IMPAIRED PRESSURE NATRIURESIS RESULTING IN SALT SENSITIVE
HYPERTENSION IS CAUSED BY TUBULOINTERSTITIAL IMMUNE CELL
INFILTRATION IN THE KIDNEY

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Short Title: Renal inflammation and pressure natriuresis

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Abstract.

Immune cell infiltration of the kidney is a constant feature in salt sensitive hypertension (SSHTN). We evaluated the relationship between the renal inflammation and pressure natriuresis in the model of salt-sensitive hypertension that results from transient oral administration of NO-nitro-L-arginine methyl ester (L-NAME). Pressure natriuresis was determined in Sprague-Dawley rats that received 4 weeks of a high salt (4% NaCl) diet starting one week after stopping L-NAME that was administered alone (SSHTN group, n=17) or in association with mycophenolate mofetil (MMF group, n=15). The administration of MMF in association with L-NAME is known to prevent the subsequent development of SSHTN. Control groups received a high (n=12) and normal (0.4%) salt diet (n=20). Rats with SSHTN had increased expression of inflammatory cytokines and oxidative stress. The severity of hypertension correlated directly (p<0.0001) with the number of tubulointerstitial immune cells and angiotensin II-expressing cells. Pressure natriuresis was studied at renal arterial pressures (RAP) of 90, 110, 130 and 150 mmHg. GFR was similar and stable in all groups and renal blood flow was decreased in the SSHTN group. Significantly decreased natriuresis (p<0.05) was found in the SSHTN group at RAPs of 130 and 150 mmHg and there was an inverse correlation (p<0.01) between the urinary sodium excretion and the number of tubulointerstitial inflammatory cells (lymphocytes and macrophages) and cells expressing angiotensin II. We conclude that tubulointerstitial inflammation plays a key role in the impairment of pressure natriuresis that results in salt dependent hypertension in this experimental model.
Arterial hypertension is a major cause of morbidity and mortality worldwide. Salt sensitivity, characterized by greater than expected blood pressure changes in response to salt loading and restriction, is a feature of the majority of adult and elderly hypertensive patients (53) and is associated with renal tubulointerstitial inflammation in experimental models of hypertension (37, 38) and in primary hypertension in humans (11, 14, 49). The relevance of the renal inflammation in the pathogenesis of salt sensitive hypertension is underlined by the demonstration that treatments that suppress the renal inflammation result in amelioration or prevention of salt-driven hypertension (11, 38, 51).

It is assumed that the role played by renal inflammation is related to the impairment in the renal capacity to excrete sodium since the central event in the pathogenesis of salt-induced hypertension is impairment in the pressure natriuresis relationship (9). Pressure natriuresis refers to the increment in urinary sodium excretion that occurs when blood pressure rises, and is the consequence of complex modifications in renal interstitial hydrostatic pressure, medullary blood flow, nitric oxide, reactive oxygen species, prostaglandins and angiotensin II activity (8, 30, 36) that result in coordinated decreases in the surface distribution of apical sodium-hydrogen exchangers and basolateral Na-K ATPase activity (27). In salt sensitive hypertension the pressure natriuresis relationship is less steep and shifted to the right so that higher blood pressure levels are necessary to drive the natriuretic response required to maintain sodium balance (41).

Tubulointerstitial inflammation may impair urinary sodium excretion via several mechanisms. Inflammation may lead to a loss of peritubular capillaries, tubular injury and fibrotic interstitial changes. If these changes are severe and extensive they may cause salt wasting, but milder changes that do not render inoperative the sodium reabsorption
mechanisms are associated with salt-sensitive hypertension. This may be due to reduced oxygen delivery to regions of the kidney that function normally under relative hypoxia, causing a further reduction in pO2 with the generation of oxidative stress and impaired pressure natriuresis (16). In addition, the infiltration of immune competent cells in the renal interstitial space is associated with inflammation-induced generation of reactive oxygen species and increased local angiotensin II activity (7, 41, 42, 51) that suppress natriuresis.

