been associated with ROS production[20, 21].

2. Methods

2.1. Cell culture

A human endothelial cell culture line (ECV304) was used. Confluent cultures were carried out using 6-well tissue culture plates in Medium 199 containing 10% fetal calf serum (FCS), supplemented with 100 $U.mL^{-1}$ penicillin, 100 $g.mL^{-1}$ streptomycin, and 2 mmol/L glutamine at 37ºC.

2.2. Peripheral blood cells preparation

Heparin blood samples from healthy volunteers were collected. Peripheral Blood Mononuclear Cells (PBMC) were isolated using Ficoll-Isopaque according to standard methods, washed in Hank's Balanced Salt Solution (HBSS) and resuspended in tissue culture medium (RPMI 1640).

2.3. Cell Co-culture

Endothelial cell cultures were washed and incubated for 12hs in RPMI 1640 serum free with PBMC ($1x10^6$ $cells.mL^{-1}$ per well) using medium with different salt concentrations, as listed below:

- 154.00 $mmol.L^{-1}$ (physiological amount of NaCl): indicated as “Standard”,
- 154.77 $mmol.L^{-1}$ of NaCl: increase of 0.5%,
- 155.54 $mmol.L^{-1}$ of NaCl: increase of 1.0%,
- 157.08 $mmol.L^{-1}$ of NaCl: increase of 2.0%,
- 158.62 $mmol.L^{-1}$ of NaCl: increase of 3.0%.

Endothelial cells were also cultured without PBMC in standard medium.

After overnight incubation, the supernatants with PBMC were separated from the monolayer of endothelial cells and reserved for analysis (See “Reactive Oxygen Species production” section). The endothelial cell monolayer was washed with HBSS and carefully harvested from the 6-well plates using a disposable rubber cell scraper (Corning Inc). Cell viability, as estimated by dye exclusion (Tripan Blue), showed approximately 97% viable cells. Three sets of experiments were performed

in order to assess the expressions of CD39 and AT1R using flow cytometry, Western blot and immunohistochemistry.

2.4. CD39 and AT1R expression

2.4.1. Flow cytometry

In order to evaluate the expression of CD39 and AT1R, the endothelial cells were subsequently washed (x2) in phosphate buffered saline supplemented with 1% bovine serum albumin (PBS/BSA) and incubated with 2µL monoclonal anti AT-1 IgG (clone TONI, Abcam) or 2µL monoclonal anti-apyrase (anti CD39) on ice. After 60 min cells were washed (x2) in PBS/BSA buffer and incubated with goat-anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (DAKO) for 30 min on ice. Control cells were incubated with 2µL of the second antibody only (i.e. goat-anti mouse-IgG FITC). After washing (x2) with PBS/BSA cells were fixed with 2% paraformaldehyde in PBS. The fluorescence was measured by flow cytometry using a fluorescence activated cells sorting (FACS) device (Calibur Beckton Dickinson, USA). According to standard methods, the data were processed using a standard software program (Winlist 6.0, Verify Software House, Topsham, ME).

2.4.2. Western blot Analysis

After washing the endothelial cell monolayer with PBS, 300µL of cold RIPA buffer was added to each well (150mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8.0) plus protease inhibitors (250mM EDTA, 20mg.mL^{-1} leupeptin, 1/100 phenylmetylsulphonyl fluoride). The cells were carefully harvested from the 6-well plates using a cell scraper and transferred to 1.5mL eppendorf tubes, and incubated on ice for 45min. Then each sample was centrifuged at maximum (20.000 RCF) speed for 5 minutes at 4°C, and the supernatant was transferred to a new 1.5mL tube. Total protein was measured in Varioskan plate reader) (Thermo Electro Corporation) with pyrogallol red-molybdate complex method [22]. Loading buffer 2x was added in a proportion of 1:1 (sample:buffer) (2.5mL Tris/HCl pH 6.8, 4mL 10%SDS, 2ml glycerol, 1ml β-mercapto-ethanol, 0.5mL water, beads of bromophenol blue). After homogenizing, the tubes were placed in a heatblock at 100ºC during 10min. Aliquots of 20 µL from each sample were separated by SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) on 8% polyacrylamide gels using the BioRad

electrophoresis system. Proteins were electroblotted (BioRad blotting system) onto PVDF membranes (Immobilon, Millipore). Blots were blocked 1 hour in TBS (10mM Tris-HCl) containing 2% skim milk solution. Blots were then incubated overnight at 4°C with antibodies recognizing AT1 receptor (polyclonal anti-AGTR1, Sigma) and monoclonal mouse anti-CD39 in 5ml TBS containing 0.05% Tween 20 (TBS-T 0.05%) supplemented with 1% skim milk. After washing with TBS-T, secondary probing was done with a 60 minutes incubation with peroxidase-labeled anti-rabbit IgG (Garpo; Dako) and rabbit anti-mouse IgG (Rampo; Dako) antibodies respectively (both at 1:1000) diluted in 5 mL of TBS-T plus 1% skim milk. Blots were washed and membrane-bound antibodies were visualized using Bio-Rad method as follows: Two tubes were prepared, one containing 20 ml of 0.1M Tris (pH 8.5) plus 12.5 µL of 30% H_2O_2, and the other containing 20 ml of 0.1M Tris (pH 8.5) plus 200 µL of 250 mM luminol and 88 µL of 90 mM p-coumaric acid. The tubes were then mixed and blots were incubated for 1 minute with this solution before developing. The density of the respective bands was quantified by densitometric scanning of the blots using a Chemidoc MP Imaging system and Image Lab analyzer software (BioRad). Then, the blots were washed with TBS-T, and the antibodies were incubated for 1 hour in stripping buffer (0.94g glycine, 50ul 10% SDS, 500mL water, pH 2.0 with 1N HCl). After washing again with TBS-T, the same blots were incubated with beta-actin (1:2000, Santa Cruz Biotehcnology) for 1 hour, used as an internal standard. After washing with TBS-T, secondary probing was done with a 60 minutes incubation with peroxidase-labeled anti-rabbit IgG at a dilution of 1:1000 in 5 mL of TBS-T plus 1% skim milk, Garpo; Dako). Detected bands were quantified and normalized for the beta-actin signal on the same blot. Protein levels were expressed as a ratio between the mean intensity of CD39 or AT1R and β-actin bands to avoid inaccuracies in protein loading.

