

ORIGINAL ARTICLE

Effects of salt status and blockade of mineralocorticoid receptors on aldosterone-induced cardiac injury

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The mineralocorticoid aldosterone regulates sodium and water homeostasis in the human body. The combination of excess aldosterone and salt loading induces hypertension and cardiac damage. However, little is known of the effects of aldosterone on blood pressure and cardiac pathophysiology in the absence of salt loading. We have now investigated the effects of salt status and blockade of mineralocorticoid receptors (MRs) on cardiac pathophysiology in uninephrectomized Sprague-Dawley rats implanted with an osmotic minipump to maintain hyperaldosteronism. The rats were fed a low-salt (0.0466% NaCl in chow) or high-salt (0.36% NaCl in chow plus 1% NaCl in drinking water) diet in the absence or presence of treatment with a subdepressor dose of the MR antagonist spironolactone (SPL). Aldosterone excess in the setting of low salt intake induced substantial cardiac remodeling and diastolic dysfunction without increasing blood pressure. These effects were accompanied by increased levels of oxidative stress and inflammation as well as increased expression of genes related to the renin–angiotensin and endothelin systems in the heart. All of these cardiac changes were completely blocked by the administration of SPL. On the other hand, aldosterone excess in the setting of high salt intake induced hypertension and a greater extent of cardiac injury, with the cardiac changes being only partially attenuated by SPL in a manner independent of its antihypertensive effect. The combination of dietary salt restriction and MR antagonism is thus a promising therapeutic option for the management of hypertensive patients with hyperaldosteronism or relative aldosterone excess.

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INTRODUCTION

The mineralocorticoid aldosterone contributes to the regulation of sodium and water homeostasis in the human body, but it has also been shown to induce cardiac remodeling and fibrosis as well as impairment of endothelial function.^{1,2} Both clinical and experimental evidence suggests that aldosterone or blockade of the mineralocorticoid receptor (MR) with spironolactone (SPL) or eplerenone modulates the pathophysiology of cardiovascular disease as well as hypertension.^{3,4} For example, the Randomized Aldactone Evaluation Study (RALES),⁵ the Eplerenone Post Acute Myocardial Infarction Heart failure Efficacy and Survival Study (EPHESUS),³ and Eplerenone in Mild Patients Hospitalization And Survival Study (EMPHASIS)⁶ have provided evidence that in the broad spectrum of patients with left ventricular (LV) systolic dysfunction and various degrees of heart failure, the addition of MR antagonism to angiotensin-converting enzyme (ACE) inhibition and/or β -blockade results in significant beneficial effects. Excess aldosterone in individuals with primary

aldosteronism is associated with LV hypertrophy and changes to the myocardium.⁷

Both the MR and 11 β -hydroxysteroid dehydrogenase 2, which limits activation of the MR by cortisol, are expressed in the human heart,⁸ consistent with the notion that aldosterone might affect the heart directly as well as through its pressor effects. Although *in vivo* studies implicate aldosterone in the development of LV hypertrophy, it is difficult to distinguish between direct effects of aldosterone on cardiomyocyte growth and secondary effects due to changes in blood pressure mediated through its regulation of renal sodium and potassium homeostasis. For instance, peripheral infusion of aldosterone in rats induces cardiac hypertrophy and fibrosis without increasing blood pressure.⁹ Furthermore, cardiac hypertrophy and fibrosis are attenuated by SPL at doses that do not ameliorate hypertension.¹⁰

Mineralocorticoid administration in the presence of excess dietary salt and unilateral nephrectomy induces a marked increase in blood

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pressure as well as in cardiac damage in animal models.¹¹ The combination of aldosterone and high salt in uninephrectomized rats has also been shown to induce inflammatory changes in the heart.¹² Most previous studies on cardiac injury induced by aldosterone infusion have been performed with animals subjected to unilateral nephrectomy, salt loading or both. The effects of aldosterone on blood pressure and cardiac pathophysiology in the absence of salt loading have thus remained largely uncharacterized. Animals with excess aldosterone did not develop cardiac hypertrophy on a low-salt (LS) diet, whereas they developed cardiac hypertrophy and fibrosis without blood pressure elevation when fed a normal-salt diet.^{13,14} Moreover, aldosterone-infused rats maintained on a LS diet did not develop hypertension, an increase in the heart weight to body weight ratio or cardiac fibrosis.¹ It therefore remains unclear whether aldosterone excess causes hypertension and cardiac injury in the absence of salt loading. We have now investigated the effects of high or low salt intake and MR blockade on cardiac pathophysiology in uninephrectomized rats with hyperaldosteronism.

METHODS

Animals and experimental protocols

Six-week-old male inbred Sprague-Dawley rats ($n=32$) were obtained from Japan SLC (Hamamatsu, Japan) and were handled in accordance with the guidelines of Nagoya University Graduate School of Medicine as well as with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). They were fed a 0.36% NaCl (normal-salt) diet after weaning. At 7 weeks of age, 26 animals were anesthetized with sodium pentobarbital (50 mg kg^{-1} , i.p.) and subjected to unilateral nephrectomy. At 1 week after the surgery, an Alzet 2004 osmotic minipump (Alza, Palo Alto, CA, USA) was implanted subcutaneously into the animals for the delivery of *d*-aldosterone (Sigma, St Louis, MO, USA) at a rate of $0.75 \mu\text{g h}^{-1}$ for 4 weeks. The aldosterone was dissolved in 0.9% NaCl containing 0.5% ethanol. The uninephrectomized rats were then randomly allocated to four groups: (1) the LS group ($n=6$), in which the animals received a LS diet (0.0466% NaCl in chow) and were treated with aldosterone; (2) the LS + SPL group ($n=6$), in which the animals were fed a LS diet and were treated with both aldosterone and SPL; (3) the HS group ($n=8$), in which the animals were maintained on a high-salt (HS) diet and were treated with aldosterone; and (4) the HS + SPL group ($n=6$), in which the animals received a HS diet and were treated with both aldosterone and SPL. In the HS and HS + SPL groups, the rats were provided with normal-salt (0.36% NaCl) chow supplemented with 1.0% NaCl solution as drinking water. Spironolactone (Sigma) was administered orally via a gastric tube at a dose of 20 mg kg^{-1} per day, which was determined on the basis of results of a previous study.¹⁵ Rats not subjected to surgery and fed a normal-salt diet were studied as controls (CNT group, $n=6$). Both diets and drinking water were provided *ad libitum* throughout the experimental period. At 12 weeks of age, the animals were anesthetized by i.p. injection of ketamine (50 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and were subjected to echocardiography. The heart was subsequently excised, and the LV tissue was separated for analysis.

