ORIGINAL ARTICLE

Influence of Long-Term Salt Diets on Cardiac Ca^{2+} Handling and Contractility Proteins in Hypertensive Rats

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BACKGROUND
High sodium intake contributes to the pathogenesis of hypertension and adversely affects cardiac function. Conversely, sodium reduction is associated with a blood pressure decrease and improved cardiovascular function. However, the mechanisms that underlie the cardiac effects induced by salt intake in hypertension have not been fully elucidated. Ca^{2+} handling is critical for efficient myocardial function; thus, we aimed to investigate the long-term effects of diets with different salt contents on cardiac function and Ca^{2+} handling proteins in spontaneously hypertensive rats (SHRs).

METHODS
Cardiac function was evaluated by catheterization. Ca^{2+} handling and contractile proteins were evaluated by immunoblotting in hearts from SHRs fed for 6 months with diets containing high (HS, 3%), low (LS, 0.03%), or normal salt content (NS, 0.3%). Diets were introduced immediately after weaning. Tail cuff pletismography was assessed at the 3rd and 7th months of follow-up.

RESULTS
Compared to the NS group, the HS group exhibited worsened hypertension, increased cardiac expression of β-myosin heavy chain (MHC), a decreased α/β-MHC ratio and reduced expression of both phospholamban (PLB) and Na^+/Ca^{2+} exchanger (NCX). LS intake attenuated the blood pressure increase and left ventricle hypertrophy, slightly decreased the cardiac contractility and relaxation index, and increased the α/β-MHC ratio. These effects were accompanied by increased cardiac PLB expression and decreased Ca^{2+} L-type channel and NCX expression.

CONCLUSIONS
These findings indicate that the modulation of Ca^{2+} handling may be one of the molecular mechanisms underlying the effect of salt intake on myocardial function in hypertension.

Keywords: blood pressure; calcium handling; cardiac function; hypertension; salt diet; SHR.

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Evidence has indicated the deleterious effects of high sodium intake, including hypertension development and damage to target organs.[1–3] In parallel with its influence on blood pressure, salt loading promotes left ventricular remodeling and fibrosis, impairs coronary hemodynamics, and induces cardiomyocyte apoptosis and oxidative stress.[4–6] Furthermore, the exposure to extremely high-salt diets is particularly associated with left ventricular diastolic dysfunction in hypertensive animals.[4–7] Conversely, a low sodium diet has been shown to reduce blood pressure and promote renoprotection.[3,8] However, the underlying molecular mechanisms of how different concentrations of sodium in the diet affect the cardiovascular system in hypertension, particularly the mechanisms that underlie the beneficial effects of a low-salt diet on the heart, remain subject of major discussion.

The sodium concentration in myocytes has a pivotal role in excitation-contraction coupling (EC coupling) because changes in Na^+ regulate Na^+/Ca^{2+} exchange (NCX), thus affecting the cytosolic and sarcoplasmic reticulum (SR) Ca^{2+} concentrations and myocardial contractility.[9,10] Although studies have described the adverse effects of salt intake on cardiac function, the molecular effects of a high-salt diet on hypertensive myocardium, particularly its effects on Ca^{2+} handling proteins, have not yet been determined.

The spontaneously hypertensive rat (SHR) is a helpful genetic model of hypertension with progressive cardiovascular failure induced by cardiac pressure overload.[11,12] In this model, changes in myocardial Ca^{2+} handling have been described at very early stages before the development of heart failure.[13,14] Furthermore, evidence has shown that cardiac remodeling as a result of workload in SHRs affects EC coupling and Ca^{2+} handling.[15,16] SHRs exhibit a markedly prolonged action potential duration and increased systolic Ca^{2+} transient, which occur before the development of hypertrophy.[13] However, the involvement of salt intake and cardiomyocyte Ca^{2+} handling in hypertensive diseases has been poorly explored. Therefore, this study aimed to investigate the long-term effects of diets with different salt contents on cardiac structure and function in SHRs and the potential effects of this dietetic intervention on the expression of key proteins involved in myocardial EC coupling.

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METHODS

Animals and experimental groups

The study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and the Ethical Principles of the Brazilian College of Animal Experimentation (COBEA), and it was approved by the Institutional Ethics Committee on Animal Research and the Institutional Animal Research Committee (Process nº 053/2012). The experimental groups were composed of male SHRs divided into 3 groups that were fed isocaloric and isoproteic diets that differed only in their sodium content. The standard normal-salt diet (NS) contained 0.3% (w/w) NaCl. The low-salt (LS) and the high-salt diet (HS) were prepared by reducing the salt content by 10-fold (0.03% NaCl) or increasing it by 10-fold (3% NaCl), respectively (PragSoluções, São Paulo, Brazil). These diets were introduced immediately after weaning (4 weeks) and maintained for the next 6 months. The animals were maintained in a 12-h light-dark cycle in a temperature-controlled room (22–25 °C) and had free access to water and chow during the entire experimental period.