2.4.3. Immunohistochemistry

Cytospins were prepared from endothelial cells, and stained for either CD39 or AT1R expression according to standard procedures. Cytospin preparations were fixed with icecold acetone (100%; room temperature) for 10 minutes, air dried and subsequently stained for CD39 using monoclonal antiapyrase antibody at a dilution of 1:150 for 60 minutes, and peroxidase-conjugated goat antimouse IgM (GaM/IgM/po; Pierce), as a second step, at a dilution of 1/50 for 30 minutes; or for AT1R, using monoclonal anti AT-1 IgG (clone TONI, Abcam) at a dilution of 1:75 for 60 minutes, peroxidaseconjugated rabbit antimouse IgG (Rampo; Dako) as a second step at a dilution of 1:50 for 30 minutes, followed by goat antirabbit IgG

conjugated with peroxidase (Garpo; Dako) as a third step at a dilution of 1:100 for 30 minutes. Endogenous peroxidise activity was blocked by addition of 0.3% of H2O2 in PBS for 30 minutes, between the first and the second antibodies. The reaction product was visualized using 3-amino-9-ethyl-carbazol (AEC) according to standard methods. The sections were counterstained using hematoxylin.

2.5. Reactive Oxygen Species (ROS) production

In order to evaluate the production of ROS in PBMC, the PBMC containing supernatant previously separated from the co-cultures was washed (x2) and the cells were labelled with dihydrorhodamine 123 (DHR, Invitrogen) for 60 min in a water bath at 37ºC. Cells were then washed again (2x) and resuspended in PBS/BSA. Unlabelled cells were used as negative controls. ROS production as reflected by the DHR signal was detected using the software program described under flow cytometry.

2.6. Statistics

Flow cytometry and immunohistochemistry statistical analysis were performed using Mann Whitney test. Western blot statistical analysis was performed using ANOVA followed by Dunnett's multiple comparison test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. CD39 and AT1R expression

3.1.1. Flow cytometry

The expression of CD39 on endothelial cells after co-culture with PBMC is shown in figure 1A. As it can be observed, an increase from 0.5% to 3.0% of salt presents significant lower mean expression of CD39, as detected by flow cytometry. Figure 1B, shows the mean expression of AT1R after co-culture with PBMC in standard versus high salt medium. Significantly decreased mean expression of AT1R after increasing the salt content of the medium with 0.5%, 1.0% or 2.0% can be seen.

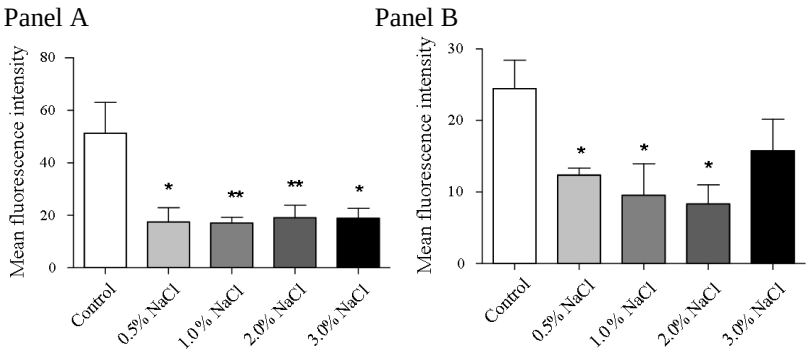


Figure 1. Panel A. Expression of CD39 on endothelial cells as measured by flow cytometry. Figure 1B. Expression of AT1R on endothelial cells as measured by flow cytometry. Columns represent mean expression of CD39 and AT1R after co-culture with PBMC in standard medium (154.00 mmol.L^{-1} NaCl) or medium supplemented with salt (0.5-3.0% increase). Bars represent mean ± SEM; n= 6-10; *p<0.05, **p<0.01 vs standard medium.

3.1.2. Western Blot

Figure 2 shows protein expression of CD39 and AT1R on endothelial cells after treatment, as measured by Western blot. In figure 2A, total protein expression of CD39 on endothelial cells was significantly down-regulated in the presence of 2% and 3% of salt, as it is shown in panel A (*p<0.05). Figure 2B shows the total protein expression of AT1R, where a slight tendency towards down-regulation is observed, although it is not significant.

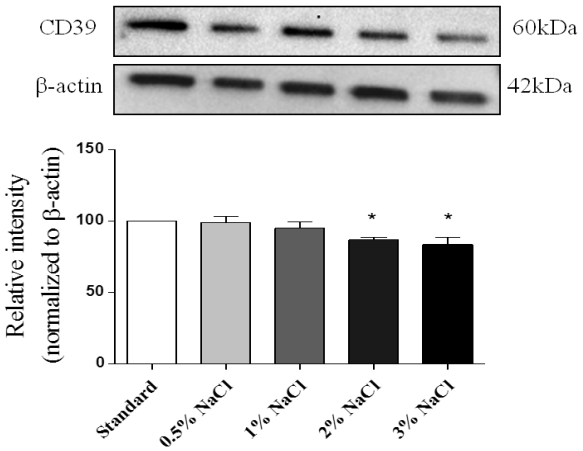


Figure 2A. Protein expression of CD39 on endothelial cells as measured by Western blot. Columns represent mean expression of CD39 after co-culture with PBMC in standard medium (154.00 mmol.L^{-1} NaCl) or medium supplemented with salt (0.5-3.0% increase). Bars represent Mean ± SEM; n= 5; *p<0.05 vs standard medium.

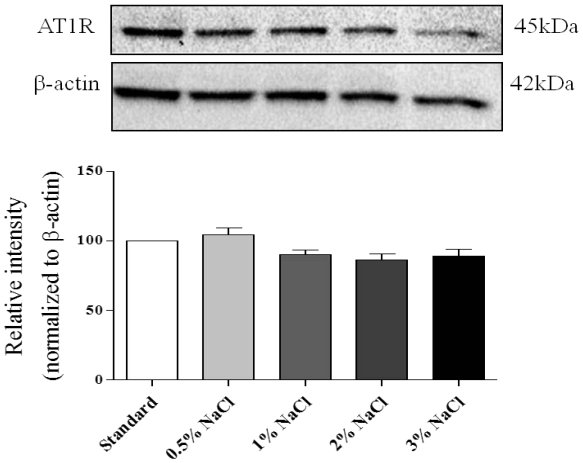


Figure 2B. Protein expression of AT1R on endothelial cells as measured by Western blot. Columns represent mean expression of AT1R after co-culture with PBMC in standard medium (154.00 mmol.L^{-1} NaCl) or medium supplemented with salt (0.5-3.0% increase). Bars represent Mean ± SEM; n= 6.

3.1.3. Immunohistochemistry

Immunostaining in cytospins of endothelial cells following co-culturing with PBMC in high salt (from 0.5% to 3.0% increase) versus standard medium, presents decreased expression of CD39 and AT1R as showed in figures 3 and 4. In figure 3B, clear reduction of stainability for CD39 is shown in cells cultured in medium with increased salt content (1.0% NaCl) versus standard medium which shows more reaction product (Figure 3A). Figure 4B shows a decreased expression of AT1R after culture with high salt (1% NaCl) versus standard medium (Figure 4A).