Echocardiographic analysis

Systolic blood pressure (SBP) was measured weekly in conscious animals by tail-cuff plethysmography (BP-98 A; Softron, Tokyo, Japan). At 12 weeks of age, rats were subjected to transthoracic echocardiography, as described previously.¹⁶ In brief, M-mode echocardiography was performed with a 12.5-MHz transducer (Xario SSA-660A; Toshiba Medical Systems, Tochigi, Japan). In addition, the pulsed Doppler echocardiographic data were analyzed for the assessment of LV diastolic function.¹⁷

Histological and immunohistochemical analysis

LV tissue was fixed in ice-cold 4% paraformaldehyde for 48–72 h, embedded in paraffin and processed for histology as described.¹⁸ Sections were stained either with hematoxylin-eosin for routine histological examination or with Azan-Mallory solution for evaluation of the extent of fibrosis. For the evaluation of

macrophage infiltration into the myocardium, frozen sections were subjected to immunostaining with antibodies to the monocyte-macrophage marker CD68.¹⁹ The number of immunoreactive macrophages in the myocardial interstitium was counted in five separate high-power fields of each section and is presented as CD68-positive cells per square millimeter. Image analysis was performed with NIH Scion Image software (Scion, Frederick, MD, USA).

Assay of superoxide production

NADPH-dependent superoxide production by homogenates prepared from freshly frozen LV tissue was measured with an assay based on lucigenin-enhanced chemiluminescence as described previously.^{20,21} The chemiluminescence signal was sampled every minute for 10 min with a microplate reader (Wallac 1420 ARVO MX/Light; Perkin-Elmer, Waltham, MA, USA), and the respective background counts were subtracted from experimental values.

Superoxide production in tissue sections was also examined as described.²⁰ Sections stained with dihydroethidium (Sigma) were thus examined with a fluorescence microscope equipped with a 585-nm long-pass filter. As a negative control, sections were incubated with superoxide dismutase (300 U ml^{-1}) before staining with dihydroethidium; such treatment prevented the generation of fluorescence signals (data not shown). The average of dihydroethidium fluorescence intensity values was calculated with NIH ImageJ software.²²

Quantitative reverse transcription-PCR analysis

Total RNA was extracted from LV tissue and subjected to quantitative reverse transcription (RT) and polymerase chain reaction (PCR) analysis as described,^{23–25} with primers and TaqMan probes specific for rat cDNAs encoding atrial natriuretic peptide,²³ brain natriuretic peptide,²³ collagen type I or type III,²² monocyte chemoattractant protein-1,²⁵ ACE,²³ the p22^{phox},²² gp91^{phox},²² p47^{phox},²⁶ p67^{phox},²⁶ or Rac1²⁶ subunits of NADPH oxidase, transforming growth factor- β 1,²⁷ the angiotensin II type 1A receptor (AT_{1A})²³ or serum- and glucocorticoid-regulated kinase-1.²² Sequences of primers and TaqMan probes specific for rat cDNAs encoding fibronectin, endothelin (ET)-1, the ET type A receptor, the ET type B receptor or ET-converting enzyme-1 are listed in Table 1. Reagents for detection of human

Table 1 Sequences of oligonucleotide primers (forward and reverse) and detection probes for RT-PCR analysis

Target cDNA	Sequence	GenBank accession no.
<i>Fibronectin</i>		NM_010233
Forward	5'-CTACTCAAGCCTGAGGATGGA-3'	
Reverse	5'-TGACTGTGTAAGCAGAACCCGG-3'	
TaqMan probe	5'-TTTCCCTGCGCCTGATGGTGA-3'	
<i>ET-1</i>		NM_012548
Forward	5'-CTGGAGACCCCGCAGGTC-3'	
Reverse	5'-GATGTCCAGGTGGCAGAAGTAG-3'	
TaqMan probe	5'-TTGCTCCTGCTCCTCTGATGGACAA-3'	
<i>ET_A</i>		NM_012550
Forward	5'-CAAGGGCGAGCAGCACAG-3'	
Reverse	5'-GAGCCACCAGTCCCTCACG-3'	
TaqMan probe	5'-CATGCTCAACGCCACGCCAAGTTATC-3'	
<i>ET_B</i>		NM_017333
Forward	5'-CCTGCATTAATCCAATCGCTCTG-3'	
Reverse	5'-GCTTCTCCTCTAAGGACTGTTTTTC-3'	
TaqMan probe	5'-TGGCACCAGCAGCACAAACACGACTT-3'	
<i>ECE-1</i>		NM_053596
Forward	5'-TCTACGACAAGGAATACCTCAGAC-3'	
Reverse	5'-TTCCGTACCAGGTTCCACATC-3'	
TaqMan probe	5'-TCTCCACACTCATCAACAGCACCAGCA-3'	

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were obtained from Applied Biosystems (Foster City, CA, USA) and were used to quantify rat GAPDH mRNA as an internal standard.