Noninvasive blood pressure measurement

Systolic blood pressure was measured by noninvasive tail cuff pletismography at the ages of 3 and 7 months as previously described.[17]

Hemodynamic parameters

Cardiac function was evaluated by catheterization at the end of the 7th month on the experimental diets. For detailed information about hemodynamic parameters, please, see Supplementary Information.

Metabolic parameters

During the last week on experimental diets, the animals were accommodated in individual metabolic cages to evaluate metabolic parameters and renal function (see Supplementary Information). At the end of hemodynamic recordings, an arterial blood sample was collected and immediately centrifuged at 4 °C for 15 minutes at 1400 g for analysis of creatinine, sodium and potassium serum concentration. For detailed information about it, please, see Supplementary Information.

Histological analysis

The animals were euthanized with an overdose of anesthetic. The ventricles, lungs, kidneys, and liver were excised and weighed. The ventricles were then rapidly flushed in cold saline solution and fixed in paraformaldehyde (4%, pH 7.4) for at least 24 hours before they were embedded in paraffin for histological analysis. Slices at 4-µm thickness were deparaffinized with xylene, rehydrated with a descending ethanol gradient and stained with hematoxylin/eosin or picrosirius red. The latter was used to evaluate the extent of interstitial fibrosis as determined by the hot-pink color fraction and its intensities as quantified by ImageJ software. Perivascular collagen was excluded from this determination. Morphometric analyses were performed, as previously reported,[18,19] by measuring the area of myocyte nuclei per high-power field. These analyses were performed using the histological analysis software Motic Plus (Motic Inc., Canada). The images were acquired using an AxioCam (Carl Zeiss MicroImaging, Germany) attached to a camera at 400× magnification, and the images were collected with AxioVision Imaging System 4.8 software (Carl Zeiss MicroImaging, Germany). For each sample, we evaluated at least 10 high-power fields. A single examiner blinded to diet groups performed all histological measurements.

Immunoblot analysis

Left ventricular tissues were homogenized and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting as previously described.[20] The entire blots of all proteins and specific primary antibodies are presented in the Supplementary Information.

Statistical analysis

Data are presented as mean ± SEM. Comparisons among the means were assessed using 1-way analysis of variance followed by the Bonferroni *post hoc* test. All statistical analyses were performed with the PRISMA 13.0 software (Chicago, IL). Statistical significance was set at $P < 0.05$.

RESULTS

Morphologic parameters and blood pressure

Table 1 shows the morphologic parameters of the heart at the end of treatments. The body weight of the LS group was lower than that of the other 2 groups. Moreover, the crude and relative weight of the ventricles were smaller in the LS group, and the differences between the HS and NS groups were not significant. Absolute weights of the lungs, liver, and kidneys of the rats in the LS group were lower than those in the other 2 groups. However, the relative weights of these organs were similar among the 3 groups.

The tail cuff blood pressure was attenuated in the rats fed the LS diet (Figure 1) during the follow-up period (3rd month: NS: 178 ± 4; LS: 153 ± 5; and HS: 178 ± 5 mm Hg). The blood pressure of the HS group was significantly higher than that of the NS group (7th month: NS: 211 ± 3; LS: 167 ± 2; and HS: 230 ± 5 mm Hg) only at the last evaluation.

Hemodynamic data and metabolic parameters

Table 2 shows the hemodynamic data obtained during arterial and LV catheterization in the anesthetized animals. The LS group showed the lowest systolic pressure and a lower LVSP than the other 2 groups, but the DBP of the LS group was only lower than the HS group. The HR was slightly higher in the LS group than in the HS group. LV catheterization

Table 1. Long-term effects of salt diets on ponderal parameters in SHR

	NS	LS	HS
Body weight (g)	310 ± 5	245 ± 4*#	312 ± 8
Ventricles (mg)	1262 ± 32	851 ± 39*#	1372 ± 34
Ventricles/BW (mg/g)	4.1 ± 0.2	3.5 ± 0,1*#	4.2 ± 0.1
Lungs (mg)	1999 ± 74	1467 ± 60*#	2045 ± 11
Lungs/BW (mg/g)	6.5 ± 0.3	6.1 ± 0.2	6.3 ± 0.3
Liver (mg)	11940 ± 281	9543 ± 343*#	12370 ± 343
Liver/BW (mg/g)	39 ± 1	40 ± 1	38 ± 1
Kidney (mg)	1161 ± 37	952 ± 28*#	1235 ± 46
Kidney/BW (mg/g)	3.7 ± 0.1	3.9 ± 0.1	3.9 ± 0.1

Values are presented as the mean ± SEM. NS: normal-salt diet (N = 13). LS: low-salt diet (N = 14). HS: high-salt diet (N = 11). *P < 0.05 vs. NS; #P < 0.05 vs. HS. Abbreviations: BW, body weight; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat.