Panel A Panel B

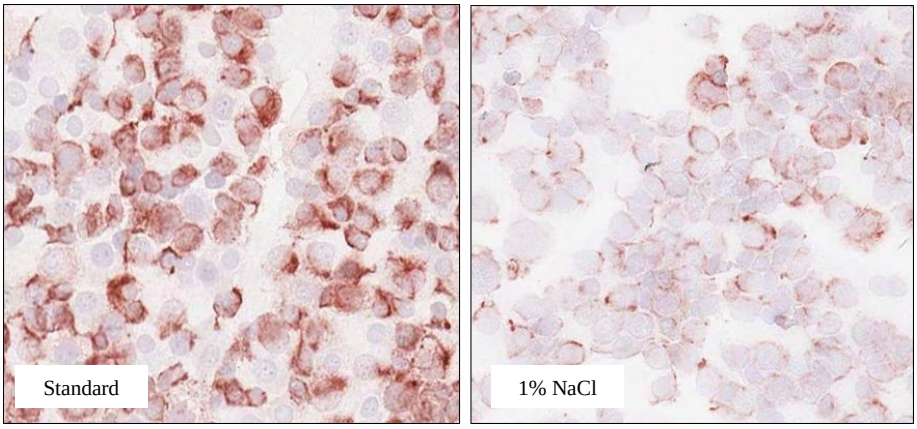
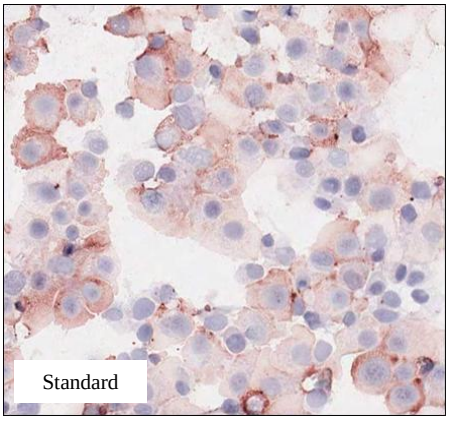


Figure 3A. Endothelial cells following co-culture with PBMC in standard medium after immunostaining for CD39. It can be seen that reaction product in cytoplasm as well as along cell membranes is present in most of the cells. Final magnification x 200. Figure 3B. Endothelial cells following co-culture with PBMC in medium supplemented with salt (1.0% increase) after immunostaining for CD39. It can be seen that a clear reduction of reaction product occurs as compared with standard medium (fig 3A). The standard method 3-amino-9-ethyl-carbazol (AEC) was used. Final magnification x 200.

Panel A



Panel B

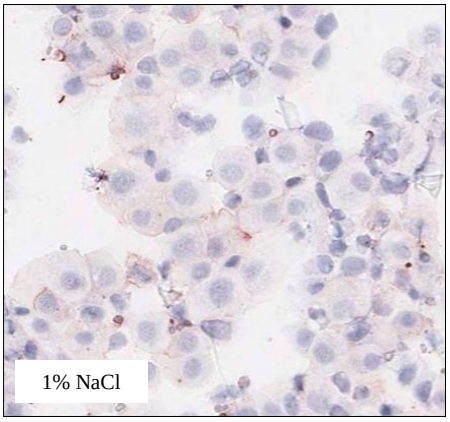


Figure 4A. Endothelial cells following co-culture with PBMC in standard medium after immunostaining for AT1R. It can be seen that in most cells reaction product is present. Final magnification x 200. Figure 4B. Endothelial cells following co-culture with PBMC in medium supplemented with salt (1.0% increase) after immunostaining for AT1R. It can be seen that a clear reduction of reaction product occurs as compared with standard medium (Fig 4A). The standard method 3-amino-9-ethyl-carbazol (AEC) was used. Final magnification x 200.

3.2. Reactive Oxygen Species production

In order to evaluate the production of ROS by PBMC after co-culturing with endothelial cells and various increments of salt into the medium, we analyzed the mean fluorescence intensity in these cells using flow cytometry. It can be seen in Figure 5 that, after salt is added to the culture medium, a mean increase of ROS in PBMC occurs, as compared to standard medium, in particular after increasing the salt content of the medium with 1% of NaCl. Although 0.5%, 2.0%, or 3.0% salt increase seems also to induce a mean increase of ROS production, these differences were not statistically significant as compared to standard medium.

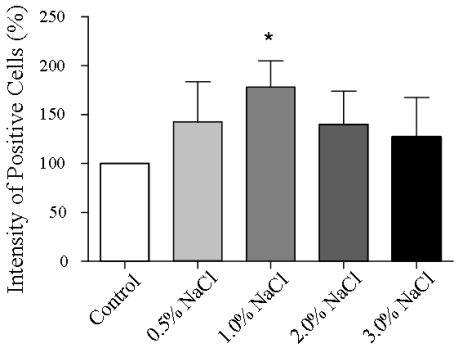


Figure 5. Staining for ROS in PMBC using flow cytometry, with dihydrorhodamine as a label. Columns represent mean staining for ROS in PBMC after co-culture with endothelial cells in either standard medium or medium supplemented with salt (0.5-3.0% increase). It can be seen that 1% increase of salt induces a significant increase of ROS generation as compared with standard medium. Bars represent mean % ± SEM; n= 5-8; *p<0.05 vs control (standard medium).

4. Discussion

The major observation of the present experiments is that minor increments of salt in the culture medium caused significant alterations along the endothelial cell surface i.e. loss of CD39 (fig. 3B) and AT1R (fig. 4B).

In the present in vitro study, where we used a concentration range of salt increase (0.5%-3%), no dose response could be observed (figs. 1A and 2A) for CD39 expression. The reason for this is obscure. Since it has been shown that CD39 is highly sensitive for oxidant stress[20, 23, 24], it is likely that ROS production induced by enhanced salt in the culture system causes the down regulation of endothelial CD39. Indeed, 1% NaCl increase causes a significant rise of ROS as compared with standard medium (fig. 5). Again no dose-dependency could be shown. The reason for this remains to be established but may be related with kinetics of ROS production as detected by the DHR 123 label and the incubation time. It is conceivable that a part of the ROS induced by the 2% salt increment has already diffused out of the cells before measurement occurs following overnight incubation. It is clear, however, that 1% increase of salt in the medium is associated with significant ROS production and CD39 affection in the present co-culture, which makes it highly likely that this type of endothelial injury is due to oxidative stress

caused by salt. This is in line with recent studies which demonstrated that salt may induce ROS mediated damage to the vessel wall in vivo[20, 21].

