Longitudinal Changes in Blood Pressure are Preceded by Changes in Albuminuria and Accelerated by Increasing Dietary Sodium Intake

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Abstract

Background: Dietary sodium is a well-known risk factor for cardiovascular and renal disease; however, direct evidence of the longitudinal changes that occur with aging, and the influence of dietary sodium on the age-associated alterations are scarce.

Methods: C57BL/6 mice were maintained for 13 months on a low (LS, 0.02% Na^+), normal (NS, 0.3% Na^+) or high (HS, 1.6% Na^+) salt diet. We assessed 1) the longitudinal trajectories for two markers of cardiovascular and renal dysfunction (blood pressure (BP) and albuminuria), as well as hormonal changes, and 2) end-of-study cardiac and renal parameters.

Results: The effect of aging on BP and kidney damage did not reach significance levels in the LS group; however, relative to baseline, there were significant increases in these parameters for animals maintained on NS and HS diets, starting as early as month 7 and month 5, respectively. Furthermore, changes in albuminuria preceded the changes in BP relative to baseline, irrespective of the diet. Circulating aldosterone and plasma renin activity displayed the expected decreasing trends with age and dietary sodium loading. As compared to LS – higher dietary sodium consumption associated with increasing trends in left ventricular mass and volume indices, consistent with an eccentric dilated phenotype. Functional and molecular markers of kidney dysfunction displayed similar trends with increasing long-term sodium levels: higher renovascular resistance, increased glomerular volumes, as well as higher levels of renal angiotensin II type 1 and mineralocorticoid receptors, and lower renal Klotho levels.

Conclusion: Our study provides a timeline for the development of cardiorenal dysfunction with aging, and documents that increasing dietary salt accelerates the age-induced phenotypes. In addition, we propose albuminuria as a prognostic biomarker for the future development of

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hypertension. Last, we identified functional and molecular markers of renal dysfunction that associate with long-term dietary salt loading,

1. INTRODUCTION

Hypertension is a leading preventable risk factor for cardiovascular morbidity and mortality[1,2]. Blood pressure (BP) elevation strongly correlates with aging, and age-dependent development of hypertension has been shown to accelerate most at mid-life[3,4]. The underlying causes for age-related BP progression are poorly understood; prevailing models to explain the pathogenesis of hypertension include the age-induced alterations in systemic vascular resistance, as well as renal dysfunction leading to volume expansion[5,6]. However, long-term BP increases have also been described as a leading cause of chronic kidney disease due to the deleterious effects of hypertension on renovascular function[7]. Further, age-related BP elevations are paralleled by fluid and electrolyte abnormalities, likely the consequence of decreases in plasma renin activity (PRA) and aldosterone (Aldo)[8,9]. Thus, a major hurdle that needs to be addressed is the unclear timelines in the etiology of age-induced cardiovascular and renal risks.

Studies in humans and animal models have documented a causal relationship between dietary sodium (Na^+) intake and a range of chronic diseases, including hypertension, cardiovascular and kidney disease[10–14]. Consistently, aging-associated BP elevations are not detected in cultures that do not consume salt, supporting the notion that dietary salt is a critical contributor to aging-induced BP elevation and hypertension[15–17]. However, most data come from cross-sectional observational and short-term dietary intervention studies, in part due to the inherent challenges in performing controlled diet, longitudinal studies, as well as to the slow progression of the phenotypes over the lifetime (particularly in humans). Thus, there is little information regarding the effects of controlled Na^+ intake on longitudinal trajectories for cardiovascular and renal risk, from an aging perspective.

The current study aims to address this gap, by assessing, in mice, 1) the effect of long-term exposure to three levels of salt intake (low, moderate and high) on the monthly longitudinal development of cardiovascular and renal risk (BP and albuminuria), and 2) the hormonal, functional and molecular mechanisms that may mediate the salt/aging-induced alterations in cardiorenal homeostasis.

2. MATERIAL AND METHODS

2.1. Animals

Animals were housed in the animal facility in 12:12-h light-dark cycle at 22°C ambient temperature and maintained on *ad libitum* regular rodent chow and tap water until study initiation. All studies were conducted according to protocols approved by the Institutional

Animal Care and Use Committee (IACUC) at Brigham and Women's Hospital Boston, MA, USA.