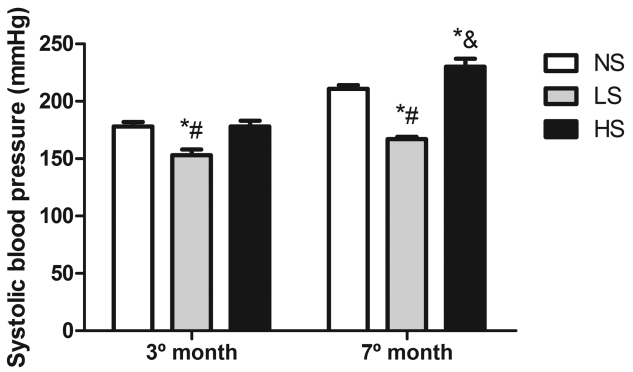

Figure 1. Systolic blood pressure evaluated by tail pletismography. Values are presented as the mean ± SEM. SBP: systolic blood pressure. NS (N = 8): normal-salt diet. LS (N = 6): low-salt diet. HS (N = 8): high-salt diet. *P < 0.05 vs. NS; #P < 0.05 vs. HS; and &P < 0.05 vs. LS.

showed that the indexes of systolic and diastolic performance (assessed by +dP/dt max and −dP/dt max, respectively) were slightly reduced in the SHRs fed the low-salt diet compared with those fed the standard normal-salt diet.

Changes in the sodium content of the diet affected water intake, urinary output, and urinary sodium concentration in the HS diet without influence in the glomerular filtration rate or serum sodium concentrations in the SHRs, suggesting a preservation in hydroelectrolytic balance and kidney autoregulatory function (see in Supplementary Information).

Histological analysis

As we identified reduced systolic blood pressure and DBP values in the LS group that were also associated with a slight reduction in the LV diastolic and systolic performance, we investigated whether morphological alterations in the myocardium could explain the functional findings. The interstitial area covered by collagen was unaffected by the dietary salt content (NS: 3.9 ± 0.2%; LS: 3.8 ± 0.2%; and HS: 4.1 ± 0.3%, % Picrosirius stain, Figure 2). Regarding the morphological analysis, the long-term HS diet did not increase cardiac hypertrophy in the SHRs. However, in accordance with the ventricles/body weight index, a significant reduction in cardiac hypertrophy appeared to occur in the animals fed the LS diet, as indicated by the significant reduction in the area of myocyte nuclei (NS: 913 ± 14; LS: 702 ± 46; and HS: 983 ± 59, μm^2, P < 0.05, Figure 2).

Table 2. Long-term effects of salt diets on hemodynamic parameters in SHR

	NS	LS	HS
HR (bpm)	198 ± 4	209 ± 9#	185 ± 5
SBP (mm Hg)	135 ± 4	119 ± 5*#	140 ± 4
DBP (mm Hg)	83 ± 3	78 ± 2#	92 ± 3
LVSP (mm Hg)	147 ± 5	127 ± 5*#	146 ± 3
dP/dt+ (mm Hg/s)	5430 ± 146	4681 ± 170*	5052 ± 117
dP/dt− (mm Hg/s)	−4615 ± 195	−3928 ± 231*	−3983 ± 140

Values are presented as the mean ± SEM. NS: normal-salt diet, N = 12. LS: low-salt diet, N = 13. HS: high-salt diet, N = 10. *P < 0.05 vs. NS; and #P < 0.05 vs. HS. Abbreviations: DBP, diastolic blood pressure; HR, heart rate; LVSP, left ventricular systolic pressure; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat.

Expression of contractile proteins

The expression of proteins directly related to the myocardial contractile performance (α-MHC and β-MHC isoforms) was determined in the left ventricle. As depicted in Figure 3, the SHRs fed the LS diet displayed a higher α/β-MHC protein ratio. The HS diet increased the β-MHC expression, which resulted in a decreased α/β-MHC ratio.

Expression of proteins involved in Ca^{2+} handling

The expression of 5 proteins (LTCC, PLB, SERCA2a, NCX, and RYR) involved in calcium homeostasis and myocardial contractility was also compared among the SHRs that were fed diets with different sodium contents. The SERCA2a and RYR expression was unaffected by the changing sodium levels in the diet. The SHRs that were fed the LS diet showed a reduced expression of LTCC and NCX as well as an increased expression of phospholamban (PLB). Moreover, the HS diet decreased the PLB and NCX expression without influencing LTCC (Figures 4 and 5). A summary of these findings is shown in Figure 6.

