

High-Salt Intake Induces Cardiomyocyte Hypertrophy in Rats in Response to Local Angiotensin II Type 1 Receptor Activation^{1–3}

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Abstract

Many studies have shown that risk factors that are independent of blood pressure (BP) can contribute to the development of cardiac hypertrophy (CH). Among these factors, high-salt (HS) intake was prominent. Although some studies have attempted to elucidate the role of salt in the development of this disease, the mechanisms by which salt acts are not yet fully understood. Thus, the aim of this study was to better understand the mechanisms of CH and interstitial fibrosis (IF) caused by HS intake. Male Wistar rats were divided into 5 groups according to diet [normal salt (NS; 1.27% NaCl) or HS (8% NaCl)] and treatment [losartan (LOS) (HS+LOS group), hydralazine (HZ) (HS+HZ group), or *N*-acetylcysteine (NAC) (HS+NAC group)], which was given in the drinking water. Tail-cuff BP, transverse diameter of the cardiomyocyte, IF, angiotensin II type 1 receptor (AT1) gene and protein expression, serum aldosterone, cardiac angiotensin II, cardiac thiobarbituric acid-reactive substances, and binding of conformation-specific anti-AT1 and anti-angiotensin II type 2 receptor (AT2) antibodies in the 2 ventricles were measured. Based on the left ventricle transverse diameter data, the primary finding was the occurrence of significant BP-independent CH in the HS+HZ group (96% of the HS group) and a partial or total prevention of such hypertrophy via treatment with NAC or LOS (81% and 67% of the HS group, respectively). The significant total or partial prevention of IF using all 3 treatments (HS+HZ, 27%; HS+LOS, 27%; and HS+NAC, 58% of the HS group, respectively), and an increase in the AT1 gene and protein expression and activity in groups that developed CH, confirmed that CH occurred via the AT1 in this experimental model. Thus, this study unveiled some relevant previously unknown mechanisms of CH induced by chronic HS intake in Wistar rats. The link of oxidative stress with CH in our experimental model is very interesting and stimulates further evaluation for its full comprehension. *J. Nutr.* 144: 1571–1578, 2014.

Introduction

Cardiovascular diseases make up the leading cause of death worldwide. Among these diseases, cardiac hypertrophy (CH)⁶ has been highlighted, especially as an important risk factor for heart failure. The Framingham Heart Study showed that in-

dividuals who had CH and were diagnosed via electrocardiogram changes had a 6-fold increase in their risk of death than the general population. Thus, many studies addressed this pathology with goals of prevention and treatment, thereby reducing the risk of death (1–3). Generally, CH is the term used to define a set of molecular, cellular, and interstitial changes that are manifested clinically as changes in the size, mass, geometry, and function of the heart. These alterations are accompanied by increased protein synthesis, changes in the organization of the structure of the sarcomeres, and the activation of the early genes (C-jun, C-fos, and C-myc) and fetal genes (e.g., atrial natriuretic factor, β -myosin heavy chain, and skeletal α -actin), which in turn are used as hypertrophy markers (4,5). CH can be caused by hemodynamic (hypertension), environmental (diet), and humoral [renin-angiotensin system (RAS)] or genetic (idiopathic hypertrophy) factors. The ingestion of large amounts of sodium chloride [NaCl (salt)] was identified as an important contributor to CH (6). Although numerous clinical and experimental studies have detected its hypertensive effect (7,8), salt can also induce CH independent of its hemodynamic effects (7,9). Therefore,

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³ Supplemental Tables 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

⁶ Abbreviations used: All, angiotensin II; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; BP, blood pressure; CH, cardiac hypertrophy; ERK1/2, extracellular signal-regulated kinases 1/2; HS, high salt; HS+HZ, HS + hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS + losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS + *N*-acetylcysteine (600 mg/L); HZ, hydralazine; IF, interstitial fibrosis; LOS, losartan; LV, left ventricle; NAC, *N*-acetylcysteine; NS, normal salt, 1.27% NaCl; RAS, renin-angiotensin system; ROS, reactive oxygen species; RV, right ventricle; TcBP, tail-cuff blood pressure.

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many studies have attempted to elucidate the mechanisms by which salt induces CH. One of the pathways more closely related to CH is RAS, and it is well established that all RAS components are locally synthesized in the cardiac tissue (10–13). These components are distributed in cardiomyocytes, fibroblasts, and endothelial and vascular smooth muscle cells, and, interestingly, are regulated independently of circulating RAS (9,14).

Angiotensin II (AII) acts on cardiac tissue in a similar way to a proinflammatory cytokine, stimulating the release of other cytokines, inducing apoptosis, increasing the generation of reactive oxygen species (ROS), and stimulating cell growth and proliferation. The majority of the effects of AII on cardiomyocytes are mediated by angiotensin II type 1 receptors (AT1s). When coupled, this ligand–receptor connection may be a potent stimulator of myocardial hypertrophy (15), increasing the production of a variety of proteins that are related to CH and inducing extracellular matrix accumulation and fibrosis formation. The increase of fibrous tissue increases myocardial stiffness and impairs diastolic filling, predisposing the patient to diastolic failure (16).

In addition to the AT1, AII can also stimulate the angiotensin II type 2 receptor (AT2). The effects that this receptor has on cardiac tissue remain a matter of controversy. Some studies suggest that this receptor possesses antiproliferative effects and thus the opposite of the effect observed because of the binding of AII to the AT1 (17). However, there is evidence in the literature suggesting that the *in vitro* stimulation of the AT2 causes constitutive cardiomyocyte hypertrophy via an extracellular signal-regulated kinases 1/2 (ERK1/2) MAPK-independent pathway (18).