The present investigations were directed to test the assumption that a consequence of tubulointerstitial inflammation is the blunting of the pressure natriuresis response. In these studies we selected an experimental model of salt-sensitive hypertension that follows recovery from nitric oxide synthase inhibition with \( N_\omega \)-nitro-L-arginine methyl ester (L-NAME) (34). In this model, three weeks of L-NAME administration cause hypertension and significant renal injury and inflammation during the period L-NAME is given. Blood pressure returns to normal one week after L-NAME is omitted but if a high salt diet is subsequently administered, hypertension develops in the following weeks. Treatment with mycophenolate mofetil (MMF) during L-NAME administration does not modify the blood pressure elevation due to inhibition of nitric oxide synthase, but ameliorates renal histological damage and inflammation caused by L-NAME and prevents the subsequent development of salt-sensitive hypertension (34). Important for the selection of this model in the present experiments are the findings that the histological damage that could, by itself, cause impairment in sodium excretion, improves after L-NAME is discontinued and several weeks later the LNAME-treated groups (with or without MMF treatment) present mild and comparable renal injury. At this time, the salient feature is that salt-induced hypertension is
associated with tubulointerstitial infiltration of immune cells, a characteristic that is common to most, if not all models of salt-sensitive hypertension (6, 34, 37-40, 42, 43).

MATERIAL AND METHODS

Male Wistar rats (250-320g) were housed in institutional animal facilities with free access to food and water. All studies were performed in accordance with the Mexican Federal Regulation for Animal Experimentation and Care (NOM-062-ZOO-2001) and the investigation was approved by Bioethics and Investigation Committees of Instituto Nacional de Cardiología Ignacio Chavez. The following experimental groups were studied:

1) Salt sensitive hypertension group (SSHTN group, n=17), that received L-NAME (Sigma, St. Louis, MO) in the drinking water at a concentration of 70 mg/100 ml for 3 weeks, followed, after a washout period of one week and return to normal blood pressure, by the administration of a high salt diet (4% NaCl diet, Harlan Teklad, Madison, WI) for 4 weeks.

2) Mycophenolate mofetil group (MMF group, n=15) received L-NAME as in the SSHTN group but, in addition, received MMF (CellCept, Roche Pharmaceutical) by gastric gavage in daily doses of 20 mg/kg during the initial 3 weeks of the study (nitric oxide synthesis inhibition) and then stopped, so than this drug was not given during the subsequent 5 weeks that preceded the pressure natriuresis studies (1 week of washout and 4 weeks of a high salt diet). MMF is relatively insoluble in water and therefore was suspended in water by vigorous agitation before administration as in previous studies (38, 44). Control groups received a high (4% NaCl) salt diet (C-HSD, n=12) and normal (0.4% NaCl) salt diet (C-NSD, n=20).
Additional groups of rats were studied to assess the renal injury resulting from L-NAME administration. In these studies, rats that received L-NAME (n=6), and L-NAME +MMF (n=7) were sacrificed at the end of 3 weeks of L-NAME administration and renal histology and immunohistology were examined. Control rats (n=5) were studied at the same time. All these rats were receiving a normal salt diet.

Finally, while MMF was given only during the L-NAME administration and therefore had been stopped 5 weeks after the pressure natriuresis studies (1 week of wash out and 4 weeks of high salt diet), the possible lingering effects of MMF administration on the pressure natriuresis were evaluated in separate experiments done in normal rats studied 4 weeks after they received MMF for 3 weeks. These studies showed a normal pressure natriuresis response (data not shown).

**Pressure natriuresis experiments.** Pressure natriuresis experiments were performed after 4 weeks of a high salt diet as described by Wang et al (52). Rats were anesthetized with pentobarbital sodium (30 mg/kg intraperitoneally) and placed on a surgical table with temperature control to maintain body temperature at 37°C. A patent airway was maintained with a PE-240 tube inserted in the trachea. A catheter in the left jugular vein was used for fluid administration and a catheter inserted in the left femoral artery to measure arterial pressure that was continuously monitored with a pressure transducer (model P23LX; Gould Hatto Rey, PR) and recorded on a polygraph (Grass Instruments, Quincy MA) as well as for take blood samples. The left kidney was exposed and placed in a lucite holder, covering the kidney surface with Ringer solution. A 2 mm flow probe was placed around the left renal artery for measuring renal blood flow (RBF) by an electromagnetic flow meter (Model T106, Transonsics System Inc. Ithaca NY) in 7-10 rats.
of each experimental and control groups. An adjustable aortic clamp was placed above the left renal artery and used to control distal blood pressure. In order to obtain data at renal arterial pressure (RAP) of 150 mmHg in normotensive rats (7 rats from the C-NSD group, 5 rats of the C-HSD group and 6 rats of the MMF group), the blood pressure was elevated by transient occlusion of carotid arteries (1) 20 minutes before to begin the first pressure-natriuresis evaluation and was maintained for the rest of the experiment. Progressive reduction in renal arterial pressure in steps of about 20 mmHg was maintained at a stable level by means of the aortic clamp, urine and blood collections were obtained at 150, 130, 110 and 90 mmHg. The left ureter was catheterized for urinary collections. Rats were maintained euvoletic by infusion of 10 ml/kg body wt of isotonic rat plasma during surgery, followed by an infusion of 10% polyfructosan (Inutest, Laevosan-Gesellschaft, Austria) in 0.9% sodium saline solution, at a 2.2 ml/h rate and replacement of blood samples obtained for analysis. The measurements were started after 1 hour of stabilization. After 10 minutes equilibration at each blood pressure level, 30-minute urinary collections were obtained for analyses. Plasma samples were obtained at mid point in urinary collections. At the end of the experiment the kidneys were removed, weighted and harvested and the animals were sacrificed by a pentobarbital overdose and the kidneys were weighed and harvested for histology and immunohistology studies.