Statistical analysis

Data are presented as means \pm s.e.m. Differences among groups of rats at 12 weeks of age were assessed by one-way factorial analysis of variance followed by Fisher's multiple-comparison test. The time course of SBP was compared among groups by two-way repeated-measures analysis of variance. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Physiological analysis

Body weight was significantly smaller in rats fed the HS diet compared with those in the CNT group (Table 2). Whereas the LS diet did not affect SBP, the HS diet induced a substantial increase in SBP that was apparent at 9 weeks of age and thereafter (Figure 1, Table 2). SBP was not affected by SPL administration. Heart rate and tibial length did not differ significantly among the five experimental groups (Table 2). Salt intake was reduced by 77% in rats fed the LS diet and was increased 9- to 12-fold in those fed the HS diet, compared with the CNT group, but it was not affected by SPL treatment (Table 2).

LV geometry and cardiac function

The ratio of LV weight to tibial length, an index of LV hypertrophy, was increased in LS rats compared with CNT rats, and this increase was prevented by SPL administration (Table 2). On the other hand, the LV hypertrophy apparent in HS rats was attenuated significantly but not completely by SPL (Table 2).

Echocardiography revealed that interventricular septum thickness and LV posterior wall thickness were significantly greater, whereas LV

end-diastolic dimension and LV end-systolic dimension were significantly shorter in HS rats than in CNT or LS rats (Table 3). SPL treatment did not affect these parameters in LS rats, whereas it significantly attenuated the increase in interventricular septum thickness in HS rats (Table 3). LV fractional shortening was similar among CNT, LS and LS+SPL rats, whereas the HS diet increased this parameter in a manner insensitive to SPL administration (Table 3). The E/A ratio was significantly decreased and both deceleration time and isovolumic relaxation time, which are indices of LV relaxation, were significantly increased in LS rats compared with CNT rats, and these changes were prevented by SPL (Table 3). The E/A ratio was decreased and both deceleration time and isovolumic relaxation time were increased to a greater extent in HS rats than in LS rats,

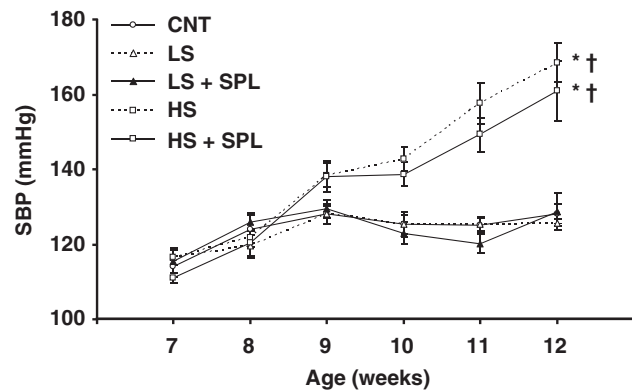


Figure 1 Time course of SBP in rats of the five experimental groups. Data are means \pm s.e.m. ($n=6, 6, 6, 8$ and 6 rats for CNT, LS, LS+SPL, HS and HS+SPL groups, respectively). **P* <0.05 vs. CNT; †*P* <0.05 vs. LS.

Table 2 Physiological parameters for rats in the five experimental groups at 12 weeks of age

Parameters	CNT	LS	LS+SPL	HS	HS+SPL
Body weight (g)	400 \pm 9	414 \pm 8	377 \pm 8†	358 \pm 7*,†	351 \pm 12*,†
SBP (mm Hg)	128 \pm 3	126 \pm 1	129 \pm 5	169 \pm 5*,†	161 \pm 8*,†
Heart rate (beats per min)	334 \pm 12	355 \pm 16	339 \pm 12	348 \pm 16	351 \pm 8
LV weight (mg)/tibial length (mm)	19.3 \pm 0.6	21.4 \pm 0.6*	19.0 \pm 0.2†	23.2 \pm 0.5*,†	21.3 \pm 0.7*,†
Tibial length (mm)	38.6 \pm 0.2	38.0 \pm 0.3	38.0 \pm 0.3	37.8 \pm 0.3	37.2 \pm 0.6
Salt intake (mg per day)	92 \pm 6	21 \pm 2*	21 \pm 1*	861 \pm 200*,†	1158 \pm 182*,†

Abbreviations: CNT, control; HS, high salt; LS, low salt; SPL, spironolactone.

Data are means \pm s.e.m. ($n=6, 6, 6, 8$ and 6 rats for CNT, LS, LS+SPL, HS and HS+SPL groups, respectively).

**P* <0.05 vs. CNT; †*P* <0.05 vs. LS; ‡*P* <0.05 vs. HS.

Table 3 Cardiac morphological and functional parameters for rats in the five experimental groups at 12 weeks of age

Parameters	CNT	LS	LS+SPL	HS	HS+SPL
IVST (mm)	1.58 \pm 0.05	1.60 \pm 0.04	1.58 \pm 0.03	2.05 \pm 0.05*,†	1.83 \pm 0.11*,†,‡
LVdD (mm)	8.33 \pm 0.22	8.65 \pm 0.13	8.27 \pm 0.12	7.83 \pm 0.19*,†	7.54 \pm 0.10*,†
LVDs (mm)	4.40 \pm 0.14	4.35 \pm 0.17	4.26 \pm 0.15	3.35 \pm 0.25*,†	3.13 \pm 0.25*,†
LVPWT (mm)	1.64 \pm 0.05	1.65 \pm 0.05	1.62 \pm 0.03	2.00 \pm 0.04*,†	1.90 \pm 0.05*,†
LVFS (%)	47.2 \pm 0.7	49.7 \pm 1.9	48.5 \pm 1.7	57.7 \pm 2.1*,†	58.5 \pm 3.0*,†
E/A	1.95 \pm 0.03	1.76 \pm 0.08*	1.91 \pm 0.05†	1.47 \pm 0.03*,†	1.62 \pm 0.05‡
DcT (ms)	37.1 \pm 1.4	46.3 \pm 1.6*	38.2 \pm 1.4†	51.9 \pm 2.2*,†	41.6 \pm 2.5‡
IRT (ms)	20.9 \pm 0.7	24.0 \pm 1.0*	20.5 \pm 0.7†	26.6 \pm 0.9*,†	21.5 \pm 1.1‡