A rise of salt (0.5%-2%) in the culture medium also reduces endothelial expression of AT1R (fig.1B). An increase of 3% of salt showed no statistically significant drop of AT1R expression. The mean AT1R loss between the ranges of 0.5% to 2.0% of salt increment shows a tendency to a dose-dependent response, as observed in both flow cytometry (fig. 1B) and western blot analysis (fig. 2B). Reduced expression of AT1R on endothelial cells after increasing the salt content of the medium up to 1.0% was also shown by immunostaining on cytospins (fig. 4B). Whether AT1R down regulation due to increased salt content of the medium is due to either a direct effect of sodium upon the cell membrane, or reduced AT1R production reflected by a reduced mRNA signal for this receptor, cannot be concluded from the present data.

However, it is likely that loss of AT1R in this particular in vitro model is also a result of oxidant injury, as seems the case for diminished CD39 expression. This is in line with data from Nickering *et al.*, who found ROS dependent down regulation of AT1R in vascular smooth muscle cells[25]. In addition, it was recently found that small increases of sodium resulted in another form of endothelial injury i.e. endothelial stiffness, although this endothelial alteration in vitro appeared dependent of aldosterone present in the culture medium[3]. The suggestion of these authors that small changes in plasma sodium concentration (possibly in cooperation with other unknown factors) can affect endothelial function supports also our present data.

Thus, slightly increased salt concentrations affect an important endothelium protecting ecto enzyme i.e. CD39, which prevents local proinflammatory and proaggregatory reactions which can be induced by extracellular ATP and ADP[6]. Down regulation of endothelial CD39 in vivo greatly affects endothelial function as also observed in diseases with endothelial dysfunction like preeclampsia, characterized by hypertension and proteinuria[15, 26].

Loss of AT1R from endothelial cells may lead to vascular non-responsiveness to angiotensin II, resulting in vasodilatation. This mechanism has been suggested in healthy pregnancy (after week 10). In this condition, expansion of the vascular bed occurs concomitantly with loss of endothelial AT1R due to enhanced activity of plasma hemopexin[27]. However, as the interaction of AT1R with angiotensin II may also induce NO release through eNOS activation, it is conceivable that downregulation of endothelial AT1R by salt may promote increase of blood pressure as well[16]. It is clear that extrapolation of the present data to the in vivo situation is complicated by the fact that besides endothelium, the vessel wall also contains vascular smooth muscle cells carrying AT1R[28].

In conclusion, we feel that loss of endothelial CD39 and AT1R due to small increase of sodium, mediated by ROS production may reflect significant endothelial injury which in vivo potentially may result in alterations in vascular tone. Although it has been suggested recently that small increases of plasma sodium may be associated with hypertension[1], the potential contribution of loss of endothelial CD39 and AT1R to salt dependent hypertension, requires further investigations in vivo.

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Summary, General Discussion & Future Perspectives

Salt in health and disease

The body's fluid balance is influenced by two primarily variables: water and sodium. Salt is an essential nutrient, since the body cannot produce it by itself. It is a requirement for good health[1]. Sodium is involved in muscle contraction, nerve impulses. Sodium is also the major extracellular electrolyte responsible for regulating water balance, pH, and osmotic pressure. Angiotensin and aldosterone adjust the system in the event of consumption of insufficient amounts of salt, which would threaten the body's nerves and muscles and interfere with the sodium-potassium pump that adjusts intra- and extra-cellular pressures[2].

For several million years humans consumed a diet containing less than 1g of salt per day, which means that humans are genetically programmed to this amount of salt intake. However, with the development of agriculture and farming, salt was utilized to store food for prolonged periods of time, leading to an increase of salt intake. High sodium ingestion has been related to hypertension, stroke, coronary heart disease and renal fibrosis[3-5]. Although the deleterious effects of a high sodium intake are obvious, the underlying mechanisms are still not resolved, especially regarding their role in kidney damage.

Models of sodium overload

In order to evaluate the consequences of the excessive salt intake, we utilized several models of sodium overload. We first tested the *in vivo* effects of sodium excess in normal rats, in a chronic model using a high salt diet, and in an acute model by administering high salt both in anaesthetized and unanaesthetized animals. As an *in vitro* model, we used endothelial cells exposed to high salt.

It is known that excess intake of salt increases water and more sodium retention[6]. To investigate the role of sodium excess in water retention, we studied the expression of the main water channels: aquaporin-1 (AQP-1) and aquaporin-2 (AQP-2) (Water Balance). High extracellular sodium concentration also constitutes a challenge to normal cell function. Renal acute inflammation initiated by sodium overload, suggests an active participation of sodium and hyper-osmolarity in the pathogenesis of the inflammatory process[7-11]. We analyzed the expression of inflammation markers in models of acute and chronic sodium overload (Inflammation Markers).

We also evaluated the role of tubular Ang II-induced AT1 receptor (AT1R) activation by using the AT1R blocker losartan. Furthermore, we tested the participation of the oxidative stress and NO in the development of renal pro-

inflammatory response, by administration of a scavenger of superoxide radicals, tempol (SOD mimetic)[12].

Water Balance

In the high salt diet model (Chapter 1), we demonstrated that chronic administration of an excess of salt down-regulates AQP-1 and AQP-2 in proximal tubules and collecting ducts respectively. We observed that blockade of AT1R prevents the increase in Angiotensin II (Ang II) staining and the decrease of endothelial nitric oxide synthase (eNOS) staining. It also augments AQPs expression and promotes urinary sodium concentration. It has been suggested that Ang II plays a role in AQPs down-regulation, and that this is clinically associated with the inability of the kidney to concentrate the urine[13-15]. In addition, the activation of a cGMP-dependent pathway, mediated by different cGMP pathway activators, such as atrial natriuretic peptide (ANP), L-arginine and nitric oxide, results in AQP-2 insertion to the cell membrane[16-17]. Therefore, we hypothesize that the decrease of eNOS expression (and thus less nitric oxide) elicited by elevated intrarenal Ang II levels, could be responsible for AQP-2 down-regulation in the renal tubules of these animals, and that losartan administration could exert an antidiuretic effect through Ang II–AT2–nitric oxide–cGMP signalling pathway.