**Chemical analyses.** Blood and urine samples were collected for determination of glomerular filtration rate, urinary sodium excretion and fractional excretion of sodium. Urine volume was determined gravimetrically. Polyfructosan concentrations were determined as described by Davidson and Sackner (4) and sodium concentrations by flame photometry.
**Histology and immunohistology.** All histological evaluations were done without prior knowledge of the experimental group being studied. Histological studies were done in kidney sections of 9 rats of the C-NSD group, 8 rats from the C-HSD group, 10 rats from the SSHTN group and 10 rats from the MMF group. Light microscopy was performed using formalin-fixed sections stained with periodic acid-Schiff (PAS) and hematoxylin and eosin. Glomerulosclerosis index score and tubulointerstitial damage were evaluated as described in previous studies. (34, 39, 40). The sclerosis in the glomeruli was graded from 0 to +4: Grade 0 = normal, grade +1 = <25% involvement of the glomerular tuft, grade +2 = 25–50% involvement of the glomerular tuft, grade +3 = 50–75%, and grade + 4 = sclerosis occupying >75% of the glomerular tuft. The glomerulosclerosis score was obtained as follows: \[(1 \times n \text{ glomeruli with } +1) + (2 \times n \text{ glomeruli with } +2) + (3 \times n \text{ glomeruli with } +3) + (4 \times n \text{ glomeruli with } +4)\] \times 100/total number of glomeruli examined.

The tubulointerstitial was evaluated in successive fields examined at 20x magnification in the entire cortical and juxtamedullary areas of each specimen using computer-assisted image analysis (Olympus BX51 System Microscope and DP70 microscope digital camera with software, Sigma Pro, Leesburg, Va., USA). Tubulointerstitial damage (tubular dilatation, tubular atrophy and interstitial fibrosis) was graded in a 0-5 scale: 0 = no changes, grade 1 = <10%, grade 2 = 10–25%, grade 3 = 25–50%, grade 4 = 50–75%, grade 5 = 75–100.

**Identification and quantification of tubulointerstitial immune cell infiltration.** Immunoperoxidase methodology was used to identify lymphocytes and macrophages in the glomeruli (positive cells/glomerular cross section) and tubulointerstitial regions (positive cells per mm²). Macrophages were identified with mouse anti CD68 monoclonal
antibody, lymphocytes with mouse anti rat CD3 (Biosource, Camarillo, Ca). CD4 and CD8 positive cells were identified with the corresponding monoclonal antibodies (anti-Rat CD4, Clone W3/25, mouse IgG1 and anti-Rat CD8 No Azide, Clone OX-8, mouse IgG1) obtained from Cedarlane Labs, Hornby, Canada. Angiotensin II positive cells were identified with rabbit anti-ANG II-human IgG (Peninsula Laboratories). Peroxidase conjugated goat anti-mouse and IgG (Stressgen Bioreagents Glandford, Canada) and donkey anti-rabbit (Accurate Chemical and Scientific Labs) were used as secondary antibodies. Infiltrating immune cells and angiotensin II positive cells were rarely found in the glomeruli and only the data in tubulointerstitial areas is given.