Abbreviations: IVST, interventricular septum thickness; LVdD, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVPWT, left ventricular posterior wall thickness; LVFS, left ventricular fractional shortening; DcT, deceleration time; IRT, isovolumic relaxation time; CNT, control; HS, high salt; LS, low salt; SPL, spironolactone.

Data are means \pm s.e.m. ($n=6, 6, 6, 8$ and 6 rats for CNT, LS, LS+SPL, HS and HS+SPL groups, respectively).

**P* <0.05 vs. CNT; †*P* <0.05 vs. LS; ‡*P* <0.05 vs. HS.

and SPL treatment again prevented these changes (Table 3). These results thus indicated that SPL administration was effective in preventing the development of LV diastolic dysfunction in both LS and HS rats.

Cardiomyocyte hypertrophy and cardiac fibrosis

The cross-sectional area of cardiac myocytes was greater in LS rats than in CNT rats and was greater in HS rats than in LS rats (Figures 2a, b). SPL administration completely blocked the development

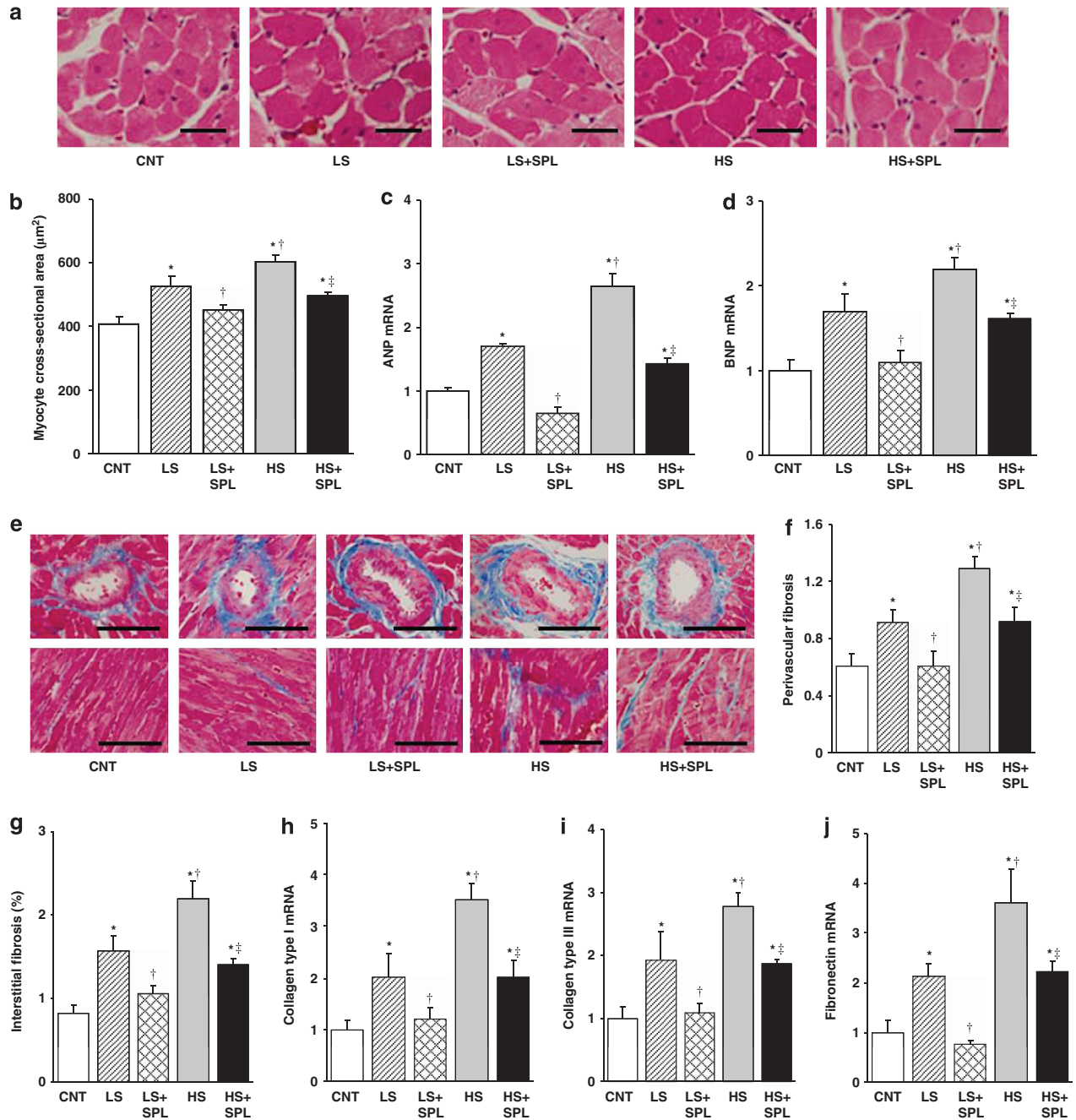


Figure 2 Cardiomyocyte size and fibrosis in the left ventricle of rats in the five experimental groups at 12 weeks of age. (a) Hematoxylin-eosin staining of transverse sections of the LV myocardium. Scale bars, 50 µm. (b) Cross-sectional area of cardiac myocytes determined from sections similar to those in (a). (c, d) Quantitative reverse transcription-PCR (RT-PCR) analysis of ANP and BNP mRNAs, respectively, in LV tissue. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the corresponding mean value for the CNT group. (e) Representative microscopic images of collagen deposition (blue) in perivascular (upper panels) and interstitial (lower panels) regions of the LV myocardium as revealed by Azan-Mallory staining. Scale bars, 50 µm (upper panels) or 100 µm (lower panels). (f, g) Relative extents of perivascular and interstitial fibrosis, respectively, in the LV myocardium as determined from sections similar to those in (e). (h-j) Quantitative RT-PCR analysis of collagen type I (h), collagen type III (i) and fibronectin (j) mRNAs in LV tissue. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the corresponding mean value for the CNT group. Data in (b) through (d) and in (f) through (j) are means ± s.e.m. ($n = 6, 6, 6, 8$ and 6 rats for CNT, LS, LS+SPL, HS and HS+SPL groups, respectively). * $P < 0.05$ vs. CNT; † $P < 0.05$ vs. LS; ‡ $P < 0.05$ vs. HS.

of cardiomyocyte hypertrophy in LS rats, whereas it significantly but only partially blunted that in HS rats (Figures 2a, b). The expression of atrial natriuretic peptide and brain natriuretic peptide genes in the LV myocardium of rats in the various experimental groups showed a pattern of changes similar to that for myocyte cross-sectional area (Figures 2c, d).

Fibrosis in perivascular and interstitial regions of the LV myocardium, as assessed by Azan-Mallory staining, was increased in LS rats compared with CNT rats and was increased to a greater extent in HS rats than in LS rats (Figures 2e, g). The increase in myocardial fibrosis was completely suppressed by SPL in LS rats, whereas it was significantly but incompletely attenuated by the drug in HS rats (Figures 2e, g). The amounts of collagen type I and type III as well as fibronectin mRNAs in the left ventricle of rats in the various experimental groups showed a pattern of changes similar to that for myocardial fibrosis (Figures 2h, j).

Cardiac oxidative stress and inflammation

Superoxide production in myocardial tissue sections revealed by staining with dihydroethidium as well as the activity of NADPH oxidase in LV homogenates were both increased in LS rats compared with CNT rats as well as in HS rats compared with LS rats (Figures 3a, c). The increase in cardiac oxidative stress associated with the LS diet was prevented by SPL treatment, whereas that associated with the HS diet was significantly but incompletely attenuated by the drug (Figures 3a, c). The expression of genes for the p22^{phox} and gp91^{phox} membrane components of NADPH oxidase was upregulated in LS rats compared with CNT rats and these effects were completely blocked by SPL treatment (Figures 3d, e). The expression of gp91^{phox} gene, but not p22^{phox} gene, was significantly increased in HS rats compared with LS rats. This increase in gp91^{phox} gene in HS rats was significantly suppressed by SPL. The expression of genes for p47^{phox}, p67^{phox} and Rac1 cytosolic components of NADPH oxidase was similar among all groups (data not shown).