DISCUSSION

This study was designed to investigate the long-term effects of diets that contain different salt contents on cardiac function in SHRs and their potential effects on the expression of key proteins involved in myocardial EC coupling. The main findings of this study are summarized as follows: (i) Prolonged high dietary salt intake increases systolic blood pressure; however, this moderately HS content (3%) does not lead to

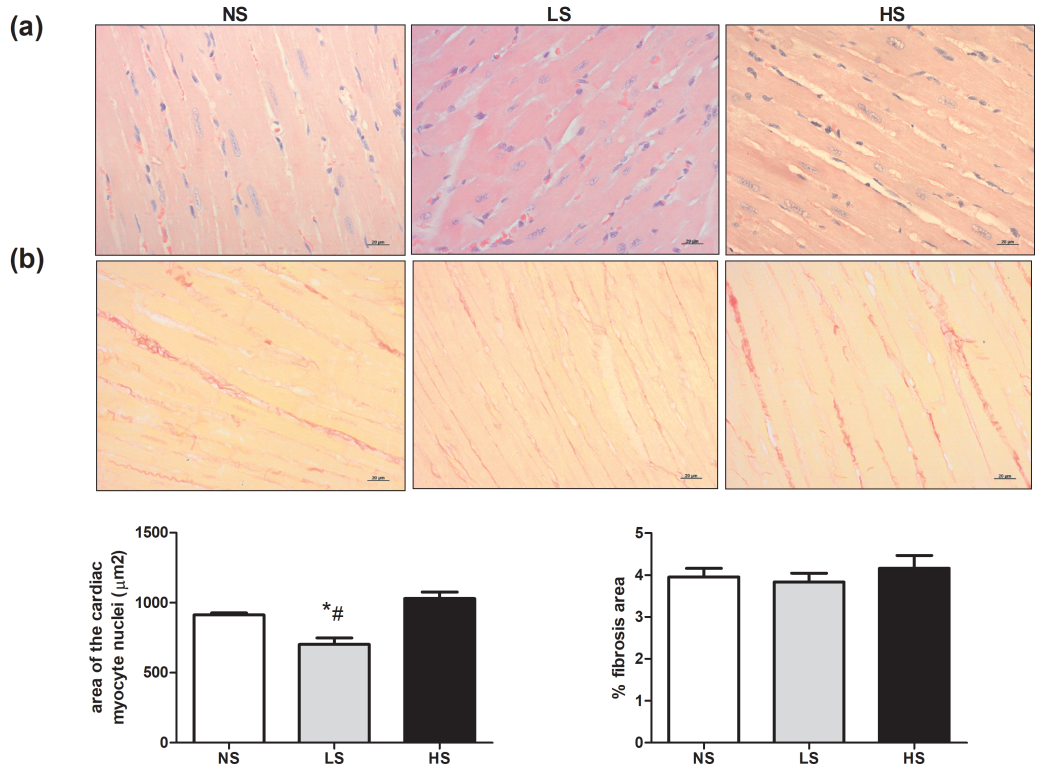

Figure 2. Histological analysis. (**a**) Photomicrographs of ventricular sections stained with hematoxylin/eosin to detect hypertrophy (area of cardiac myocyte nuclei) in the groups. (**b**) Photomicrographs of ventricle sections stained with picrosirius red (PC) to determine interstitial collagen deposition. Values are presented as the mean ± SEM. NS: normal-salt diet ($N = 4$). LS: low-salt diet ($N = 6$). HS: high-salt diet ($N = 5$). *$P < 0.05$ vs. NS; #$P < 0.05$ vs. HS Scale bar: 20 µm.

an increase in ventricular weight or affect LV hypertrophy or fibrosis, whereas salt restriction attenuates hypertension, which is associated with a decrease in ventricular mass and an improvement in hypertrophy. (ii) HS intake promotes a shift of the MHC phenotype toward an upregulation of β-MHC protein, which results in a decreased α/β-MHC ratio. In contrast, SHRs fed a low-salt diet exhibit an increased α/β-MHC ratio. (iii) A long-term high-salt diet reduces cardiac NCX and PLB protein expression, whereas SHRs subjected to prolonged salt restriction present a reduction in cardiac LTCC and NCX protein expression as well as an increased PLB expression. These results indicate that the effects of salt intake on cardiac function during hypertension are complex and may explain some contractility disturbances in these conditions.

As observed in our study, prolonged HS intake (3% for 6 months) in SHRs increased the systolic blood pressure by the end of the follow-up without worsening LV hypertrophy or fibrosis, which suggests that the hypertrophic response occurs after the blood pressure increase and changes in EC coupling.[13] It is worth nothing that previous studies using a very high (8% NaCl) salt content in the diet for shorter periods of time reported the development of major ventricular dysfunctions in SHRs with concomitant myocardial fibrosis and coronary circulation impairment.[5,6] However, Leenen *et al.*[21] previously showed that a 2% sodium diet for 4, 8, and 12 weeks did not change the LV weight or mean arterial pressure in SHRs; changes were observed only when the sodium intake was excessively high (8% salt diet).