**Inflammatory cytokines and oxidative stress.** Renal cortex from 5 animals of experimental (SSHTN and MMF) and control (C-HSD) groups were used for Western blots (IL-2, IL-6 and nitrotyrosine abundance) and malondialdehyde (MDA) determinations. For the Western blot analyses 40 μg of protein were treated with 12% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was washed and probed with polyclonal antibody (1:1,000) against Il-2, IL-6 and nitrotyrosine (Santa cruz CA USA), and (1:1,000) goat horseradish peroxidase-labeled anti-rabbit IgG (Santa cruz CA USA). Finally, enhanced chemiluminescence (ECL) detection solution was added, and hyperfilm ECL (GE Healthcare) was exposed to the membrane. Each membrane was stripped of bound antibody and reprobed with anti β-actin on the same membrane for quantitative comparison. The bands were analyzed with a Kodak electrophoresis documentation and analysis system (EDAS 290).

MDA content was determined by the method of Ohkawa et al. (31). Briefly Kidney slices for MDA were placed in a cold mixture of 100mM KCL and 0.003 EDTA, homogenized,
and centrifuged at 600 g for 15 min. Supernatants of this preparation were used for MDA determination as previously described (39, 45)

Statistical Analyses. Glomerular filtration rate (GFR), renal blood flow (RBF), urinary sodium excretion (U\textsubscript{Na}V) and the fractional (percent of filtered) sodium excreted (F\textsubscript{Na}E) are expressed in relation to left kidney weight. Multi-group ANOVA and Tukey Kramer post-tests were used to examine differences between groups. Serial changes were studied with paired T tests. Correlations were analyzed with Pearson’s correlation coefficient. Data are shown as mean ± SE. Two-tailed p values <0.05 were considered significant. A commercially available statistical package (Instat, GraphPad, San Diego, CA) was used for statistical calculations.

RESULTS.

Blood pressure and histological findings induced by 3 weeks of L-NAME administration are shown in Table 1. As in previous studies (34), MMF administration did not modify the hypertension induced by inhibition of nitric oxide synthase or the return to normal blood pressure one week after the discontinuation of L-NAME (\textit{L-NAME}: Basal=120±1.7 mmHg, 1 week= 154±3.1, 2-weeks=163±3.2, 3-weeks=175.5±3.05, 1 week after discontinuation of L-NAME= 136±3.0; \textit{LNAME+MMF}: Basal=117±1.4, 1 week=151±3.6, 2 weeks=151±3.6, 3 weeks= 176±3.6, 1 week after discontinuation of L-NAME= 139±2.4). As shown in Table 1, L-NAME administration resulted in renal injury (glomerulosclerosis and tubulointerstitial damage) and renal infiltration of lymphocytes (CD3 + cells), macrophages (CD68 + cells) and angiotensin II + cells in tubulointerstitial
areas. Treatment with MMF during the L-NAME resulted in a significant reduction (albeit
not normalization) of renal injury and tubulointerstitial inflammation

General data obtained prior to the pressure natriuresis experiments (5 weeks after
discontinuation of L-NAME) are shown in Table 2. As expected, systolic blood pressure
was higher in the rats from the SSHTN group. As in previous studies (34) MMF treatment
prevented the subsequent development of salt-sensitive hypertension and the systolic blood
pressure in the MMF group was not significantly different than in the control groups on a
normal (C-NSD) or high (C-HSD) sodium diet (Table 2). Food intake, urine volume and
proteinuria were similar in all experimental groups. (Table 2).

Renal immunohistologic findings present in kidneys harvested immediately after the
pressure natriuresis experiments are shown in Table 3. During the 5 weeks that elapsed
after discontinuation of L-NAME (with and without MMF) and the pressure natriuresis
studies, the renal injury and tubulointerstitial inflammation were reduced with respect to the
findings at the end of L-NAME treatment (Table 1). At the time when the pressure
natriuresis studies were performed (Table 3), the glomerulosclerosis and tubular damage,
while higher in the SSHTN group, were not significantly different in any of the study
groups and essentially comparable in the SSHTN group and the MMF group. In contrast,
interstitial macrophages (CD68 positive cells), lymphocytes (CD3 positive cells, as well as
CD4 and CD8 T cells) and angiotensin II positive cells were several folds higher in the
group with SSHTN than in the control groups and the MMF group. Representative
microphotographs are shown in Figure 1.
Renal inflammatory cytokines and oxidative stress measurements are shown in Figure 2. IL-2 (Figure 2A), IL-6 (Figure 2B), nitrotyrosine (Figure 2C) and MDA (Figure 2C) were increased in the SSHTN group and was suppressed in the group that had been previously administered MMF.