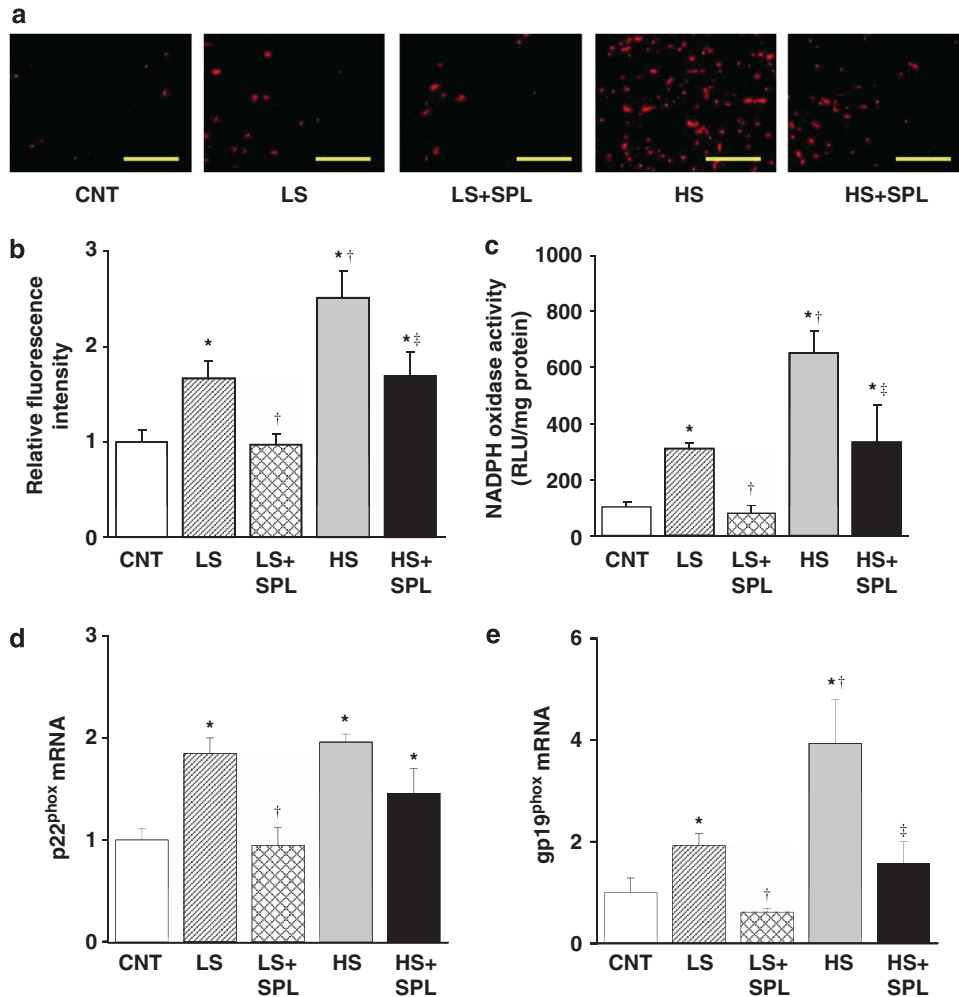


Figure 3 Oxidative stress and inflammation in the left ventricle of rats in the five experimental groups at 12 weeks of age. (a) Representative microscopic images of superoxide production in the LV myocardium as revealed by staining with dihydroethidium, the oxidation of which to ethidium by superoxide generates red fluorescence. Scale bars, 100 μ m. (b) Dihydroethidium fluorescence intensity as determined from sections similar to those in (a) and expressed relative to the value for the CNT group. (c) NADPH-dependent superoxide production in LV homogenates. Data are expressed as relative light units (RLU) per milligram of protein. (d, e) Quantitative RT-PCR analysis of p22^{phox} (d) and gp91^{phox} (e) mRNAs in LV tissue. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the corresponding mean value for the CNT group. Data in (d) and (e) are means \pm s.e.m. ($n=6, 6, 6, 8$ and 6 rats for CNT, LS, LS + SPL, HS and HS + SPL groups, respectively). * $P<0.05$ vs. CNT; † $P<0.05$ vs. LS; ‡ $P<0.05$ vs. HS.

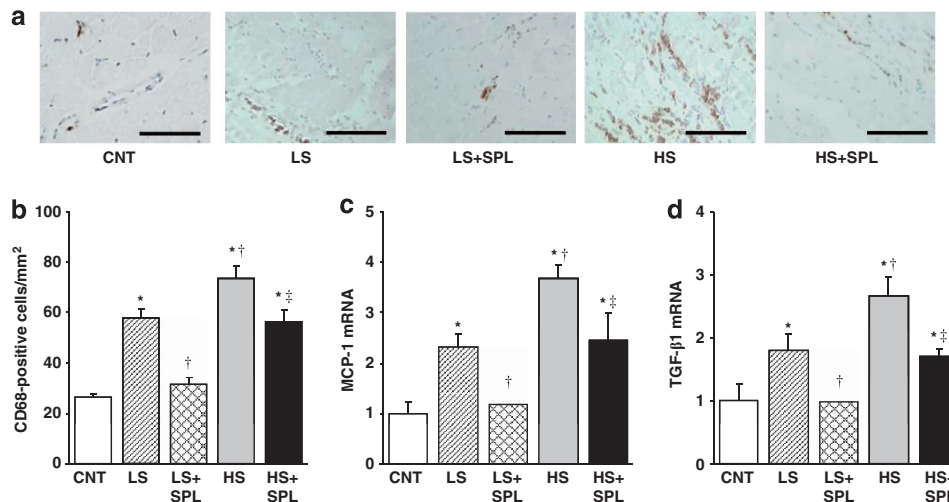


Figure 4 Representative immunohistochemical staining (brown) for the monocyte–macrophage marker CD68 in the LV myocardium. Scale bars, 50 μm. (a) Density of CD68-positive cells in the LV myocardium as determined from sections similar to those in (d). (b, c) Quantitative RT-PCR analysis of monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β1 (TGF-β1) mRNAs, respectively, in LV tissue. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the corresponding mean value for the CNT group. Data in (b) and (c) are means ± s.e.m. ($n=6, 6, 6, 8$ and 6 rats for CNT, LS, LS+SPL, HS and HS+SPL groups, respectively). * $P<0.05$ vs. CNT; † $P<0.05$ vs. LS; ‡ $P<0.05$ vs. HS.