In addition to the classic RAS pathway, alternative routes have been described for the synthesis of AII in cardiac tissue. An important component of this pathway is an enzyme called chymase, which is capable of cleaving angiotensin I, resulting in AII production (19). Chymase is present in higher concentrations in the human heart and is accountable for up to 90% of the generation of AII; there are studies that also demonstrate the presence of chymase in the hearts of rats (20).

Oxidative stress is related to CH and is characterized by an imbalance between the production and removal of ROS. Several factors can induce cardiac oxidative stress such as increased AII concentrations (21), mechanical stress (22), and inflammatory processes (23). In isolated cardiomyocytes, a moderate increase of ROS was observed to induce hypertrophy and apoptosis (24). It is known that the use of antioxidants such as vitamin E can inhibit the hypertrophy induced by AII in neonatal rat cardiomyocytes (25), and *N*-acetylcysteine (NAC) can also attenuate CH in Wistar rats subjected to 5/6 nephrectomy (26).

A previous study in our laboratory (9) found that dietary salt overload induced blood pressure (BP)–independent CH and increased left ventricular mass and interstitial fibrosis (IF). There was a decrease in plasma renin activity and serum aldosterone in all rats that were administered dietary salt overload, suggesting that the circulating RAS may not be involved in the development of CH. However, all groups fed a high-salt (HS) diet had higher cardiac AII contents.

Understanding how HS intake induces CH can aid in lowering CH prevalence and reducing the morbidity and mortality rates associated with this disease. Therefore, to better understand the effects of high dietary salt intake on the structural and functional changes of the myocardium and the mechanisms involved in this process, this study aimed to verify the involvement of cardiac RAS and oxidative stress in an experimental model.

Materials and Methods

The experiments were previously approved by the Research Projects Evaluation Committee of the University of São Paulo School of Medicine, Brazil (certificate no. 1132/09).

Rats. Male Wistar rats obtained from the Institutional Animal Facility of the University of São Paulo School of Medicine were housed in a temperature-controlled environment at 25°C on a 12-h light/dark cycle and with free access to food and water.

The rats were fed a normal-salt (NS) (1.27% NaCl) or HS (8% NaCl) diet (Harlan Teklad) from weaning (3 wk of age) to adulthood (18 wk of age). All diets were pelleted. The only difference between the diets was their sodium chloride content (9).

At 7 wk of age, 3 HS diet subgroups were administered hydralazine (HZ) (HS+HZ; 15 mg·kg⁻¹·d⁻¹), losartan (LOS) (HS+LOS; 20 mg·kg⁻¹·d⁻¹), or NAC (HS+NAC; 600 mg/L) in their drinking water. The dose of HZ and LOS was corrected by the body weight on a weekly basis. All doses were based on previous studies (9,27).

Experimental protocol. Body weight was measured weekly from 3 to 18 wk of age. BP was measured at 6 wk of age and every 2 wk from 7 to 18 wk of age. At 18 wk of age, the rats were individually housed in metabolic cages (model 650–0100; Nalgene Brand Products) for 24-h urine collection for volume and sodium determination. Blood was collected from the tail vein to measure serum aldosterone and sodium concentrations, as well as hematocrit. At 18 wk of age, the rats were killed and decapitated for organ collection or perfused for histologic analysis.

BP measurement. Tail-cuff BP (TcBP) was measured via an oscillometric method (model RTBP 2045 with the RTBP 001 acquisition system; Kent Scientific) in conscious rats. TcBP was recorded every 2 wk from 6 to 18 wk of age, and the means of all values were used in the calculations.

Serum sodium, potassium, and aldosterone, and urine sodium and potassium concentrations. Serum and urinary sodium and potassium were evaluated by using a flame spectrophotometer (model FC 280; CELM). Serum aldosterone was assayed by using a commercial RIA kit (Coat-a-Count; Siemens).

Histologic analysis. At 18 wk of age, the rats were anesthetized with sodium pentobarbital (40 mg·kg⁻¹ body weight⁻¹ *i.p.*). Cardiac arrest was induced at diastole with 14 mM intracardiac KCl followed by perfusion fixation with Dubosque-Brasil. The heart was quickly removed and weighed. Both ventricles were dissected and weighed separately and transversely sectioned at the midpoint between the apex and the base and fixed in 10% buffered formalin and embedded in paraffin blocks.

Five- μ m-thick sections were cut from the blocked tissue and stained with periodic acid-Schiff reagent for cardiomyocyte transverse diameter determination. Cardiomyocyte transverse diameter was measured by using a microscope connected to a video monitor with an adapted millimeter ruler. The measurements were taken along the short axis of the cardiomyocytes at the level of the nucleus.

At least 50 left ventricle (LV) and 30 right ventricle (RV) cardiomyocytes were analyzed in each section with a magnification of 400 \times (Nikon).

For the quantification of IF areas using a point-counting method, the sections were stained with Masson's Trichrome. The tissue slices were examined under a magnification of 400 \times (Nikon). The amount of fibrosis was expressed as the percentage of the total area of the specified section of the heart.