The MHC protein is a dimer formed by α and β filaments. Several pathological stimuli may shift the MHC composition in the rat heart,[22,23,24] and changes in the proportion of these proteins are also directly related to myocardial mechanical performance.[25] Pressure overload, as observed in hypertension, is associated with the development of cardiac hypertrophy, which, in turn, shifts the isoform distribution toward β-MHC with a simultaneous downregulation of the α-MHC isoform. However, when the load is removed, these alterations are normalized.[22,23,26] Here, we showed that SHRs subjected to a long-term high-salt diet decreased the α/β-MHC ratio, whereas a low-salt diet for the same period of time increased this ratio. The mechanisms by which salt intake shifts MHC gene expression remain unclear. A study in cultured cardiac atrial myocytes showed that a small increase in the intracellular Na^+ concentration activated myocyte enhancer factor (MEF)2/nuclear factor of activated T cell (NFAT) transcriptional activity, which led to an increase in the expression of MHC genes. Salt-inducible kinase 1 (SIK1) activity was crucial for the β-MHC increase in response to increased intracellular sodium.[27] Thus, it is tempting to speculate that in response to salt intake, myocardial cells are able to trigger myocyte signaling and modulate transcriptional activation as well as gene expression.

We have also shown that SHRs fed the LS diet exhibited an attenuation of hypertension with a concurrent and slight decrease in d*P*/d*t* values. It has been shown that in the

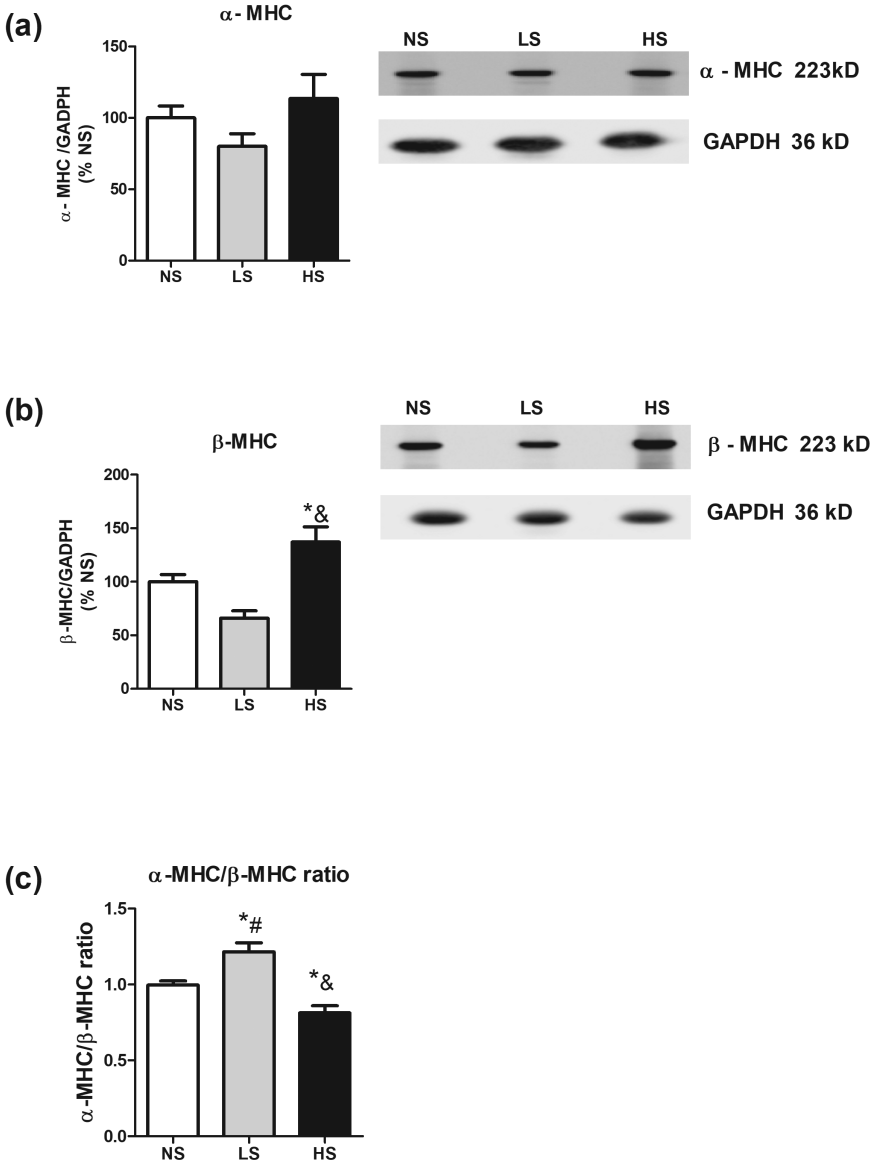

Figure 3. Left ventricular á-MHC and â-MHC protein expression and ratio. (**a**) α-MHC. (**b**) β-MHC. (**c**) α/β-MHC ratio. Values are presented as the mean ± SEM. $N = 6$. $^{*}P < 0.05$ vs. NS. $^{\#}P < 0.05$ vs. HS. $^{\&}P < 0.05$ vs. LS. Abbreviations: HS, high-salt diet; LS, low-salt diet; MHC, myosin heavy chain; NS, normal-salt diet.