Immunostaining of the LV myocardium for the monocyte–macrophage marker CD68 revealed that the number of CD68-positive cells was increased in LS rats and, to an even greater extent, in HS rats compared with CNT rats (Figures 4a, b). Such macrophage infiltration was completely suppressed in LS rats and partially attenuated in HS rats by SPL administration. The expression of monocyte chemoattractant protein-1 and transforming growth factor-β1 genes in the left ventricle of rats in the various experimental groups showed a pattern of changes similar to that for macrophage infiltration (Figures 4c, d).

Cardiac renin–angiotensin and ET systems

Cardiac ACE, angiotensin II type 1A receptor and serum- and glucocorticoid-regulated kinase-1 mRNA levels were increased in LS rats compared with CNT rats as well as in HS rats compared with LS rats (Figures 5a, c). SPL blocked the increases in ACE and in angiotensin II type 1A receptor mRNAs associated with the LS diet, whereas it partially inhibited the upregulation of all three mRNAs associated with the HS diet.

Expression of ET-1, ET type A receptor, ET type B receptor and ET-converting enzyme-1 genes in the left ventricle was upregulated in LS rats and, to a greater extent, in HS rats compared with CNT rats (Figures 5d, g). SPL prevented the increased expression of these genes associated with the LS diet, whereas it significantly but incompletely inhibited that associated with the HS diet.

DISCUSSION

We have examined the effects of excess aldosterone on the heart under conditions of high or low salt intake as well as the pathophysiological role of MRs in Sprague-Dawley rats. We found that a LS diet induced LV hypertrophy, fibrosis and diastolic dysfunction, without increasing blood pressure, in rats administered exogenous aldosterone and that SPL completely blocked these cardiac effects. The LS diet also promoted cardiac oxidative stress and inflammation as well as activation of the renin–angiotensin and ET systems in the heart in a manner sensitive to SPL treatment. Cardiac damage induced by

aldosterone in the presence of high salt intake was more pronounced than that in the presence of low salt intake. Importantly, the cardiac changes in rats fed the HS diet were only partially attenuated by SPL administration.