Cross-sectional pilot study.—In preliminary studies, we used male and female CL57Bl/6 mice (Charles River, Wilmington, MA, USA) in three age groups (Table S1): young adult (< 20-week-old), middle aged (45–55 week-old) and old (> 65 week-old). Animals were housed in the animal facility in 12:12-h light-dark cycle at 22°C ambient temperature and maintained on *ad libitum* regular rodent chow and tap water. BP measurements were performed using tail-cuff plethysmography as described below. As age showed a significant effect on BP in male (but not female) mice (Table S1), the subsequent longitudinal studies were performed using male animals only.

Longitudinal study.—Fifteen-week-old male mice CL57Bl/6 (Charles River, Wilmington, MA, USA) were randomized to one of three levels of Na^+ intake (Purina, St. Louis, MO) for 13 months: low (LS, 0.03% Na^+, #5885), normal (NS, 0.26% Na^+, #5755), or high (HS, 1.6% Na^+, #26661). Body weight (BW) and BP were measured every month. Blood samples were collected at months 1, 5 and 13 for the assessment of plasma Aldo and PRA. Animals were also placed every month in metabolic cages, for 24h food and water intake measurements; 24h urine was collected bimonthly for the assessment of the albumin (Alb) and creatinine (Cr)), and urine output was measured. Fluid balance was calculated as the difference between water intake and urine output. At the end of the study, animals underwent an echocardiography and Doppler ultrasound assessment (cardiac and renal); the following morning, mice were euthanized under isoflurane anesthesia and heart and kidney tissues were collected.

2.2. Blood pressure measurements

Systolic (SBP) and diastolic BP (DBP), as well as heart rate (HR) were measured in conscious animals every month, for 13 months, using tail-cuff plethysmography and the CODA noninvasive BP system (KENT Scientific Corporation, Torrington, CT), as previously described by us[18,19]. Mice were warmed at 30°C and allowed to rest quietly. BP measurements were taken in a quiet room in the morning, and the mice were kept calm and handled by the same person. No sedative was used. We have previously demonstrated excellent correlation between SBPs assessed simultaneously by tail cuff and telemetry in mice[19,20].

2.3. Blood collection

On months 1, 5 and 13, mice were slightly anesthetized, and blood was collected from the submandibular plexus in purple-top BD Microtainer tubes (EDTA), as previously described by us[21]. Plasma was separated by centrifugation, and samples stored at −80C until processed for Aldo and PRA assessments.

2.4. Urine and plasma analyte measurements.

Plasma Aldo and PRA were measured in duplicate using a solid-phase radioimmunoassays (IBL International GMBH, Hamburg, Germany; DiaSorin, Stillwater, MN) as previously described[22]. Urine Alb and Cr were measured using DCA 2000 microalbumin reagent kit

(Bayer, Elkhart, IN), as reported by us[22], and are shown as Alb/Cr ratios (mg/g). The detectable Alb/Cr range was 1–2000 μg/mg. The inter-assay CVs were < 5%.

2.5. Histological analysis

Following euthanasia, one kidney was dissected, and the cortex portion sectioned and placed in liquid nitrogen immediately after collection, for protein analysis. The other kidney was fixed in 10% formalin and embedded in paraffin blocks for histology analyses. Five-μm-thick sections were cut and stained with Periodic Acid-Schiff reagent for glomerular volume determination. Glomerular volumes measurements were carried out in a blinded fashion as previously described[23], using 30 glomeruli per section.

2.6. Binding of conformation specific antibodies in the kidney.

To determine whether renal angiotensin II receptors are activated by long term dietary sodium intake, conformation state-sensitive anti-AT_1R and anti-AT_2R antibodies[24,25] (Proteimax Biotechnology, Cotia, São Paulo, Brazil) were used. These antibodies are sensitive to activity-mediated conformational changes in the receptors being able to specifically recognize the activated state of the receptor, as previously documented[26–29]. Deparaffinized and rehydrated sections were incubated with blocking solution (1% bovine serum albumin + 5% sucrose in PBS) and then with conformation-specific antibodies against the angiotensin II receptors 1 and 2 (AT_1R and AT_2R), conjugated with DY-682 (red) and DY-800 (green) fluorophores, respectively. Sections were then washed with PBS, and fluorescence intensity measured using Odyssey equipment (LI-COR Biosciences) as described by us in previous studies[24,25]. The intensity of each sample was normalized to negative control (i.e. sections treated only with secondary antibody labelled with fluorescent Alexa Fluor dyes DY-682 and DY-800).