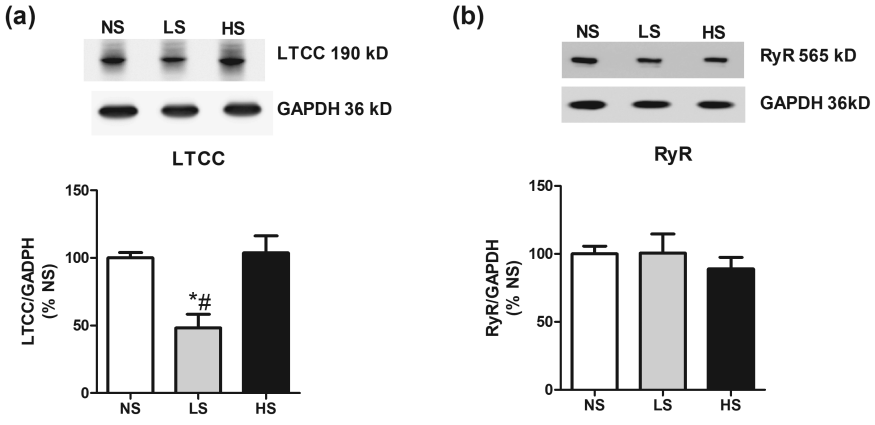

Figure 4. Left ventricular LTCC and RYR protein expression. Values are presented as the mean ± SEM. (**a**) LTCC, L-type Ca^{2+} channel. (**b**) RYR, ryanodine receptor. $N = 6$. $^{*}P < 0.05$ vs. NS. $^{\#}P < 0.05$ vs. HS. Abbreviations: HS, high-salt diet; LS, low-salt diet; NS, normal-salt diet.