Our finding that excess aldosterone in the context of a LS (0.0466% NaCl) diet did not induce hypertension in Sprague-Dawley rats is consistent with the previous observation that blood pressure was not increased significantly in such rats with hyperaldosteronism fed an extremely low salt (0.00004% NaCl) diet.²⁸ Similarly, mice with hyperaldosteronism fed a normal-salt (0.3% NaCl) diet were also found to be normotensive.²⁹ Together, these findings suggest that high salt intake is necessary for the development of aldosterone-induced hypertension, with aldosterone excess alone not being sufficient to trigger an increase in blood pressure.

The effects of aldosterone on cardiac morphology in the absence of salt loading have remained unclear. For instance, wild-type C57BL/6J mice treated with the MR ligand deoxycorticosterone acetate did not develop cardiac hypertrophy when maintained on a low-sodium (0.05% Na⁺) diet.¹³ On the other hand, 11β-hydroxysteroid dehydrogenase 2 transgenic mice fed a normal-salt (0.32% NaCl) diet were normotensive but spontaneously developed cardiac hypertrophy and fibrosis.¹⁴ In addition, aldosterone infusion induced marked cardiac hypertrophy accompanied by only a small increase in blood pressure in rats fed a normal-salt (0.5% NaCl) diet.³⁰ Whether or not aldosterone excess results in the development of cardiac hypertrophy and fibrosis independently of salt loading has thus remained to be determined definitively. In the present study, aldosterone administration in the setting of low salt intake did not increase blood pressure but induced LV hypertrophy and fibrosis, indicating that aldosterone excess *per se* may have detrimental cardiac effects that are independent of the development of hypertension. The fact that the extent of LV hypertrophy associated with primary aldosteronism is greater than that associated with hypertension of similar severity owing to other causes³¹ also suggests that aldosterone might directly affect cardiomyocyte growth. In addition, the LV hypertrophy and fibrosis induced by the HS diet in the present study

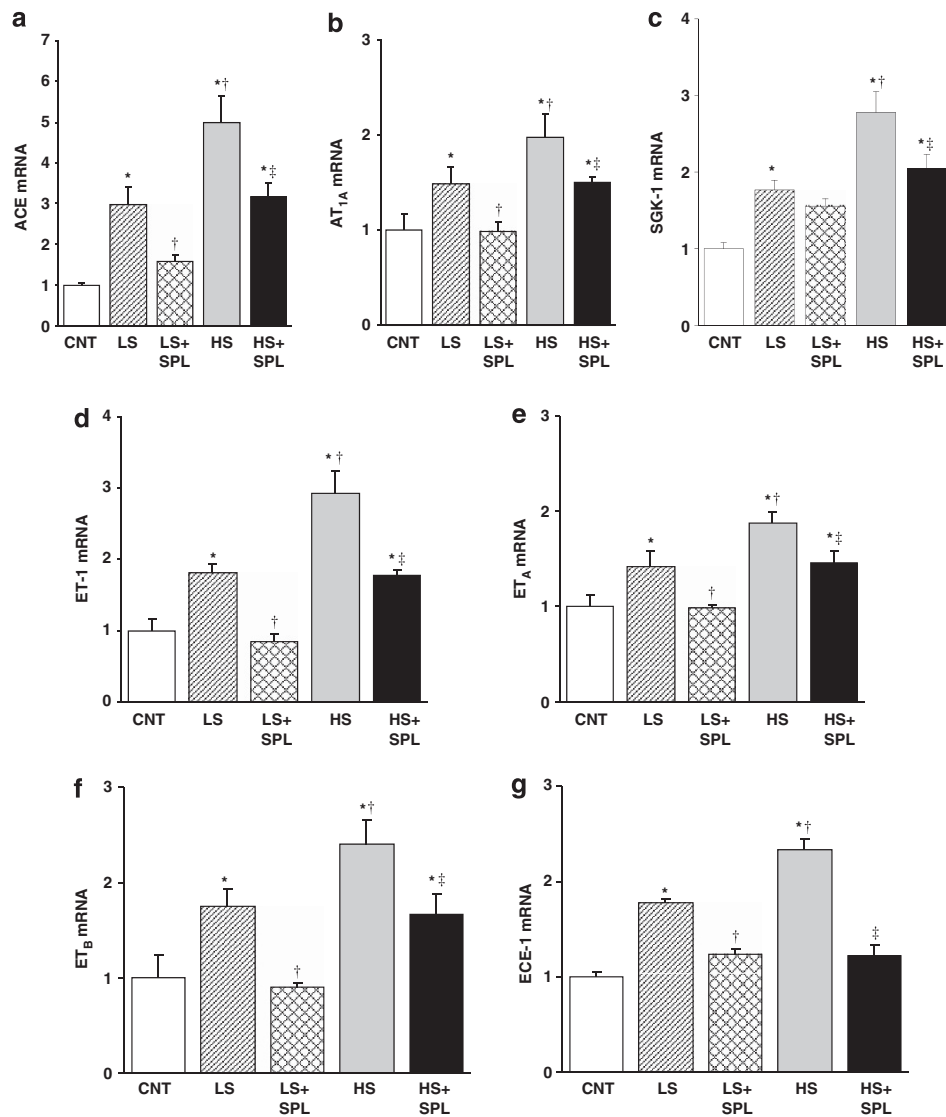


Figure 5 Expression of genes related to the renin-angiotensin and ET systems in the left ventricle of rats in the five experimental groups at 12 weeks of age. The amounts of ACE (a), angiotensin II type 1A receptor (AT_{1A}) (b), serum- and glucocorticoid-regulated kinase-1 (SGK-1) (c), ET-1 (d), ET type A receptor (ET_A) (e), ET type B receptor (ET_B) (f) and ET-converting enzyme-1 (ECE-1) (g) mRNAs were determined by quantitative RT-PCR analysis, normalized by that of GAPDH mRNA and then expressed relative to the corresponding mean value for the CNT group. Data are means \pm s.e.m. ($n=6, 6, 6, 8$ and 6 rats for CNT, LS, LS+SPL, HS and HS+SPL groups, respectively). * $P<0.05$ vs. CNT; † $P<0.05$ vs. LS; ‡ $P<0.05$ vs. HS.