2.7. Electrophoresis and Western blotting

Protein expression was measured as previously reported by us[22]. In brief, kidney cortex homogenates (20 μg total protein) were size-fractionated by electrophoresis on 10% SDS-PAGE gels, then transferred onto nitrocellulose membrane by electroblotting. The membranes were first blocked with 5% non-fat dried milk in Tris-buffered saline (TBS)-Tween (USB Corporation, Cleveland, OH) for 1 h at room temperature, then incubated overnight at 4°C with the following primary antibodies: anti-AT_1R, anti-AT_2R, anti-MR (Santa Cruz Biotechnology), anti-Klotho (Abcam). Membranes were then washed 3×15 min with TBS-Tween and incubated with peroxidase-conjugated secondary antibody (Dako) for 2 h. The membranes were thoroughly washed, and immunocomplexes detected using enhanced chemiluminescence (Denville Scientific Inc., Holliston, MA). To correct for loading, blots were re-probed with mouse anti-β-actin antibody (Sigma Aldrich). Results were analyzed with optical densitometry; data are presented as fold change relative to measurements in the LS group.

2.8. Imaging protocol for echocardiogram and renal resistive index

At the end of the study, animals were imaged at the Cardiovascular Physiology Rodent Core at Brigham and Women's Hospital as previously described[30,31]. In brief, a MS550D probe

with center frequency of 40 and 21 MHz was used to capture images of the heart and kidney. Images were acquired using Vevo3100 system and the data were analyzed using the Vevo Lab software (Fujifilm/Visual sonics, Toronto, ON, Canada). Mice were under controlled anesthesia and kept warm on a prewarmed platform to ensure that the body temperature was maintained at physiologic levels. The probe was positioned using the rail system to obtain the left ventricular diastolic and systolic dimensions for the internal diameter (LVID) and posterior wall (LVPW), as well as the calculated ejection fraction (EF), fractional shortening (FS), left ventricle mass index (LVMI), end diastolic (EDVI) and systolic volume indices (ESVI), cardiac output (CO), and left renal artery – resistance index (LRA RI). The LRA RI was calculated by the formula: (peak systolic velocity – end diastolic velocity)/peak systolic velocity. The concentricity was calculated during diastole, using the following formula: 2*LVID/LVPW.

2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). The longitudinal effects were tested using repeated measures ANOVA. Comparisons between three or more groups were performed using one-way ANOVA followed by post-hoc analyses. Correction for multiple comparisons was performed using the Dunnett test (repeated measures ANOVA) or the Sidak test (one-way ANOVA). Trend analyses were performed via simple linear regression, using the concentration of sodium in the three diets as independent variable. Data were presented as mean ± SEM, and a P value < 0.05 was considered statistically significant.

3.   RESULTS

3.1.   Aging and dietary sodium effects on body weight and metabolic parameters

As expected, BW progressively increased throughout the study, irrespective of diet (Table S2). Consistent with the increase in BW, 24h food intake and urine output also increased over time in all diet groups; however, 24h water intake remained relatively constant (Table S2). While moderate Na^+ loading (NS) did not lead to any changes in food or water intake, or urine output versus LS, HS consumption associated with reduced food intake, yet increased water intake and urine output as compared to the LS diet group. These changes became significant in month 1 (**Mo 1**) for water intake and **Mo 5** for food intake and urine output and maintained significance throughout the study (Table S2). Consistent with previous reports[32,33], fluid balance, calculated as the difference between water intake and urine output, progressively declined with aging in the LS and NS groups, but stayed relatively constant in the HS group (data not shown), suggesting potential water retention in this group.

3.2.   Aging and dietary sodium effects on blood pressure homeostasis

At the initiation of the study, there were no significant differences between the three diet groups for either SBP (mmHg, LS: 116.2±3.27; NS: 116.3±5.92; HS: 118.9±3.44) or DBP (mmHg, LS: 87.4±5.22; NS: 88.2±7.15; HS: 83.4±4.0). As shown in Fig. 1, long-term LS diet did not induce a significant increase in either SBP or DBP with aging. As compared to **Mo 1**, animals maintained on a NS diet progressively increased their SBP and DBP, reaching

significance starting with **Mo 11** and **Mo 12** respectively. The HS group displayed a steeper increase in both SBP and DBP, which reached significance (relative to **Mo 1**) at earlier time points: **Mo 6** and **Mo 7**, respectively. As compared to values on a LS diet, SBP (but not DBP) on a NS diet only reached significant differences in **Mo 11** and **Mo 12** (Fig. S1–S2); however, SBP and DBP on a HS diet were both significantly higher vs LS in **Mo 6–13** (Fig. S1–S2). Thus, at the end of the study (**Mo 13**), SBP levels were similar in the LS and NS diet groups, but significantly higher in the HS diet group (mm Hg, LS: 123.0±2.52; NS: 130.7±5.25; HS: 138.5±2.40, p =0.0001 vs. LS); the end of study DBP showed a similar trend, with HS (but not NS) values being significantly greater than the LS ones (Fig. S1–S2). Neither aging nor diet had a significant effect on heart rate (HR) or pulse pressure (data not shown).