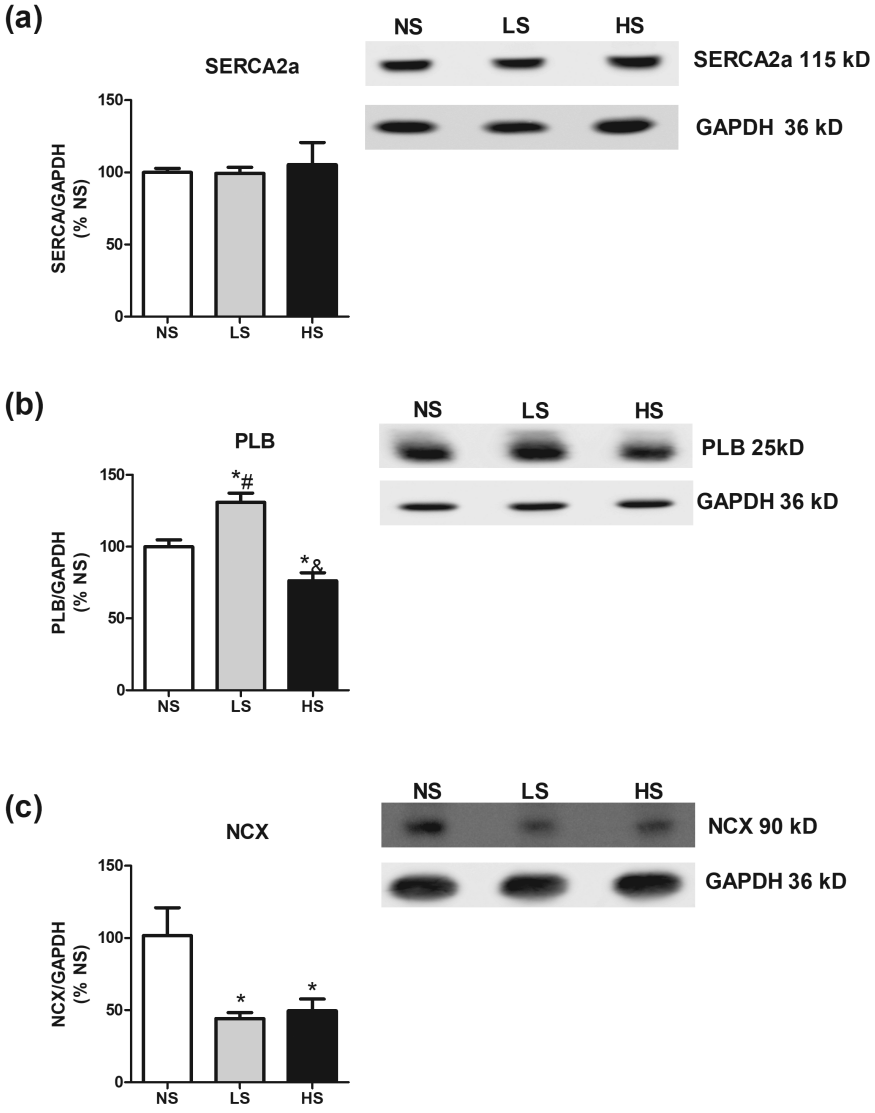

Figure 5. Left ventricular SERCA2a, PLB, and NCX protein expression. Values are presented as the mean ± SEM. (**a**) SERCA2a: RS Ca^{2+}-ATPase. (**b**) PLB: phospholamban. (**c**) NCX: Na^+/Ca^{2+} exchanger. $N = 6$. $^*P < 0.05$ vs. NS. $^{\#}P < 0.05$ vs. HS. $^{\&}P < 0.05$ vs LS. Abbreviations: HS, high-salt diet; LS, low-salt diet; NS, normal-salt diet.

compensation phase, the heart in SHR shows an enhanced contractility and Ca^{2+} transient, which may partially compensate for a greater workload to maintain cardiac output.[13,28] Thus, our findings suggest a reflex of reduced overload in the left ventricle by a decrease in blood pressure. Furthermore, as previously proposed *in vitro* studies with cultured heart cells, when contractility is inhibited, α-MHC RNA expression is upregulated, and β-MHC levels are downregulated.[29,30] Thus, the changes in the contractility index identified in the LS group in our study may be related to the shift in α- and β-MHC expression.

Ca^{2+}-regulating proteins exert direct effects on myocardial function. The L-type Ca^{2+}-channel (LTCC) is responsible for the characteristic plateau of the action potential. Ca^{2+} entry induces ryanodine receptors (RYR), which triggers Ca^{2+} release from the SR, thus increasing the level of free intracellular Ca^{2+} and enabling contractile activation. During diastole, Ca^{2+} is mainly pumped from the cytosol by SERCA2a, which takes Ca^{2+} back into the SR (this determines Ca^{2+} stores and cardiac contractility). In parallel, Ca^{2+} is also removed from the cytosol by sarcolemmal NCX-1, which mediates Ca^{2+} and Na^+ exchange and the contribution of Na^+ to EC coupling regulation.[31]

SHRs exhibit altered Ca^{2+} handling even before the appearance of heart failure. Early cardiac compensation in response to hypertension includes the lengthening of the action potential duration and an EC coupling gain,[13] which may be a result of changes in the interaction between RYR and LTCC in junctional SR, as previously reported.[28] Moreover, in adult SHR hearts, Zwaldlo *et al.*[14] identified disease-associated changes in major cardiac ion channels, ion exchangers, and cytoskeletal proteins, including an enhancement of SERCA2a, LTCC, NCX, and PLB mRNA expression.

In the present study, in addition to hypertension attenuation, long-term salt restriction decreased the