On the other hand, anaesthetized rats under acute sodium overload or unanesthetized rats subjected to the i.p. administration of a hypertonic saline solution without access to water, exhibited unchanged or decreased expression of these AQPs (Chapter 2). Therefore, our results demonstrate that sodium overload could be involved in an early down-regulation of AQP-2 expression in the collecting ducts. It is known that nuclear factor-kappaB (NF-κB) modulates AQP-2 expression by binding to its promotor[18]. As we have shown in Chapters 1, 2 and 3, there is an imbalance between Ang II (increased) and eNOS (decreased) expression in sodium overloaded rats. These two conditions activate the NF-κB transcription factor which was elevated in our experiments and in accordance to literature[19, 20]. In order to investigate the participation of Ang II-NF-κB signal on AQPs expression, we studied the effects of the inhibition of Ang II by losartan or the oxidative stress by tempol (Chapter 2), as discussed in the section "Inflammation markers". Our results indicate that the Ang II-oxidative stress-NF-κB pathway could be a repressor mechanism to diminish AQP-2 expression in rats under high sodium[21]. Alternatively, a recent report showed that the hypertonicity-induced activation of the NFATc factor may also increase AQP-2 transcription. In this order, it has been demonstrated that NO can enhance

the nuclear import of NFATc and decrease its export via PKG by NO signalling[22]. In agreement with this finding, our data show that sodium overload lowered eNOS expression, probably lowering NO production, while losartan and tempol administration caused a marked increase in eNOS expression.

Renal function and Mean Arterial Pressure

Following the study of water channels in a high sodium condition, we evaluated arterial pressure and renal function. In the acute model of sodium overload in anaesthetized normal rats (Chapters 2 and 3), the animals showed no significant alterations in the mean arterial pressure (MAP) and exhibited hyperfiltration, increased diuresis, natriuresis, and preserved glomerular function and tubular morphology. Both, tempol and losartan did not increase blood pressure and glomerular filtration rate (GFR) but produced greater sodium fractional excretion, suggesting that they are predominantly involved in sodium tubular transport.
A high salt diet in normal rats during three weeks produced a slight but significant increase in MAP and urinary sodium excretion (Chapters 1, 4 and 5). Our studies showed that losartan exerted a natriuretic effect at the tubular level, independently of its hypotensive effect and of GFR changes. It is well documented that renal AT1R, via activation of renal sodium transporters, produce antinatriuretic effects[23], whereas AT2R activation results in rise of urinary sodium excretion through the nitric oxide-cGMP pathway[24-25] as commented in the previous section (Water Balance). Finally, tempol improved renal hemodynamic and electrolyte excretory function in this model of high salt diet. It normalized MAP levels observed in the high salt group, and showed a potent diuretic and natriuretic effect, as we could also see in the acute model. We conclude that in every case, losartan and tempol could regulate blood pressure by improving sodium excretion.

Inflammation markers

In Chapters 2 and 3, we showed an early renal expression of inflammatory markers, such as Ang II, NF-κB, transforming growth factor-beta1 (TGF-β1), alpha-smooth muscle actine (α-SMA) and RANTES, in rats submitted to acute sodium overload. This was associated with the simultaneous decrease in eNOS expression and increase in hypoxia inducible factor-1alpha (HIF-1α) expression, suggesting greater oxidative stress and hypoxia, respectively. We tested the effect of tempol and losartan on the expression of the aforementioned markers. Tempol showed an anti-

inflammatory effect accompanied by an antioxidant action. On the contrary, losartan did not prevent the over-expression of inflammatory markers in renal tubules. Therefore, superoxide (O^{-}_{2}) up-regulation, as well as NO lower availability, may contribute to stimulate TGF-β1 production. RANTES, a pro-inflammatory chemokine, was over-expressed in collecting ducts but not in proximal tubules in the acute model (Chapter 3). Under a high salt diet (Chapters 1, 4 and 5), TGF-β1 expression increased in the distal nephron but not in proximal tubules, whereas Ang II showed the opposite pattern. Tempol and losartan administration prevented these changes. It seems that the profibrogenic response bears no association with Ang II localization. Recently published data have shown that, despite the positive-feedback loop between TGF-β1 and nitric oxide under physiological conditions, the inhibitory effect of nitric oxide on TGF-β1 production was reduced after salt intake in Dahl salt sensitive rats, supporting our results[26-27]. We hypothesize that 1) the profibrogenic response (showed by α-SMA and TGF-β1 enhanced staining) observed in our experiments could be related to the disbalance of oxidative stress and nitric oxide, and not to renal Ang II expression, and that 2) the role of elevated levels of Ang II observed in proximal tubules of animals fed with a high salt diet could be to regulate sodium transport. In this way, Ang II could stabilize nephron functions, avoiding a sodium overload to distal segments.

In Chapter 5, we evaluated the expression of ANP, a hormone that increases sodium excretion and decreases blood pressure and blood volume, in animals under high salt diet. It has been demonstrated that the activation of ANP/cGMP/PKG signalling pathway disrupts TGF-β1-induced downstream events, including myofibroblast transformation and the proliferation and expression of extracellular matrix molecules[28-29]. In our experiments, the increase in renal ANP levels coincided with TGF-β1 over-expression in glomeruli, thick ascending limb and cortical collecting ducts. These results suggest that the elevated expression of ANP in these animals could constitute a protective counter-regulatory mechanism against anti-natriuretic and/or pro-fibrotic actions of TGF-β1. In addition, the plasmatic concentration of ANP was not affected by dietary salt, suggesting that these animals did not change cardiac ANP secretion[30]. Renal ANP expression levels are independent of circulating ANP levels, which may be subjected to a different regulation. In accordance with our findings, it has been demonstrated that dietary salt supplementation may selectively increase ANP levels in the kidney by down-regulating its clearance receptor (NPR-C)[31]. One of the endothelium protecting enzymes in inflammation is the E-NTPDase, which converts the pro-inflammatory ATP to ADP released by damaged cells. Thus in Chapter 6, we tested the effect of

small increases of salt in vitro on the expression of endothelial AT1R and E-NTPDase by co-culturing an endothelial cell line with peripheral blood mononuclear cells (PBMCs). These experiments showed that minor increments of salt in the culture medium caused significant alterations along the endothelial cell surface, where AT1R and E-NTPDase where down-regulated and ROS production by the PBMCs was increased. This is in line with recent studies which demonstrated that salt may induce ROS mediated damage to the vessel wall in vivo and in vitro[32-36]. According to our results, it is likely that loss of AT1R in this particular in vitro model is a result of oxidant injury and may lead to vascular non-responsiveness to angiotensin II in endothelial cells, resulting in vasodilatation as it has been shown[37]. Down regulation of endothelial E-NTPDase in vivo greatly affects endothelial function as is also observed in diseases with endothelial dysfunction like preeclampsia, characterized by hypertension and proteinuria[38-39].