3.3. Aging and dietary sodium effects on circulating aldosterone and plasma renin activity

To begin to understand the timeline of the hemodynamic changes described above, we then assessed elements of the renin-angiotensin-aldosterone system (RAAS) at the beginning of study (**Mo 1**), prior to the earliest BP elevation timepoint (**Mo 5**) and at the end of study (**Mo 13**) (Fig. 2). As anticipated, dietary Na^+ loading was associated with decreased circulating Aldo levels, as compared to the LS group, even after only 1 month of diet. Further, aging induced a progressive decrease in plasma Aldo levels in all groups. This decline was particularly strong on a HS diet, where Aldo suppression at **Mo 13** relative to **Mo 1** was ~ 18-fold, while only ~4 and ~2 fold in the NS and LS groups. Aging did not significantly affect PRA levels in the LS and NS groups (data not shown) but induced a ~3-fold decrease in the HS group (ng/ml/h, **Mo 1**: 16.15±0.5; **Mo 5**: 11.64±0.4; **Mo 13**: 5.62±2.6, p=0.004 vs **Mo 1**). Thus, the aging-induced suppression was greater for Aldo than for PRA, particularly on a HS diet.

3.4. Long-term dietary sodium – effects on cardiac structure and function

As shown in Table S2, BW were not different between the three diet groups in month 13. As compared to LS, long-term NS diet had no significant effect on heart weight (mg, LS: 195.0±6.35; NS: 199.0±6.40) or kidney weight (mg, LS: 249.2±5.36; NS: 247.5±9.21). However, the HS group displayed significant cardiac hypertrophy (228.3±6.13 mg, p=0.001 vs. LS, p=0.01 vs. NS). Consistently, the echocardiographic assessment (Fig. 3A) showed no difference between LVMI in the LS and NS groups; however, there was a positive trend towards higher LVMI levels with increasing dietary Na^+ intake, which – relative to LS – only reached significance in the HS group. Further, the left ventricular volume indices (ESVI and EDVI) also displayed significant increases on a HS, but not NS, diet (Fig. 3B–C); however, linear regression analyses revealed a significant trend for higher volume indices with dietary sodium only for EDVI (Fig. 3C). Calculated concentricity values showed no difference between diets (LS: 0.44±0.03; NS: 0.44±0.02; HS: 0.44±0.03), suggesting the hypertrophy seen in the HS group may belong to the "eccentric" dilated phenotype group, as often seen in models of volume expansion[34]. The cardiac output displayed a significant increase in the HS vs. LS diet groups (Fig. 3 D).

3.5.   Aging and dietary sodium effects on albumin/creatinine ratio

As BP increases often lead to kidney damage, we then focused on the longitudinal effects of aging and dietary Na^+ on Alb/Cr. At the initiation of the study, there were no significant differences between the three diet groups for Alb/Cr (mg/g, LS: 31.0±1.92; NS: 28.9±1.30; HS: 29.8±1.79). As shown in Fig. 4A, aging on a LS diet was associated with an increasing trend in Alb/Cr but did not reach significance (relative to **Mo 1**) until **Mo 13**, where significance was marginal (p=0.051). As compared to baseline, animals maintained on a NS diet progressively increased their Alb/Cr, reaching significance starting with **Mo 7** and continuing till the end of study (Fig. 4B). However, there were no significant differences between the LS and NS diets at any time during the study (Fig. S3). The HS group displayed a greater progression for Alb/Cr, which became significant (relative to baseline) starting with **Mo 5** (Fig. 4C) and maintained significance until the end of the study. Thus, an all diets, significant upward progression of Alb/Cr relative to baseline occurred earlier than the respective progression in BP: on a LS diet, Alb/Cr progressed in **Mo 13** whereas BP did not; on a NS diet, Alb/Cr progressed in **Mo 7** while BP – in **Mo 11**; and on a HS diet, Alb/Cr progressed in **Mo 5** whereas BP – in **Mo 6**. Alb/Cr values on a LS and NS diets were similar throughout the experiment (Fig. S3); however, on a HS diet Alb/Cr reached significantly higher levels in **Mo 5**, as compared to both other diets. Alb/Cr also displayed positive trends with higher levels of sodium intake in **Mo 5–9**; however, at the end of the study there was no difference in albuminuria between the three diet groups (mg/g, LS: 42.17±6.27; NS: 46.78±3.78; HS: 48.20±6.51)(Fig. S1–S2).