Renal blood flow in the experimental groups at renal artery pressure (RAP) of 90, 110, 130 and 150 mmHg are shown in Figure 3. The rats with SSHTN had lower values of RBF and this was corrected by MMF treatment (Figure 3A). Differences in GFR in the groups under study were not significantly different (Figure 3B) and consequently, Filtration Fraction was increased in rats of the SSHTN group (Figure 3C). The control groups (C-NSD and C-HSD) and the MMF group did not show significant modifications in the GFR. In contrast, rats with SSHTN increased the GFR from 110-150 mmHg RAP (p<0.05 Figure 3B).

Urinary sodium excretion (UNaV) and fractional sodium excretion (FNaE) at the corresponding RAPs are shown in Figures 4A and 4B. The essentially unchanged sodium excretion rate in the SSHTN group contrasts with the values in the C-NSD and C-HSD that increase from 90 to 130 mmHg of RAP. Rats from the MMF group had values of UNaV and FNaE that were not significantly different from those in rats from the control groups.

The relationship between the inflammatory infiltrate and UNaV and FNaE was examined at a RAP of 130 mmHg when a separation between experimental and control groups was present. The intensity of immune cell infiltration had a strong negative relationship with the urinary sodium excretion rate (Figure 5A) and with the fractional sodium excretion
(Figure 5B). Similar negative relationships were found between the number of angiotensin II positive cells and natriuresis (Figure 6A) and fractional sodium excretion (Figure 6B).

Not unexpectedly, a strong (p<0.0001) positive relationship exists between the severity of hypertension (determined prior to the pressure natriuresis experiments) and the intensity of tubulointerstitial infiltration of immune competent cells (Figure 7A), as well as with cells expressing angiotensin II (Figure 7B).

DISCUSSION

There is a strong association between renal inflammation and salt-sensitive hypertension (37, 38,). The novel findings of this study are the demonstration of a direct association between the degree of impairment in pressure natriuresis and the severity of tubulointerstitial inflammation. These studies, therefore, provide compelling evidence that interstitial inflammation, in the absence of significant renal damage (Table 3), plays in the impairment of pressure natriuresis that drives salt-induced hypertension.

The renal blood flow was lower in the group with salt-sensitive hypertension consistent with renal vasoconstriction, likely as the result of intrarenal oxidative stress and angiotensin II overactivity generated by inflammation (41, 51). Glomerular filtration rate did not differ significantly in experimental and control groups in the range of RAP studied. No significant changes in GFR between 90 and 150 mmHg RAP were found in control and MMF groups; in contrast, the rats in the SSHTN group had a progressive increment in GFR as RAP was varied from 110 to 150 mmHg (paired T test p<0.01). In pressure natriuresis studies done in various laboratories, GFR in control groups has been found to be at steady
levels or showing only a mild increment (15, 20, 26, 44, 52). In hypertension induced by angiotensin II, Mattson et al (26) and Wang et al (52) found a progressive increment in GFR with increasing RAP pressure, similar to what we observed in the SSHTN group. In addition, inflammatory cytokines, such as TGFβ, impair the autoregulation of glomerular hemodynamics via the generation of reactive oxygen species (12, 47) and may cause afferent arteriolar remodeling (7, 46). Taken together, these findings suggest that the loss of GFR autoregulation in the SSHTN group may be related to renal angiotensin II overactivity and renal inflammation. Since glomerular filtration rate was essentially the same in the experimental and control groups and the renal blood flow was lower in the SSHTN group, the filtration fraction in the SSHTN group was increased as has been reported in oxidative stress associated with a high salt diet (25) and in the chronic slow pressor responses induced by angiotensin II infusion (19).

The role played by oxidative stress in the medullary regions of the kidney restricting nitric oxide availability and causing dysfunction of the pressure natriuresis response has been reviewed recently (8, 30, 36). Reduction of oxidative stress by hypoxia inducible factor (HIF)-1α (23) can improve pressure natriuresis. The generation of reactive oxygen species is an obligatory consequence of inflammation (51) and reduction in nitric oxide resulting from oxidative stress is expected to restrict the vasodilation capacity and reduce interstitial cGMP which is critical for the natriuresis response (24). The increased renal nitrotyrosine and MDA content in the SSHTN group (Figures 2C and 2D) support this notion.

The pressure natriuresis in rats of the control group and rats with mild inflammatory infiltration of immune cells (MMF group) showed a progressive increment of natriuresis and at 130 mmHg RAP and 150 mmHg RAP, the urinary sodium excretion rate was more
than 3 times and 13 times, respectively, higher than the values at 90 mmHg RAP pressure.