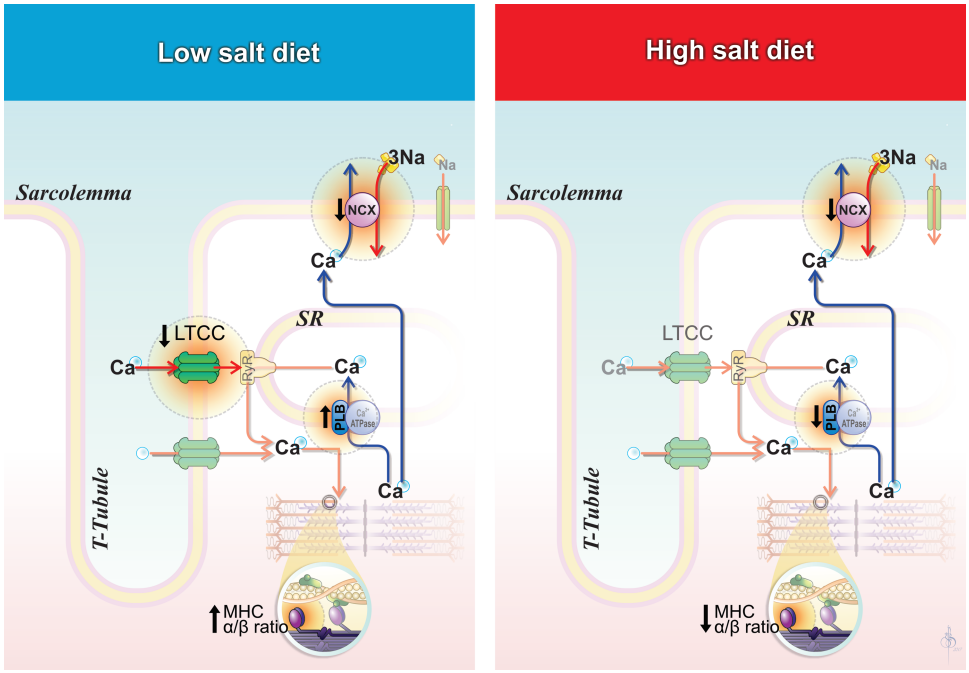

Figure 6. Effects of long-term salt diets on cardiac Ca^{2+} handling and contractility proteins in SHRs. Abbreviations: LTCC, L-type Ca^{2+} channel; β-MHC, β-myosin heavy chain, α-MHC, α-myosin heavy chain; NCX, Na^{+}/Ca^{2+} exchanger; PLB, phospholamban; RYR, Ryanodine Receptor; SERCA2a, Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

LTCC protein levels in the left ventricle, which suggests a decrease in the Ca^{2+} induced Ca^{2+} release from the SR as a result of the cardiac workload reduction. Furthermore, the PLB expression was enhanced in these animals without changes in SERCA2a levels, which was associated with a decrease in NCX protein expression. PLB has an inhibitory effect on SERCA2a activity, thereby decreasing the affinity for Ca^{2+} with consequent effects on SR Ca^{2+} stores and contractility.[32] Thus, the observed PLB enhancement may have contributed to the decrease in the SR Ca^{2+} load and may be associated with the slight decrease in the contractility index. Moreover, as previously demonstrated, PLB overexpression alters LTCC kinetics and decreases systolic Ca^{2+} levels, thus changing contractile parameters in cardiomyocytes.[33,34] At the same time, long-term salt restriction reduced NCX levels. In this condition, the reduction of Ca^{2+} removal in parallel with a reduction of the Ca^{2+} influx may restore the Ca^{2+} steady-state; that is, cellular Ca^{2+} extrusion must equally match Ca^{2+} entry.[35,36] Thus, together with the increase in the α/β-MHC ratio and the attenuation of blood pressure, it appears that an LS diet in SHRs led to a reduction in cardiac work by reducing the overload pressure with a decreased use of calcium for contraction. Moreover, the reduction of the PLB and NCX levels observed in SHRs that received the high-salt diet without changes in SERCA2a or LTCC, together with β-MHC upregulation and a blood pressure increase, suggest that a chronic 3% high-salt diet in SHRs could maintain contractility in the presence of an increased SR calcium load, which may increase cardiac energy expenditure and contribute to accelerate the impairment of ventricular function in hypertensive cardiomyopathy.

It was previously shown that adult SHRs subjected to a 4% high-salt diet for 12 weeks exhibited a left ventricular hyperkinetic state, whereas an 8% salt diet impaired ventricle contractility. In our study, the contractile index was similar between SHRs fed HS and NS diets. As PLB is a repressor of left ventricular contractility,[37] the observed decrease in PLB expression would play a key role in the maintenance of myocardial contractility in the presence of HS diets.

In addition to SR Ca^{2+} uptake by SERCA2a, Ca^{2+} extrusion by NCX contributes to myocyte relaxation, which thus contributes to diastolic function. Interestingly, previous studies have shown that high-salt diets may specifically promote relaxation impairment without major changes in systolic function.[4–6] Thus, the increase in diastolic Ca^{2+} may contribute to the diastolic dysfunction observed in many studies.

The specific mechanisms of how salt intake directly or indirectly alters calcium handling is unclear. Wang *et al.*[38] previously showed that a high-salt diet was associated with an increase in SIK1 expression in rat adrenal. Moreover, in response to an increase in intracellular sodium in atrial myocytes, SIK1 mediates the activation of MEF2/NFAT and genes associated with cardiac hypertrophy.[27] The main Ca^{2+}-dependent pathways related to the transcriptional control of several cardiac genes result in NFAT and MEF2 activation.[39] Thus, the involvement of these pathways remains to be determined.

In summary, our findings show that chronic HS intake contributes to progressive cardiac hypertensive disease with molecular cardiac changes toward pathological phenotypes. In contrast, low-salt diets not only reduced blood pressure but also affected intracellular calcium cycling, likely as a result of the load/stretch force. These findings suggest that changes in salt intake content can modify Ca^{2+}

handling, which may provide a rational for the mechanisms that underlie the cardiac effects observed following salt loading.

SUPPLEMENTARY DATA

Supplementary materials are available at *American Journal of Hypertension* online.

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DISCLOSURE

The authors declared no conflict of interest.

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