In conclusion, an acute or chronic sodium overload is able to regulate the expression of AQP-1 and AQP-2 and thus the water balance through intrarenal Ang II and oxidative stress. Early renal inflammation produced by acute sodium excess in non-physiological concentrations (2hs) is a process that can be prevented and/or reversed. Chronic inflammation may be reversible in the absence of histopathological lesions. Inflammation by sodium is linked to ROS (due to flux, stretch and/or transport) more tan to tubular Ang II, which could be more related to sodium and water transport. Triggering of the cascade of inflammatory events is due to an increase of the cellular work when it reabsorbs sodium, which produces relative hypoxia (HIF-1α), increase of renal Ang II and NF-kB expression and develops oxidative stress. This cascade can be prevented or reverted by cutting the chain in any of its links: a) decreasing sodium reabsorption (ANP), b) inhibiting local Ang II (losartan) or c) the oxidative stress (tempol).

Future Perspectives

In this thesis, we studied the deleterious effects of sodium through oxidative stress and inflammation on renal damage at the tubular level (studies *in vivo*). Studies *in vitro* were performed as well; testing vascular damage elicited by sodium at the endothelial level. Future experiments will include functional studies of vascular reactivity to demonstrate that salt can induce ROS in the vascular wall by stretching, and how this can affect the regulation of eNOS in endothelial and vascular smooth muscle cells. Moreover, we would like to study how this response can be potentiated by exogenous Ang II. The functional tests can be performed by measuring isometric tension in aortic rings placed in a perfusion bath with different testing drugs, and designing dose-response curves. The participation of NO and oxidative stress can be studied as regulators of vascular reactivity in function of 1) vascular stretching (similar to the stretch produced by increase of arterial pressure), 2) sodium concentration in the extracellular medium, and 3) of the combination of both. Also, several inhibitors can be used, such as: oxidative stress inhibitors (tempol or apocynine), inhibitors of AQP-1 as channel for NO from endothelium to the smooth muscle cells ($HgCl_2$), neutral endopeptidase inhibitors (omapatrilat) to increase the availability of endogenous ANP.

Furthermore, the ability to selectively up-regulate or inhibit AQP functions in specific tissues may lead to novel therapeutic approaches for human disease. Our current knowledge of AQPs' pore structure and function should permit the development of designer drugs that can target specific AQPs for clinical use. Small-molecule modulators of AQP expression/function could have clinical applications in the therapy of congestive heart failure and hypertension, cytotoxic and vasogenic brain edema, epilepsy, obesity, cancer, glaucoma and other conditions. AQP inhibitors, called aquaretics, could reduce urine concentration, producing a diuresis with a higher water/salt ratio, beneficial for example in severe congestive heart failure, where conventional salt-blocking diuretics are of limited efficacy[40].

There are controversial studies regarding the administration of antioxidants to prevent, for example, cardiovascular diseases. According to the American Heart Association, there is not enough evidence to whether they should recommend the administration of antioxidants to prevent the reduction of disease risk. Small studies have indicated advantages and some of them have even shown adverse effects. Clinical trials have not yet demonstrated conclusive assertions[41]. The benefit of antioxidants, in particular the nitroxides such as tempol, lays not only in differences of age, gender, origin and disease. It also depends on the dose and exposure time,

since above certain concentrations, they can be oxidants and in some cases toxic[42]. Further research of the pathophysiology of oxidative stress and the antioxidant therapy will hopefully lead to better designed clinical trials which will result in a better antioxidant therapy.

On the other hand, it would be useful to study the antioxidant capacity of endogenous atrial natriuretic peptide (ANP) locally produced for example in the aorta, utilizing an in vitro model of endothelial and smooth muscle cells or proximal tubular cells, to test its expression under the influence of oxidants and to determine ROS generation when the endogenous production of ANP is inhibited. Moreover, the pharmacological potentiation of endogenous ANP may be a therapeutic approach for the management of oxidative stress in salt-sensitive hypertension.

It is generally admitted that a functional negative crosstalk between AT1 and AT2 receptors exists in several pathophysiological conditions including hypertension[43]. AT2 receptor activation may directly antagonize AT1R-mediated actions by forming heterodimers with AT1R. Therefore, the role of AT1R blockers such as losartan could be further studied in order to find new beneficial effects other than the known anti-hypertensive properties. Moreover, in vitro experiments are needed to clarify the molecular mechanism of damage of sodium to discover the potential contribution of loss of AT1R and endothelium protective E-NTPDase to salt dependent hypertension.

A better understanding of the mechanisms involved in the regulation of water and sodium handling, especially when the body is exposed to an excess of salt, would lead to improved prevention of renal and cardiovascular diseases and would increase treatment efficacy.

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Nederlandse Samenvatting

Natrium en chloride zijn de twee elementen die samen natriumchloride (zout) vormen. In het lichaam bevindt natrium zich vooral in het bloedplasma en in de vloeistof buiten de cellen. Natrium speelt een rol bij het ontspannen van de spieren, waaronder ook de hartspier. Het neemt deel aan de overdracht van zenuwimpulsen en helpt om de bloeddruk te reguleren. Omdat zout een onderdeel van ons dieet is, komt natrium deficiëntie maar zelden voor. Langdurige diarree of braken kan echter leiden tot deze deficiëntie resulterend in misselijkheid, duizeligheid en spierkrampen. Bij gezonde mensen wordt de overmaat aan natrium uitgescheiden in de urine. Nierziekten kunnen echter natriumuitscheiding verstoren met als gevolg vochtretentie en oedeem. Tegenwoordig is wereldwijd de individuele gemiddelde zoutinname hoger dan de fysiologische behoefte. Voor mensen die gevoelig zijn voor zout, kan een natrium rijk dieet zorgen voor de ontwikkeling van zowel hypertensie als cardiovasculaire- en nierziekten.

Waterretentie veroorzaakt door een teveel aan natrium in het lichaam wordt voornamelijk gemedieerd door eiwit kanalen genaamd aquaporines (AQPs). Deze eiwitten reguleren de beweging van water over de celmembranen. De twee belangrijkste AQPs (AQP-1 en AQP-2) bevinden zich in de nier. Ongeveer 70% van ons lichaamsgewicht bestaat uit water en elke dag filtreren en reabsorberen onze nieren rond de 180 liter water. Daarom is een goede verdeling van het water nodig om de vochtbalans in de verschillende anatomische compartimenten te behouden. Bovendien heeft een renaal overschot aan natrium een ontstekingsreactie tot gevolg, welke retentie van natrium en water alleen maar meer bevordert. De moleculaire mechanismen van de ontstekingsreactie die ontstaat op zout gevoelige hypertensie is nog niet volledig uitgekristalliseerd. Verhoogde natrium reabsorptie vanuit de nierbuisjes leidt tot verhoogde doorbloeding van de nier en dus meer glomerulaire filtratie. Hyperfiltratie verhoogt de metabole vraag naar zuurstof, resulterend in een afname van de zuurstofspanning in nierweefsel (pO_2). Het verhoogde zuurstofverbruik leidt op zijn beurt tot een relatieve hypoxie. Dit laatste leidt tot een cascade van gebeurtenissen, welke de productie van vrije zuurstofradicalen (ROS) vergroot. Hierdoor ontstaat er een verhoogde expressie van pro-inflammatoire nucleaire transcriptiefactoren, zoals activator eiwit-1 (AP-1) en nucleaire factor–kappa B (NF-κB). NF-κB activeert genen die betrokken zijn bij de inflammatoire en fibrotische respons, waardoor de expressie van cytokinen, chemokinen en verwante hormonen toeneemt.