In contrast, the SSHTN group showed a suppression of the pressure natriuresis that was strikingly evident at RAP values of 130 and 150 mmHg.

In agreement with previous studies (6, 40, 43,) we found that severity of salt sensitive hypertension is directly correlated (p<0.001) with the intensity of the infiltration of immune competent cells in the interstitial areas of the kidney. The association of immune cell infiltration and inflammatory activity is confirmed by the increment in renal expression of inflammatory cytokines (Figure 2A and 2B).

As reported by several investigators (2, 34, 35), L-NAME administration induces renal injury and inflammation that is reduced after the discontinuation of L-NAME treatment. These findings were confirmed in the present studies and at the time of the pressure natriuresis experiments the hypertensive rats of the SSHTN group and the normotensive rats of the other groups do not have significantly different histological damage. In contrast, the infiltration of immune cells and the number of angiotensin II expressing cells in tubulointerstitial areas is significantly higher in the SSHTN group (Table 3). The causes responsible for the renal accumulation of lymphocytes and macrophages and its role in salt-sensitive hypertension are incompletely understood but the role of immune reactivity in hypertension has recently been reviewed (10, 37, 42, 47) and recent work has identified HSP70 overexpressed in the kidney as a relevant autoantigen in SSHTN (32). In line with these emerging observations, elegant investigations have shown that mice lacking lymphocyte responses develop increased natriuresis and blunted angiotensin II-induced hypertension (4).
In the present experiments, we have documented that the suppression of the natriuresis induced by tubulointerstitial inflammation is evident at RAP of 130 and 150 mmHg when there is a strong negative correlation between the $U_{\text{Na}}$V and $F_{\text{Na}}$E and the number of inflammatory and angiotensin II expressing cells. As described before (6, 34, 39, 40, 43), the renal inflammation was associated with accumulation of angiotensin II positive cells. The activation of a functional renin-angiotensin system in the immune competent cells (12, 17), and tubular cells (21, 28,) likely plays a major role in the blunting of pressure natriuresis induced by renal inflammation. In previous studies we have shown that in experimental models of salt sensitive hypertension the plasma angiotensin is reduced as a result of sodium retention; in contrast, the renal angiotensin activity is increased (6). These findings underline the independence of intrarenal angiotensin from physiologic modulation of plasma angiotensin (29) and indicate a primary role of intrarenal angiotensin in the pathogenesis of salt-sensitive hypertension (6). While in the present studies we only identified angiotensin II expressing cells by immunohistology, previous works from our group have established that the number of angiotensin II positive cells, the intensity of renal angiotensin II activity, and the severity of tubulointerstitial inflammation are directly correlated with one another (6, 50). Antinatriuretic effects of angiotensin II result from reduction in sodium filtered load and increased sodium reabsorption (26, 29), and are mediated by angiotensin II type 1 receptors (3, 18, 20, 21, 22, 28) while natriuresis is mediated by angiotensin III and type 2 receptors (32).

Oxidative stress and angiotensin II activity are key components of renal inflammation. The increased renal MDA content and inflammatory cytokines in the SSHTN group (Figure 3)
confirm this association and it is reasonable to assume the participation of these elements in the impairment of pressure natriuresis resulting from renal inflammation.

In conclusion, this investigation found that interstitial inflammation is associated with suppression of the natriuresis resulting from increments in renal arterial perfusion pressure. At RAP levels of 130 mmHg the urinary sodium excretion is inversely correlated with the number of inflammatory cells and angiotensin II positive cells in the kidney. These findings add insight to the pathogenesis of salt-sensitive hypertension and its relation to renal inflammation.

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Authors contributions: BR-I., RJJ, MF conception and design of research; MF, ET, UP, RB, JS performed experiments; YQ did the histology and immunohistology; RB did the Western blots; MF, ET, RB, YQ, BR-I analyzed data; MF, YQ, BR-I interpreted results of experiments; BR-I, MF, RJJ, drafted manuscript; BR-I, MF and RJJ edited and revised manuscript. All authors approved final version of manuscript.
REFERENCES.


FIGURE LEGENDS.

Figure 1. Light microscopy and immunohistology of renal sections of a rats from the control group, SSHTN group and MMF group. Column A shows light microscopy (PAS staining), Column B shows macrophage (CD68 positive cells) infiltration and Column C angiotensin II positive cells. Tubulointerstitial inflammation and tubular cells and infiltrating cells staining positive for angiotensin II are evident in the SSHTN group. Scale mark corresponds to the whole column. Staining details in Methods.