All determination in the 2 ventricles. Immunohistochemistry was performed on 5- μ m-thick sections that were mounted on glass slides precoated with 2% silane for AII content in the 2 ventricles. The sections were deparaffinized and rehydrated by using conventional techniques. Next, the sections were circled immediately with a PAP pen (Vector Laboratories) to form a waterproof barrier and were preincubated with an Avidin/Biotin blocking kit (Vector Laboratories). The sections were then washed and incubated with a protein block serum-free solution

TABLE 1 Hemodynamic variables, food and water intake, urinary volume, urinary and serum sodium, and hematocrit in 18-wk-old Wistar rats¹

	NS	HS	HS+HZ	HS+LOS	HS+NAC
TcBP, mm Hg					
6 wk of age	120 ± 1 (14)	125 ± 3 (12)	125 ± 3 (12)	128 ± 4 (11)	118 ± 2 (14)
7 wk of age	120 ± 2 ^c (14)	139 ± 2 ^b (12)	116 ± 2 ^c (12)	120 ± 1 ^c (11)	163 ± 3 ^a (14)
18 wk of age	110 ± 1 ^c (14)	143 ± 4 ^b (12)	116 ± 1 ^c (12)	116 ± 1 ^c (11)	166 ± 1 ^a (14)
Heart rate, beats/min	383 ± 12 (14)	368 ± 9 (12)	376 ± 7 (12)	388 ± 10 (11)	396 ± 4 (14)
Food intake, g/d	16 ± 1.8 ^b (8)	15 ± 0.9 ^b (7)	14 ± 0.5 ^b (8)	16 ± 0.9 ^b (7)	23 ± 0.6 ^a (8)
Water intake, mL/d	25 ± 2.5 ^c (8)	57 ± 3.6 ^b (7)	56 ± 7.2 ^b (8)	56 ± 3.2 ^b (7)	93 ± 1.8 ^a (8)
Urinary volume, mL/24 h	11 ± 1.5 ^c (8)	48 ± 2.9 ^{ab} (7)	39 ± 5.1 ^b (8)	44 ± 2.3 ^b (7)	56 ± 1 ^a (8)
Urinary sodium, mmol/24 h	2 ± 0.2 ^c (8)	14 ± 0.8 ^b (7)	12 ± 1.5 ^b (8)	13 ± 1.1 ^b (7)	19 ± 1.3 ^a (8)
Serum sodium, mmol/L	152 ± 3.4 (8)	152 ± 2.3 (8)	148 ± 2.1 (8)	149 ± 2.6 (8)	148 ± 1.9 (8)
Hematocrit, %	49 ± 0.4 (8)	49 ± 0.5 (8)	48 ± 0.5 (8)	49 ± 0.4 (8)	49 ± 0.3 (8)

¹ Values are means ± SEMs; *n* in parentheses. Means in a row without a common letter differ, *P* < 0.05. HS, high salt (8% NaCl); HS+HZ, HS + hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); NS, normal salt (1.27% NaCl); TcBP, tail-cuff blood pressure.

(Dako) for 30 min. Afterward, they were incubated overnight with the primary antibody anti-AII (Peninsula) at 4°C. The sections were washed and incubated with a biotinylated anti-rabbit IgG (Vector Laboratories) for 45 min, followed by incubation with a Vectastain ABC-AP kit (Vector Laboratories) for 30 min.

Quantification of the AII-positive areas was performed by using a point-counting method. The total area of the 2 slices per rat (both ventricles) was examined under a magnification of 200× (Nikon).

Cardiac TBARS. Cardiac concentrations of TBARS, which are markers of lipid peroxidation, were determined by using the thiobarbituric acid assay as previously described (27). Results were corrected via tissue protein content, which was measured by using a commercially available kit (Pierce BCA Protein Assay kit; Thermo Scientific).

RNA isolation and gene expression of RAS components in the 2 ventricles. Gene expression was assessed via RT-PCR as previously described (28). The primers used for the RT-PCR are included in Supplemental Table 1.

Protein expression of RAS components and chymase in the 2 ventricles. Protein expression was measured via Western blot according to the following protocol.

Samples of the LV and RV were homogenized separately by using a RIPA Lysis Buffer (Millipore). The total protein concentration was quantified in each sample by using a BCA Protein Assay kit (Thermo Scientific).

Aliquots of 100 µg of the total protein from the homogenized tissue were electrophoretically separated via 12% SDS-PAGE and were transferred to nitrocellulose membranes (Bio-Rad Laboratories) for 1.5 h at 20 V by using a semidry transfer cell (Bio-Rad Laboratories). To ensure adequate protein transfer, membranes were stained with Ponceau S (Sigma-Aldrich). Membranes were blocked for 2 h with 5% nonfat dry milk to prevent nonspecific antibody binding and incubated overnight at 4°C with the following primary antibodies: anti-angiotensinogen (1:500; Fitzgerald Industries International), anti-renin (1:250; Fitzgerald Industries International), anti-angiotensin-converting enzyme (1:300; Santa Cruz Biotechnology), anti-angiotensin-converting enzyme 2 (1:250; Santa Cruz Biotechnology), anti-AT1 (1:200; Santa Cruz Biotechnology), anti-AT2 (1:200; Santa Cruz Biotechnology), anti-chymase (1:1500; Proteimex Biotechnology), and anti-GAPDH (1:1000; Sigma).

Membranes were washed with Tris-buffered saline plus Tween and were incubated with a secondary anti-goat IgG-peroxidase conjugate antibody produced in the rabbit at 1:10,000 (Santa Cruz Biotechnology) for 2 h, anti-rabbit IgG-horseradish peroxidase conjugate antibody at 1:5000 (Dako) for 2 h, or anti-mouse IgG-horseradish peroxidase conjugate antibody at 1:3000 (Dako) for 2 h. The membranes were then thoroughly washed, and immunocomplexes were detected by using a peroxidase-luminol chemiluminescence system (ECL). The intensity of the individual bands was quantified via optical densitometry by using ImageQuant TL (GE) divided by the GAPDH density and expressed as arbitrary units (AU/GAPDH). A prestained protein marker (precision plus protein standards; Bio-Rad) was used as a molecular weight standard.