In het eerste deel van dit proefschrift, richten we ons op de regulatie van AQPs in de nieren en de veranderingen die zij ondergaan wanneer ze worden blootgesteld aan een overmaat aan natrium (hoofdstuk 1 en 2). In het tweede deel onderzoeken we het effect van de overmaat van zout op inflammatie en fibrose markers in verschillende in vivo en in vitro modellen (hoofdstuk 3-6). In de hoofdstukken 1, 4 en 5, hebben we gebruik gemaakt van een model waarbij normale ratten een chronische natriumarm dieet volgden, terwijl in de hoofdstukken 2 en 3, we acute zoutoverbelasting modellen bestudeerd hebben, zowel in in slaap gehouden als wakkere dieren. We hebben de rol van de signalering van het hormoon Angiotensine II in de niertubuli onderzocht door toediening van losartan, een remmer van de AT1 receptor. We hebben tevens gekeken naar de bijdrage van oxidatieve stress en stikstofoxide (NO) aan nierbeschadiging door ROS productie te remmen met tempol, mimetische of superoxide dismutase. Hierbij hebben we de bloeddruk en nierfunctie gemeten. Ook hebben we de intrarenale niveaus van aquaporines en grote ontstekingsmarkers zoals cytokinen, chemokinen en nucleaire factoren bepaald. In hoofdstuk 4 hebben we tevens de rol van hypoxie-induceerbare factor-1 alfa (HIF-1α) en atriaal natriuretisch peptide (ANP), welke door de nier gemaakt worden, geëvalueerd. Beiden blijken betrokken te zijn bij een adaptief mechanisme in reactie op chronische natrium overbelasting. In hoofdstuk 6 hebben we in vitro het directe effect van zout op het endotheel getest. We hebben gekeken naar de deelname van ROS in het mechanisme van schade in dit model, welke gemaakt werd van een co-cultuur met een monolaag van endotheelcellen en mononucleaire cellen in perifeer bloed (PBMC).

We concluderen dat acute of chronische natrium overbelasting, door Ang II en renale oxidatieve stress, de expressie van AQP-1 en AQP-2 kan reguleren en op die manier de waterbalans. Acute ontsteking veroorzaakt door een acute overmaat aan natrium is een proces dat kan worden voorkomen of omgekeerd. Ontsteking te wijten aan een natrium overschot is meer gerelateerd aan ROS dan aan tubulaire formatie van Ang II. De laatste is meer gerelateerd aan het transport van natrium en water. Chronische ontsteking kan reversibel zijn bij afwezigheid van histopathologische laesies. Activering van de inflammatoire cascade is te wijten aan verhoogde activiteit van cellen bij het reabsorberen van natrium, wat zorgt voor relatieve hypoxie (HIF-1α), verhoogde renale expressie van NF-kB door AngII en het ontwikkelen van oxidatieve stress. Deze cascade kan worden voorkomen of teruggedraaid door in te grijpen op één van de volgende manieren: a) het verminderen van de reabsorptie van natrium (door middel van ANP), b) het lokaal

remmen van Ang II (door middel van losartan) of c) door het inhiberen van oxidatieve stress (door middel van tempol). Bovendien kan zout schade aan de vaatwand te induceren. Met name in ons in vitro model kan het effect van de PBMC's via oxidatieve stress leiden tot het gebrek aan respons op Angiotensine II in endotheelcellen (door het verlagen van de expressie van de AT1 receptor) en een minder goede bescherming van het endotheel door de E-NTPDase, als gevolg van een lagere expressie van dit enzym.

Wanneer we de mechanismen die betrokken zijn bij het regelen van de zout en water balans beter begrijpen, vooral wanneer het lichaam wordt blootgesteld aan een overmaat aan zout, kan dit leiden tot betere preventie van renale en cardiovasculaire aandoeningen en daarnaast de effectiviteit van behandelingen vergroten.

Resumen en Español

El sodio y el cloro son los dos elementos que se combinan para formar cloruro de sodio (sal de mesa). En el organismo, el sodio se encuentra principalmente en el plasma sanguíneo y es el principal catión extracelular. El sodio es parte importante del potencial de acción de las membranas celulares, incluyendo las neuronas y el musculo, por lo tanto es vital para la neurotransmisión y la contracción muscular. De la entrada y salida de sodio y potasio se conforman los potenciales de acción tanto en la despolarización como en la repolarización, fenómenos eléctricos que suceden durante la contracción y la relajación. El sodio está involucrado en la transmisión de los impulsos nerviosos y contribuye a regular la presión arterial.

Debido a que la sal de mesa es una parte común de la dieta, es rara la deficiencia de sodio. Sin embargo, en las diarreas agudas y crónicas y en la deshidratación, la gran pérdida de sodio puede llevar a su deficiencia; lo que refleja en síntomas como náuseas, mareos y calambres musculares.

En personas sanas, el exceso de sodio es excretado, pero algunas enfermedades renales, sin embargo, interfieren con la excreción de sodio provocando retención de líquido y edema. En la actualidad el promedio individual de ingesta de sal en todo el mundo supera ampliamente las necesidades fisiológicas, y en personas que son sensibles a la sal, una dieta con alto contenido de sodio puede fomentar tanto el desarrollo de hipertensión arterial como enfermedades renales y cardiovasculares.