Figure 2. Western blots of renal content of IL-2 (2A), IL-6 (2B), nitrotyrosine (2C) and malondialdehyde (2D) in experimental (SSHTN and MMF) and control (C-NSD) groups. Western blot data and are expressed as optical density relative to β-actin. Gels pictures are samples for one run. Data correspond to n=5 in each group. * Molecular weight of nitrosylated tyrosine containing proteins. *** p<0.001 vs. the rest.

Figure 3. Renal blood flow (RBF) and glomerular filtration rate at the studied renal arterial pressure (RAP) levels in the experimental (SSHTN and MMF groups) and control groups with normal (C-NSD) and high (C-HSD) salt diet. 2A) No significant differences in RBF in control groups with normal salt diet (C-NSD), high salt diet (C-HSD) and MMF group. RBF in SSHTN group was lower. (* p<0.01 vs. the rest). 2B) No significant differences between the GFR at 90, 110, 130 and 150 mmHg RAP in experimental and control groups. GFR in the SSHTN group increased progressively from 90 mmHg to 150 mmHg. 2C) Filtration fraction in the SSHTN is significantly higher (*p<0.05, **p<0.01) than in the rest. Studies done with induced (see text) RAP of 150 mmHg in normotensive rats from
the C-NSD (n=7), C-HSD (n=5) and MMF (n=6) groups as indicated in Methods. Symbols and error bars are mean ± SE.

**Figure 4.** Variations in (4A) urinary sodium excretion rate \( \text{U}_{\text{Na}}V_\text{s} \) and (4B) Fractional sodium excretion \( \text{F}_{\text{Na}}E_\text{s} \) at the studied renal arterial pressures. The progressive increment in urinary sodium excretion and fractional sodium excretion observed in the control and MMF groups was not present in the SSHTN group. Studies done with RAP of 150 mmHg in normotensive rats from the C-NSD (n=5), C- HSD (n=5) and MMF (n=6) groups as indicated in Methods. *p<0.05 vs. the rest.

**Figure 5.** Inverse relationship between interstitial immune cell (CD68 + CD3 positive cells) infiltration and (5A) urinary sodium excretion rate \( \text{U}_{\text{Na}}V_\text{s} \) and (5B) fractional sodium excretion \( \text{F}_{\text{Na}}E_\text{s} \). Data corresponds to values at renal artery pressure of 130 mmHg. Each symbol represents one animal of the corresponding groups.

**Figure 6.** Inverse relationship between the number of angiotensin II positive cells and (6A) urinary sodium excretion rate \( \text{U}_{\text{Na}}V_\text{s} \) and (6B) fractional sodium excretion \( \text{F}_{\text{Na}}E_\text{s} \). Data corresponds to renal artery pressure of 130 mmHg. Symbols correspond to the experimental and control groups.

**Figure 7.** Direct relationship between the systolic blood pressure and the number of infiltrating immune cells (CD68 positive + CD3 positive cells) in 7A and angiotensin II positive cells in 7B. Symbols correspond to the experimental and control groups.
Table 1. Data after 3 weeks of L-NAME treatment.

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<th>L-NAME +MMF (n=7)</th>
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<td>SBP (mmHg)</td>
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<td>TI damage score (0-5)</td>
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</tr>
<tr>
<td>All + cells/mm²</td>
<td>0.8 ± 0.58</td>
<td>28.5 ±3.51 ‡***</td>
<td>12.5 ± 1.23 ‡</td>
</tr>
</tbody>
</table>

Table 1. Studies done in kidney sections harvested after 3 weeks of L-NAME administration in the drinking water (L-NAME group) and L-NAME plus daily MMF treatment (MMF group). Control groups received no treatment. All rats were in a normal (0.4%) salt diet. SBP= Systolic blood pressure. Data are mean ±SE. ‡ values higher than controls (p<0.01 or lower). Differences between L-NAME and L-NAME+MMF groups are *p<0.05, **p<0.01, ***p<0.001 (multigroup variance analysis).
Table 2. General Results