TABLE 2 Body weight, cardiac mass and index, LV and RV mass, and LV and RV mass indexes in 18-wk-old Wistar rats¹

	NS	HS	HS+HZ	HS+LOS	HS+NAC
Body weight, g					
3 wk of age	69 ± 2 (12)	69 ± 2 (12)	66 ± 2 (12)	71 ± 2 (11)	75 ± 2 (14)
8 wk of age	338 ± 5 ^a (12)	306 ± 5 ^{ab} (12)	303 ± 5 ^b (12)	316 ± 11 ^{ab} (11)	342 ± 5 ^a (14)
9 wk of age	377 ± 7 ^a (12)	340 ± 6 ^b (12)	340 ± 5 ^b (12)	343 ± 10 ^{ab} (11)	384 ± 6 ^a (14)
18 wk of age	572 ± 12 ^a (12)	489 ± 8 ^b (12)	481 ± 10 ^b (12)	514 ± 15 ^b (11)	551 ± 9 ^a (14)
Cardiac index, g/cm	0.34 ± 0.01 ^c (16)	0.51 ± 0.02 ^a (16)	0.47 ± 0.02 ^{ab} (15)	0.38 ± 0.01 ^{bc} (15)	0.43 ± 0.01 ^b (14)
Cardiac mass, g	1.59 ± 0.11 ^c (16)	2.27 ± 0.11 ^a (16)	2.11 ± 0.12 ^{ab} (15)	1.74 ± 0.05 ^{bc} (15)	1.97 ± 0.03 ^b (14)
LV index, g/cm	0.25 ± 0.01 ^c (16)	0.35 ± 0.01 ^a (16)	0.31 ± 0.01 ^b (15)	0.26 ± 0.01 ^c (15)	0.29 ± 0.01 ^{bc} (14)
LV mass, g	1.18 ± 0.02 ^c (16)	1.59 ± 0.07 ^a (16)	1.43 ± 0.06 ^b (15)	1.19 ± 0.03 ^c (15)	1.31 ± 0.01 ^{bc} (14)
RV index, g/cm	0.06 ± 0.01 ^c (16)	0.08 ± 0.01 ^a (16)	0.08 ± 0.01 ^a (15)	0.07 ± 0.01 ^b (15)	0.07 ± 0.01 ^b (14)
RV mass, g	0.29 ± 0.01 ^c (16)	0.39 ± 0.01 ^a (16)	0.39 ± 0.01 ^a (15)	0.34 ± 0.01 ^b (15)	0.35 ± 0.01 ^{ab} (14)

¹ Values are means ± SEMs; *n* in parentheses. Means in a row without a common letter differ, *P* < 0.05. HS, high salt (8% NaCl); HS+HZ, HS+hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); LV, left ventricle; NS, normal salt (1.27% NaCl); RV, right ventricle.

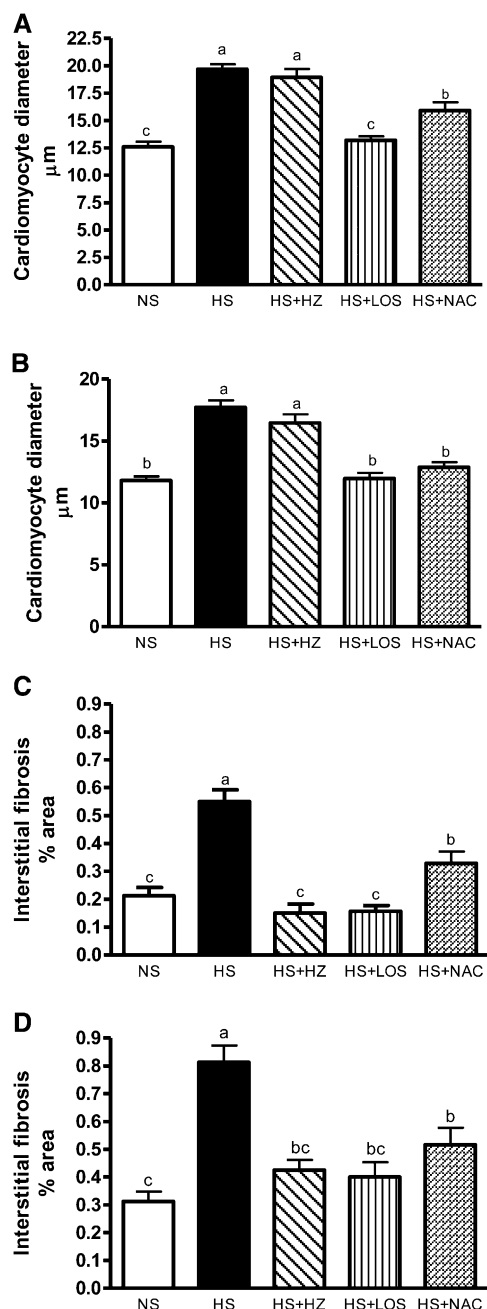


FIGURE 1 Cardiomyocyte diameter of the LV (A) and RV (B) and IF of the LV (C) and RV (D) of 18-wk-old rats fed diets of varying salt concentrations and untreated or treated with HZ, LOS, or NAC. Data are means \pm SEMs, $n = 6-8$. Labeled means without a common letter differ, $P < 0.05$. HS, high salt (8% NaCl); HS+HZ, HS+hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); HZ, hydralazine; IF, interstitial fibrosis; LOS, losartan; LV, left ventricle; NAC, N-acetylcysteine; NS, normal salt (1.27% NaCl); RV, right ventricle.

Binding of conformation-specific anti-AT1 and anti-AT2 antibodies in the 2 ventricles. The sections were deparaffinized and rehydrated by using conventional techniques. Then, the sections were circled with a PAP pen (Vector Laboratories) to form a waterproof barrier. The sections were incubated with a blocking solution (1% bovine serum albumin + 5% sucrose in PBS) to prevent nonspecific antibody binding for 3 h. The sections were then incubated with the anti-AT1 and anti-AT2 conformation-specific polyclonal antibody conjugates

with DY-682 (red) and DY-800 (green) fluorophores, respectively (Proteimax Biotecnologia).