were attenuated by the administration of SPL at a dose previously shown not to reduce blood pressure,¹⁵ suggesting that the effects of aldosterone on cardiac remodeling are mediated at least in part directly via MRs rather than through blood pressure elevation.

Previous studies have shown that aldosterone administration in rats subjected to salt loading, unilateral nephrectomy or both induces cardiac inflammation characterized by macrophage infiltration.^{1,12} The expression of several pro-inflammatory molecules that may contribute to the pathogenesis of cardiac remodeling is also modulated by aldosterone in association with salt through MR-dependent mechanisms.³² Given the central role of NaCl in the animal models examined, the possibility that the marked cardiac inflammatory responses induced by aldosterone in these previous studies were dependent on salt loading or were due to severe hypertension cannot be excluded. Indeed, it has been suggested that one or more cofactors are required for the profibrotic action of

aldosterone.^{33,34} We have now shown that aldosterone excess, in the absence of salt loading or hypertension, induced substantial cardiac inflammatory changes. The macrophage infiltration into the myocardium apparent in LS rats was associated with the upregulation of monocyte chemoattractant protein-1 gene expression in the heart as well as with the myocardial fibrosis. Our results thus suggest that aldosterone alone directly induces cardiac inflammation in the context of a LS diet.

Rats with deoxycorticosterone acetate- and salt-induced hypertension manifest increased oxidative stress.³⁵ Cardiac inflammation induced by aldosterone and salt loading has thus been attributed to oxidative stress.¹² It has remained unknown, however, how the combination of aldosterone and salt might induce oxidative stress in the heart of rats. The increased production of oxygen radicals by aortic tissue in hypertensive rats has been thought to result from the arterial pressure elevation.³⁶ However, the effects of aldosterone on

oxidative stress in normotensive rats have not previously been studied, and norepinephrine-induced hypertension was found not to be associated with oxidative stress.³⁷ Circulating neurohormones including angiotensin II and catecholamines have been implicated in the induction of oxidative stress in the rodent heart in response to aldosterone-salt administration.³⁸ We have now shown that aldosterone excess induced oxidative stress in the heart of rats fed the LS diet. Aldosterone may therefore also promote oxidative stress in a manner independent of salt loading and hypertension.

Although tempol, a superoxide dismutase mimetic, completely blocked the elevation of blood pressure by aldosterone, it failed to inhibit aldosterone-induced cardiac hypertrophy, inflammation or monocyte chemoattractant protein-1 gene expression.³⁰ These results indicate that oxidative stress has a minor role in the development of aldosterone-induced cardiac injury, and that aldosterone *per se* may influence cardiac pathology independently of oxidative stress.

A cardiac renin-angiotensin system is thought to support local angiotensin production in the heart.³⁹ Aldosterone was found to increase ACE expression and activity in neonatal rat cardiomyocytes in a manner sensitive to SPL,⁴⁰ consistent with our present results showing that ACE gene expression was increased in the heart of LS rats and that this effect was prevented by SPL administration. The upregulation of ACE activity in the heart results in an increase in the local level of angiotensin II, leading to cardiac hypertrophy and fibrosis.³⁹ We also showed that aldosterone increased cardiac expression of the angiotensin II type 1A receptor gene. Suppression of the cardiac renin-angiotensin system by SPL may thus have contributed to the prevention of myocardial hypertrophy and fibrosis by this drug in rats of the LS + SPL group. In addition to the renin-angiotensin system, the ET system was found to be activated during the transition to diastolic heart failure in hypertensive heart disease.⁴¹ A link between mineralocorticoids and ET-1 has been suggested.^{2,42} ETs exert their physiological and pathophysiological effects via two receptors, ET type A receptor and ET type B receptor. The ET type A receptor has been implicated in cardiovascular diseases, whereas ET type B receptor has been linked to the regulation of sodium balance and arterial blood pressure.⁴³ Aldosterone has been shown to induce ET-1 gene expression in the kidney and colon of rats.⁴⁴ We have now shown that the expression of genes related to the ET system was increased in the heart of LS rats in a manner sensitive to SPL administration, suggesting that SPL ameliorated cardiac injury in these animals through suppression of the ET system as well as through that of the cardiac renin-angiotensin system.

In conclusion, we have shown that aldosterone excess in the setting of low salt intake induces substantial LV remodeling and diastolic dysfunction without increasing blood pressure in rats. Furthermore, these cardiac changes were prevented by treatment with SPL. On the other hand, aldosterone excess in the setting of high salt intake induced hypertension and a greater extent of cardiac injury, with the cardiac changes being partially attenuated by SPL in a manner independent of its antihypertensive effect. The beneficial cardiac effects of SPL irrespective of sodium status were associated with reduced cardiac oxidative stress and inflammation. Our results thus suggest that dietary salt restriction in combination with the administration of an MR antagonist may prove to be an effective therapeutic strategy to limit cardiac injury in hypertensive patients with hyperaldosteronism or relative aldosterone excess.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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