<table>
<thead>
<tr>
<th></th>
<th>C-NSD (n=20)</th>
<th>C- HSD (n=12)</th>
<th>SSHTN (n=17)</th>
<th>MMF (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before L-NAME</td>
<td>301,4±4.92</td>
<td>308,0±3.09</td>
<td>302,0±1.35</td>
<td>307,5±3.85</td>
</tr>
<tr>
<td>Prior to pressure natriuresis</td>
<td>438,9±10,72</td>
<td>448,4±16,44</td>
<td>450,0±20.02</td>
<td>452,5±8,69</td>
</tr>
<tr>
<td>Left Kidney weight (g)</td>
<td>1,39±0,025</td>
<td>1,45±0,036</td>
<td>1,56±0,040</td>
<td>1,56±0,058</td>
</tr>
<tr>
<td>Food ingestion (g/day)</td>
<td>30,2±1,60</td>
<td>32,0±0,88</td>
<td>30,0±2,29</td>
<td>26,5±1,41</td>
</tr>
<tr>
<td>Urine Volume (ml/24 hs)</td>
<td>20,43±4,51</td>
<td>20,0±2,70</td>
<td>26,0±5,38</td>
<td>22,0±3,45</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>8,25±1.03</td>
<td>8.62±0.85</td>
<td>9.11±1.20</td>
<td>8.77±0.92</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>116,0±1.08</td>
<td>118,8±1.54</td>
<td>157,9±1.91 ***</td>
<td>132,7±1,64***</td>
</tr>
</tbody>
</table>

Table 2. Kidney weight at the end of pressure natriuresis experiments. Mean arterial pressure (MAP) obtained by direct intraarterial determinations prior to the pressure natriuresis experiments. Experimental (SSHTN and MMF) and C-HSD groups had been 4 weeks on a high salt diet and the C-NSD group on a normal sodium diet. Food ingestion mean during the preceding 30 days; Proteinuria and urine volume the day before pressure natriuresis studies. *** p<0.01 vs the rest.
Table 3. Histology and immunohistology at the time of pressure natriuresis studies.

<table>
<thead>
<tr>
<th></th>
<th>C- NSD (n=7)</th>
<th>C-HSD (n=7)</th>
<th>SSHTN (n=10)</th>
<th>MMF (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS index (0-400)</td>
<td>0.6±0.24</td>
<td>0.6±0.40</td>
<td>4.8±2.63</td>
<td>3.4±1.69</td>
</tr>
<tr>
<td>TI damage score (0-5)</td>
<td>0.6±0.24</td>
<td>0.8±0.37</td>
<td>1.8±0.58</td>
<td>1.2±0.20</td>
</tr>
<tr>
<td>CD68 + cells/mm²</td>
<td>7.82±0.57</td>
<td>10.14±0.66</td>
<td>35.65±2.29**</td>
<td>22.99±1.49</td>
</tr>
<tr>
<td>CD3 + cells/mm²</td>
<td>7.76±0.63</td>
<td>11.66±0.84</td>
<td>43.17±2.76**</td>
<td>26.77±2.15</td>
</tr>
<tr>
<td>CD4 + cells/mm²</td>
<td>6.78±0.78</td>
<td>2.67±1.09</td>
<td>29.3±3.80</td>
<td>17.9±2.29</td>
</tr>
<tr>
<td>CD8 + cells/mm²</td>
<td>3.16±1.08</td>
<td>6.28±0.60</td>
<td>18.9±2.12***</td>
<td>9.81±0.49†</td>
</tr>
<tr>
<td>AII + cells/mm²</td>
<td>8.09±0.60</td>
<td>10.63±0.64</td>
<td>32.06±2.39δ</td>
<td>19.25±0.42γ</td>
</tr>
</tbody>
</table>

Table 3. Histology and immunohistology in the control groups with normal (C-NSD) and high (C-HSD) salt diets and the experimental groups (SSHTN and MMF). Values are mean ± SE. ** p<0.01 vs. the rest; *** p<0.001 vs. the rest; p<0.01 vs. C-NSD and C-HSD; δ p< 0.001 vs. C-NSD and C-HSD and p<0.05 vs. MMF; † p<0.05 vs. C-NSD.
A  

**IL-2**

- 15kDa
- Control
- SSHTN
- MMF

B  

**IL-6**

- 21kDa
- Control
- SSHTN
- MMF

C  

**Nitrotyrosine**

- ~55-60 kDa
- Control
- SSHTN
- MMF

D  

**Malondialdehyde**

- Control
- SSHTN
- MMF

---

**Relative O.D.**

*** **

---
A

B

$U_{Na V}$

(μ Eq. min$^{-1}$·g LKW$^{-1}$)

$F_{Na E}$ (%)

C-NSD
C-HSD
SSHTN
MMF

p = 0.0063

p = 0.0019