The slices were washed with PBS, and fluorescence intensity was measured by using Odyssey equipment (LI-COR Biosciences).

Statistical analysis. Differences between the 5 groups were analyzed by using a 1-factor ANOVA with a Tukey post hoc test. The changes in TcBP over time were analyzed via a 2-factor ANOVA with Bonferroni's post hoc test. A Bartlett test for ≥ 3 variances was performed. The result did not show significant differences among the variances from the 5 experimental groups. Results are presented as means \pm SEMs. Calculations were performed by using Prism 4.0 (GraphPad Software). $P < 0.05$ was considered significant.

Results

TcBP and heart rate. At 6 wk of age, there was no difference in TcBP between the 5 experimental groups. However, TcBP was 15–35% higher in the HS and HS+NAC groups from wk 7 of age than in the NS, HS+HZ, and HS+LOS groups, and this profile was maintained until 18 wk of age. Additionally, TcBP was 17% higher in the HS+NAC group than in the group fed an HS diet, indicating the absence of an antihypertensive effect of NAC. TcBP did not differ between rats in the NS, HS+HZ, or HS+LOS groups (Table 1). Furthermore, heart rate did not differ between any experimental groups (Table 1).

Food and water intake, 24-h urinary volume, 24-h urinary sodium, and serum sodium and hematocrit. Food intake was 53% higher in the HS+NAC group than all other groups. Water intake and urine volume were 128% higher in the HS, HS+HZ, and HS+LOS groups than in the NS group; however, these items were 40% lower than in the HS+NAC group (Table 1).

At 18 wk of age, urinary sodium excretion was 550% higher in the 4 groups that were administered an HS diet than in the control group; however, this increase was 46% higher in the HS+NAC group than in the other groups that were fed an HS diet. Serum sodium and hematocrit at 18 wk of age did not differ between the 5 experimental groups (Table 1).

Body weight and cardiac index. Body weight between 3 and 8 wk of age was similar among the 5 groups. From 8 wk of age, body weight was lower in the HS+HZ group than in the control and HS+NAC groups. At 9 wk of age, body weight was lower in the HS group than in the NS and HS+NAC groups. At 14 wk of age, body weight in the HS+LOS group was also lower than in the NS and HS+NAC groups (data not shown). At 18 wk of age, the NS and HS+NAC groups maintained $\sim 12\%$ higher body weight than the other groups, and no differences were observed in body weight between the HS, HS+HZ, or HS+LOS groups or between the NS and HS+NAC groups (Table 2).

Ratios between cardiac mass (cardiac index), LV mass (LV index), RV mass (RV index), and tibia length measured in the 18-wk-old Wistar rats were higher in the HS and HS+HZ groups than in the NS and HS+LOS groups. The HS+NAC group had intermediate values between the HS, HS+HZ, and HS+LOS groups (Table 2). Cardiac mass not corrected for tibial length was also analyzed. The cardiac, LV, and RV masses were similar to the cardiac, LV, and RV indices (Table 2).

Cardiomyocyte transverse diameter and IF in the 2 ventricles. The cardiomyocyte transverse diameter of the 2 ventricles was larger in the HS and HS+HZ groups in than in the other groups. However, the HS+NAC group had an intermediate

TABLE 3 Serum aldosterone and LV and RV All concentrations in 18-wk-old Wistar rats¹

	NS	HS	HS+HZ	HS+LOS	HS+NAC
Serum aldosterone, <i>pg/ml</i>	64.7 ± 15.7 ^a (10)	26.7 ± 2.6 ^b (10)	34.0 ± 3.6 ^b (10)	38.9 ± 5.6 ^b (10)	29.1 ± 2.1 ^b (7)
All-LV, % area	0.03 ± 0.01 ^b (8)	0.17 ± 0.02 ^a (8)	0.16 ± 0.02 ^a (8)	0.17 ± 0.04 ^a (8)	0.17 ± 0.02 ^a (7)
All-RV, % area	0.02 ± 0.20 ^b (8)	0.15 ± 1.20 ^a (8)	0.15 ± 1.20 ^a (8)	0.15 ± 1.20 ^a (8)	0.14 ± 1.00 ^a (7)

¹ Values are means ± SEMs; *n* in parentheses. Means in a row without a common letter differ, *P* < 0.05. All, angiotensin II; HS, high salt (8% NaCl); HS+HZ, HS+hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); LV, left ventricle; NS, normal salt (1.27% NaCl); RV, right ventricle.

value in the LV; in the RV, this was not different from that of the NS group, showing partial prevention in the LV (NS group = 12.6 ± 0.5; HS group = 19.7 ± 0.4; HS+HZ group = 18.9 ± 0.8; HS+LOS group = 13.2 ± 0.3; and HS+NAC group = 15.9 ± 0.8 μm) and full prevention in the RV (NS group = 11.8 ± 0.3; HS group = 17.7 ± 0.6; HS+HZ group = 16.5 ± 0.7; HS+LOS group = 12.0 ± 0.5; and HS+NAC group = 12.9 ± 0.4 μm) (Fig. 1).

Myocardium Masson's trichrome stain revealed that IF was higher in both ventricles of the HS group than in those of the other experimental groups. The HS+NAC group had more IF than the NS, HS+HZ, or HS+LOS groups in the RV (NS group = 0.31 ± 0.03; HS group = 0.81 ± 0.06; HS+HZ group = 0.42 ± 0.03; HS+LOS group = 0.40 ± 0.05; and HS+NAC group = 0.51 ± 0.06 % area); however, in the LV, this value in the HS+NAC group was only higher than the NS group (NS group = 0.21 ± 0.02; HS group = 0.55 ± 0.04; HS+HZ group = 0.15 ± 0.03; HS+LOS group = 0.15 ± 0.02; and HS+NAC group = 0.32 ± 0.04 % area). These results suggest that IF was completely prevented via the HZ and LOS treatments and partially prevented via the NAC treatment (Fig. 1).