La retención de agua que se produce por exceso de sodio en el organismo, es mediada principalmente a través de canales proteicos llamados aquaporinas (AQPs), que son proteínas que regulan el movimiento de agua a través de las membranas celulares. Las dos principales AQPs (AQP-1 y AQP-2) se encuentran localizadas en el riñón. El agua constituye aproximadamente el 70% de nuestra masa corporal, y cada día nuestros riñones filtran y reabsorben alrededor de 180 litros de agua. Por lo tanto, se requiere una distribución apropiada de agua para mantener el equilibrio de fluidos dentro de los diferentes compartimentos anatómicos. Por otro lado, el exceso de sodio, además de favorecer directamente la retención de agua, produce una respuesta inflamatoria renal, que favorece indirectamente, aún más la retención de sodio y agua. Los mecanismos moleculares involucrados en la respuesta inflamatoria renal a la hipertensión sensible a la sal aún no se han caracterizado completamente. El aumento de la reabsorción de sodio en los túbulos renales retroalimenta incrementando el flujo de sangre renal y por lo tanto induciendo la hiperfiltración glomerular y mayor reabsorción de sodio, cerrando un círculo vicioso. Este proceso intensifica la demanda metabólica de oxígeno a nivel del túbulo renal, lo que resulta en una disminución en la tensión de oxígeno del tejido renal (pO2) debido al aumento de su consumo. A su vez, el aumento del consumo de

oxígeno conduce a una hipoxia relativa. Esta última, desencadena una cascada de eventos, magnificando la producción de especies reactivas del oxígeno (ROS) y posteriormente desencadenando un aumento de la expresión de factores de transcripción pro-inflamatorios nucleares tales como el activador de la proteína-1 (AP-1) y el factor nuclear kappa B (NF-kB). El NF-kB por su parte, activa genes relacionados con la respuesta inflamatoria y fibrótica, incrementando la expresión de citoquinas, quimioquinas y hormonas relacionadas con el proceso inflamatorio.

Nuestra hipótesis de trabajo fue que la sobrecarga de sodio por exceso de de su ingesta o provisión, desencadena a nivel tubular renal un proceso inflamatorio y profibrótico precozmente y anterior a la aparición de alteraciones funcionales glomerulares y/o histopatológicas detectables y desregular la expresión y localización de proteínas encargadas de la reabsorción de agua en el riñón. Por lo tanto el objetivo de la Tesis fue demostrar la existencia de ese proceso inflamatorio, evaluando la funcionalidad renal y a nivel molecular, la expresión de factores proinflamatorios y profibróticos en tejidos renales en ausencia de lesiones histopatológicas. Además se estudió la alteración de la expresión de las acuaporinas y la prevención/atenuación farmacológica de la inflamación renal por sobrecarga de sodio.

En la primera parte de esta tesis nos centramos en el estudio de la regulación de las AQPs en el riñón y las alteraciones que ocurren cuando los nefrones se exponen a un exceso de sodio (Capítulos 1 y 2). En la segunda parte (Capítulos 3 a 6), estudiamos los efectos del exceso de sal sobre marcadores de inflamación y fibrosis en diferentes modelos *in vivo* e *in vitro* En los Capítulos 1, 4 y 5 se utilizó un modelo con sobrecarga crónica en ratas normales mediante una dieta rica en sodio, y en los Capítulos 2 y 3 se usaron modelos de sobrecarga salina aguda tanto, en animales anestesiados como despiertos. Se evaluaron los efectos de la Angiotensina II sobre la función tubular y prevención y/o atenuación de la inflamación renal sodio dependiente, mediante la administración de un bloqueante de su receptor AT1R, losartán. También se evaluó la participación del estrés oxidativo y del oxido nítrico (NO) mediante la inhibición de la producción de ROS con tempol, un mimético de la enzima superóxido dismutasa. En el capítulo 6, se estudio *in vitro* el efecto directo del cloruro de sodio sobre el endotelio y la potencial participación de los ROS lesión endotelial. En este modelo, se utilizó un co-cultivo con una monocapa de células endoteliales y células mononucleares extraídas de sangre periférica (PBMC).

El análisis de los resultados obtenidos permite concluir que una sobrecarga aguda o crónica de sodio es capaz de regular la expresión de las AQP-1 y AQP-2 y el

balance de agua a través del aumento de síntesis de Angiotensina II (Ang II) intrarrenal y producción de estrés oxidativo.
La sobrecarga de sal produce la activación de una cascada de eventos inflamatorios, que se debe al aumento del trabajo celular cuando se reabsorbe sodio., esto produce una hipoxia relativa (expresada por el factor HIF-1α), con aumento de producción de Ang II renal y expresión de NF-kB, factor que conduce al desarrollo de estrés oxidativo. La inflamación puede ser prevenida y/o atenuada en ausencia de lesiones histopatológicas, cortando la cascada o cadena de eventos intracelulares en cualquiera de sus eslabones mediante agentes farmacológicos: a) disminuyendo la reabsorción de sodio (por el factor natriuréticos ANP), b) bloqueando a los receptores AT1 de la Ang II renal (con losartan) o c) inhibiendo el estrés oxidativo (con tempol).
Mientras que la inflamación temprana producida por un exceso de sodio en forma aguda, que es un proceso que puede ser prevenido o atenuado, está más relacionado con la producción de ROS (debido mayor flujo tubular renal, el estiramiento de los vasos y el aumento de transporte de sodio), el incremento de Ang II intratubular, estaría más relacionada con el aumento de transporte de sodio y agua.
Además, el exceso de sal puede inducir daño en la pared vascular. En nuestro modelo desarrollado *in vitro* en particular, el efecto producido por los PBMCs a través del estrés oxidativo, lleva a la ausencia de respuesta a la Ang II en las células endoteliales (por disminución de la expresión de sus receptores AT1R) y a una menor protección del endotelio por parte de la E-NTPDasa por menor expresión de esta enzima.
De esta manera, se cumplieron los objetivos de la tesis, comprobando la veracidad de la hipótesis de la misma y aportando conocimientos originales que contribuyen a comprender la fisiopatología del daño inflamatorio por exceso de aporte de sodio. Un mejor entendimiento de los mecanismos involucrados en la regulación del manejo del sodio y el agua, especialmente cuando el cuerpo está expuesto a un exceso de sal, puede colaborar a una mejor prevención de enfermedades renales y cardiovasculares, e incrementar la eficacia de los tratamientos.

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Publications

Silvana Lorena Della Penna, Pharmacist.

A slight increase of salt induces down regulation of CD39 and type-1 angiotensin II receptor in endothelial cells in vitro.
Silvana L. Della Penna, Theo Borghuis, Harry van Goor, Winston W. Bakker.
In preparation

Renal Overexpression of ANP and HIF-1α as Adaptive Response to a High Salt Diet.
Silvana L. Della Penna, Gabriel Cao, Elsa Zotta, Susana Gorzalczany, Carolina S. Cerrudo, Natalia L. Rukavina Mikusic, Alicia Correa, Verónica Trida, Jorge E. Toblli, María I. Rosón, Belisario E. Fernández.
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Effects of acute hypertonic sodium overload on the expression of aquaporins 1 and 2 in the rat kidney.
S Della Penna, G Cao, C Cerrudo, M Pandolfo, Andrea Fellet, Ana M. Balaszczuk, JE Toblli, MI Rosón, BE Fernández.
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