Serum aldosterone and LV and RV All content. Serum aldosterone concentration was 40–59% lower in the HS, HS+HZ, HS+LOS, and HS+NAC groups than in the NS group. All content was 5 times higher in the LV and 8 times higher in the RV in all HS-fed groups than in the NS group (Table 3).

Gene and protein expression of cardiac RAS components. There were no differences in the gene or protein expression of the RAS components in the 2 ventricles in the 5 groups, with the exception of the AT1. The gene expression of the AT1 in the LV but not in the RV was 53% lower in the NS and HS+LOS groups and 40% lower in the HS+NAC group than in the HS and HS+HZ groups (Fig. 2, Supplemental Table 2). The protein expression of the AT1 in the LV and RV were 39% lower in the NS and HS+LOS groups and 50% lower in the HS+NAC group than in the HS and HS+HZ groups (Fig. 2, Supplemental Table 3).

The protein expression of chymase was 55% higher in the HS+HZ group than in the other experimental groups for the LV and did not differ between the 5 groups for the RV (Supplemental Table 3).

The AT1 protein expression was higher in the HS and HS+HZ groups than in the other groups for both the LV and the RV (Fig. 2).

Cardiac TBARS. The TBARS in the myocardium was ~70% lower in the NS and HS+NAC groups than in the HS, HS+HZ, and HS+LOS groups (Fig. 3).

Binding of conformation-specific anti-AT1 and anti-AT2 antibodies in the LV and RV. The binding of the antibody that recognizes the activated conformation of the AT1 in the LV was ~66% higher in the HS and HS+HZ groups than in the other groups. Similar differences between the groups were observed in the RV.

There was no difference between the ventricles in the affinity of the conformation-specific anti-AT2 antibody (Fig. 4).

Discussion

In this study, rats fed an HS diet from weaning had higher TcBP from 7 wk of age. However, rats that were fed an HS diet as well as being administered NAC from 7 wk of age had even higher TcBP levels, and the groups that were fed an HS diet and were administered HZ or LOS in the same period had normal TcBP levels. At 18 wk of age, the body weight of the rats fed an HS diet and those treated with HZ or LOS was lower than that in the NS group. Interestingly, NAC impeded this effect. It should be noted that there were no differences in body weight between the groups at weaning. Corroborating these findings in a previous study from this laboratory, Coelho et al. (29) observed that, despite consuming a greater amount of food, rats fed an HS diet had lower body weight than rats fed a low- or normal-sodium diet. These authors observed a higher energy expenditure in rats fed an HS diet. However, in the group that was administered an HS diet and NAC, the NAC appeared to affect the appetite of the rats; their food intake was higher than in the other groups. This effect may explain the higher BP and higher body weight than in the other groups that were administered an HS diet. However, more studies are required to elucidate this finding.

The 24-h urinary sodium excretion was higher in the HS, HS+HZ, and HS+LOS groups than in the control group. As expected, sodium excretion was higher in the HS+NAC group than in the other experimental groups because the salt intake in this group was also higher.

Because serum sodium and hematocrit concentrations were not different between the 5 groups, it may be conjectured that myocardial hypertrophy is not caused by a volume expansion.

The higher cardiac index in the HS and HS+HZ groups than in the NS and HS+LOS groups is indicative of a BP-independent hypertrophic effect of salt intake, confirming the results of a previous study by our group (9). The partial prevention of an increase in the cardiac index in response to NAC is potentially due to the restricted response of myocardial fibrosis in both ventricles and the restricted response of the cardiomyocyte diameter in the LV but not the RV. In agreement with this result, the histologic analysis showed a similar profile of the transverse diameter of the cardiomyocytes. In the RV, the transverse diameter was higher in the HS and HS+HZ groups than in the other groups. Moreover, the larger transverse diameter of the cardiomyocytes in the RV induced by a HS diet also indicates that this is independent of increased BP because the RV is not subjected to systemic BP. Another finding that supports this information is that, in the RV, the HS+NAC group showed a complete prevention of the increase in the transverse diameter of the cardiomyocytes, which was dissimilar to the partial prevention that occurred in the LV (likely because of the very high BP

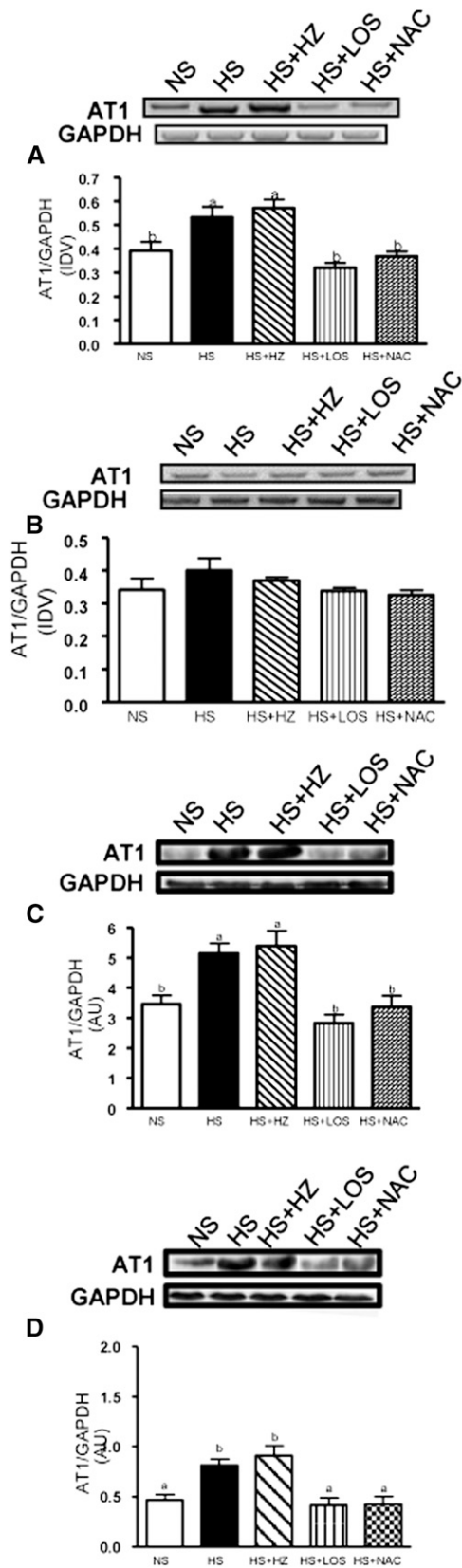


FIGURE 2 AT1 gene expression in the LV (A) and RV (B) and AT1 protein expression of the LV (C) and RV (D) of 18 wk-old-rats fed diets of varying salt concentrations and untreated or treated with HZ, LOS, or NAC. Data are means \pm SEMs, $n = 6-8$. Labeled means without a common letter differ, $P < 0.05$. AT1, angiotensin II type 1 receptor; AU, arbitrary units; HS, high salt (8% NaCl); HS+HZ, HS+hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); HZ, hydralazine; LOS, losartan; NAC, N-acetylcysteine; NS, normal salt (1.27% NaCl).

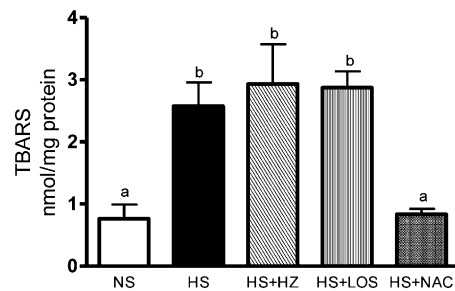


FIGURE 3 TBARS on the myocardium of 18-wk-old rats fed diets of varying salt concentrations and untreated or treated with HZ, LOS, or NAC. Data are means \pm SEMs, $n = 6$ /group. Labeled means without a common letter differ, $P < 0.05$. HS, high salt (8% NaCl); HS+HZ, HS+hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); HZ, hydralazine; LOS, losartan; NAC, N-acetylcysteine; NS, normal salt (1.27% NaCl).

levels in this group). The transverse diameter of the HS+LOS group was not different from the NS group, suggesting that the salt-induced CH was due to AT1 activation. Interestingly, the treatment with LOS prevented the formation of IF in both ventricles. In agreement with this result, a study by Krämer et al. (30) showed that LOS possesses anti-inflammatory properties via its metabolite EXP3179 and that this property is independent of the effect of the AT1 blocker. In a previous study from our laboratory using the same experimental protocol, LOS treatment did not prevent fibrosis. Further studies are needed to explain this discrepancy. One possible explanation for the observed prevention of IF but not of cardiomyocyte hypertrophy via HZ is that this drug also has some anti-inflammatory properties. Neves et al. (31) observed that HZ attenuated the deposition of type I and III collagen and perivascular fibrosis in rats administered AII infusions. Corroborating these results, a study conducted in our laboratory by Ferreira et al. (9) observed that left and right ventricular fibrosis induced via salt overload was prevented via HZ in rats subjected to the same experimental protocol, suggesting an anti-inflammatory effect of this drug.

An increased TBARS concentration in the myocardium of the groups fed an HS diet was prevented by NAC, thus confirming its antioxidant effect.

With the intent to elucidate the possible mechanisms involved in the development of cardiomyocyte hypertrophy and IF induced by salt, the present study attempted to evaluate the chronic effect of this diet on circulating and tissue RAS. In this study, salt overload caused an increase in AII content in the cardiac tissue of the HS, HS+HZ, HS+LOS, and HS+NAC groups and a decrease in serum aldosterone concentration in rats from the same groups than in the NS group, reinforcing the finding that there is a local myocardial RAS and that this is regulated independently of the circulating RAS (10,13). Furthermore, these results suggest that the circulating RAS may not be involved in the development of CH in this experimental model.

In an attempt to understand the effect of the increased myocardial AT1 gene and protein expression in both ventricles of the HS and HS+HZ groups, a functional study was performed by using anti-AT1 and anti-AT2 conformation-specific antibodies that preferentially recognize the activated form of the receptor. The binding of the conformation-specific antibody anti-AT1 was increased in

HS+N-acetylcysteine (600 mg/L); HZ, hydralazine; IDV, integrated density value; LOS, losartan; LV, left ventricle; NAC, N-acetylcysteine; NS, normal salt (1.27% NaCl); RV, right ventricle.

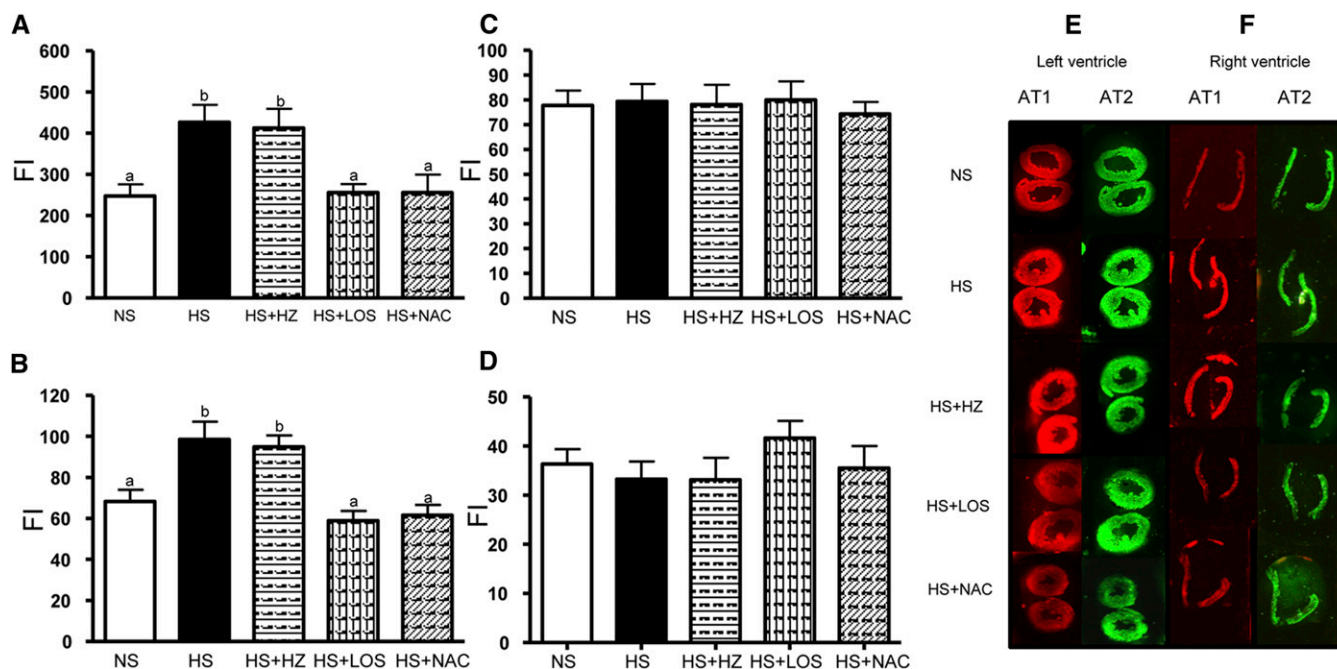


FIGURE 4 Affinity of the conformation-specific anti-AT1 antibody in the LV (A) and RV (B), affinity of the conformation-specific anti-AT2 antibody in the LV (C) and RV (D), and representative images showing binding of conformation-specific anti-AT1 and anti-AT2 antibody in the LV (E) and RV (F). Data are means \pm SEMs, $n = 7-8$. Labeled means without a common letter differ, $P < 0.05$. AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; FI, fluorescence intensity; HS, high salt (8% NaCl); HS+HZ, HS+hydralazine (15 mg·kg⁻¹·d⁻¹); HS+LOS, HS+losartan (20 mg·kg⁻¹·d⁻¹); HS+NAC, HS+N-acetylcysteine (600 mg/L); LV, left ventricle; NS, normal salt (1.27% NaCl); RV, right ventricle.

the LV of the rats in the HS and HS+HZ groups compared with the other experimental groups. When considering these findings together with the increased AII content in the groups that were fed an HS diet, it can be stated that the CH in this experimental model was at least partially due to AT1 activation by AII. The increase in the AT1 gene and protein expressions can be explained through several studies of Zou et al. (32) that showed that mechanical stress can induce CH *in vivo* by the activation and increased expression of AT1 without the involvement of AII; however, this did not appear to occur in this study. This is because, in addition to the AII increase in the groups fed an HS diet, there was no increased cardiac mechanical stress in the HS+HZ group, although the HS+HZ group had an increased AT1 expression. The opposite occurred with the HS+NAC group—despite showing an increase in cardiac mechanical stress, the rats in this group had no increase in the expression of this receptor.

Interestingly, the binding of the anti-AT1 conformation-specific antibody in the HS+NAC group was similar to that of the control group. A possible explanation for this finding is that some antioxidants such as NAC may have an important action as reducers of disulfide bonds. These antioxidants have free sulfhydryl groups that may interact with the disulfide bonds of the AT1, altering its tertiary structure and resulting in the inhibition of the interaction between AII and its AT1 (33).

The AT2 is not linked to salt-induced CH because the gene and protein expression as well as the binding of the conformation-specific anti-AT2 did not differ between groups.

We have no explanation for the higher myocardial chymase protein expression in the LV, but not in the RV, in the HS+HZ group. Further studies are required to understand this phenomenon.

The results of this study suggest a cardioprotective effect of sodium overload that is independent of BP increases. Furthermore, it can be stated that the CH in this experimental model developed via the AT1 because the transverse diameter of the cardiomyocytes, the gene and protein expression, and the activation

of the AT1 in both ventricles were not higher in the HS+LOS group than in the NS group, indicating that blocking this receptor can prevent the development of CH.

Another important observation was the involvement of oxidative stress in the development of CH in this experimental model. The treatment with NAC improved the degree of hypertrophy likely because of its ability to alter the structure of the AT1 (33) and prevent the binding of AII without reducing BP levels.

In addition to evaluating the cardiomyocyte transverse diameter, it would have been informative to measure myocardial hypertrophy markers, such as protein turnover, atrial natriuretic factor, β -myosin heavy chain, and skeletal α -actin. The determination of cardiomyocyte volume is potentially a better indicator of hypertrophy than the transverse diameter. Therefore, hypertrophy could be measured via stereology.

An additional experimental group fed an HS diet, treated with NAC, and pair-fed with the other groups would provide more precise information on the effect of this drug.

The reduction in BP in the group of rats fed the NS diet was unexpected, and there is no plausible explanation for this finding. Perhaps the method of measuring BP may have influenced the result, which warrants confirmation by other methods such as telemetry or intra-arterial BP measurement. Similarly, it would also be advisable to confirm the increase in BP in response to HS intake in non-salt-sensitive rats with another method of BP evaluation.

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primarily responsible for the final content. All authors read and approved the final manuscript.

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