3.6.   Long-term dietary sodium – effects on the kidney

As compared to LS, long-term NS diet had no significant effect on kidney weight (mg, LS: 249.2±5.36; NS: 247.5±9.21). However, the HS group displayed significant renal hypertrophy (273.7±6.51 mg, p=0.009 vs LS). Other groups have described the left renal artery resistive index (LRA RI) as an indicator of renal vascular resistance, with values <0.65 being considered normal, 0.65 – 0.70 high-normal, and values > 0.70 indicating dysfunction[35–37]. In our current report, end of study LRA RI (**Mo 13**) was within the high-normal range for animals maintained on a LS diet (0.699±0.01) but trended significantly (p(trend) = 0.03) towards the dysfunctional range for the NS and HS diets (0.710±0.01 and 0.736±0.01, respectively) (Fig. 5A). To further inquire on additional renal dysfunction traits, we then assessed the glomerular volumes in kidney cortex samples from our three diet groups. As shown in Fig. 5B–E, increasing dietary Na^+ intake associated with a positive trend for higher glomerular volumes ($x10^6 \mu m^3$, LS: 0.252±0.01; NS: 0.261±0.01; HS: 0.2868±0.01, p=0.03).

3.7.   Long-term dietary sodium – effects on renal protein expression

At the end of the study, the expression of the AT1 receptor in the LS and NS groups was similar. In the HS diet group, AT1 receptor expression was ~ 2-fold higher than in the LS and NS groups; however, this difference only reached significance relative to the NS diet group (p=0.01, Fig 6A). Another RAAS component that was investigated in this study was the mineralocorticoid receptor (MR); as shown in Fig. 6B, dietary Na^+ loading led to the progressive increase in MR expression (p (trend) = 0.003), which reached significance

only for the HS vs. LS group (p=0.01). On the other hand, the Klotho protein expression displayed an opposite trend: Klotho levels were progressively decreased by dietary sodium, but the difference only reached significance in the HS vs. LS groups (p=0.01) (Fig. 6C), consistent with the reported role of dietary sodium on Klotho levels[38–40].

3.8. Long-term dietary sodium – effects on renal AT_1R and AT_2R conformation

The conformational change of AT_1R and AT_2R is induced by the binding of angiotensin II[24–29]. To assess the effect of long-term dietary Na^+ on the activation state of these receptors in the kidney, we used conformation-specific anti-AT_1R and anti-AT_2R antibodies that recognize the activated form of the receptors. As shown in Fig. 7A–B, increasing Na^+ intake levels were associated with a trend for higher AT_1R activation levels, and reached significance in the HS vs. LS diet groups. Interestingly, the increase in AT_1R activation between the LS and HS diets was only ~ 20%, while the increase in total AT_1R protein (Fig. 6A) was much greater (~100%); this discrepancy may be explained by the expected drop in agonist (angiotensin II) levels on a HS diet, or by different rates of dimerization or internalization between the two diets. The AT_2R activation state was similar in the three diet groups (Fig. 7C–D).

4. DISCUSSION

The current study aimed to assess, in normotensive mice, the effect of long-term exposure to three levels of salt intake (low, moderate, and high) on the monthly longitudinal development of cardiovascular and renal risk (BP and albuminuria), as well as the hormonal, functional and molecular mechanisms that may mediate the salt/aging-induced alterations in cardiorenal homeostasis. Our data shows age- and dietary Na^+-dependent differences in BP and albuminuria across 13 months of the adult mouse lifespan. BP did not significantly change with aging on a LS diet but displayed an accelerated progression to significantly higher levels (as compared to baseline), after 11 and 6 months of NS and HS diet, respectively. In addition, Alb/Cr showed a similar (but earlier) pattern, with marginal changes during long-term LS, but significant progression after 7 and 5 months of NS and HS diet, respectively – supporting a role for albuminuria as a prognostic biomarker for future BP progression. Furthermore, as compared to LS, long-term exposure to high (but not moderate) salt intake was significantly associated with adverse effects on cardiovascular and renal outcomes – including an eccentric dilated cardiac hypertrophy phenotype, as well as kidney hypertrophy with higher glomerular volumes, increased MR and AT_1R (and an activated conformation for the latter), but decreased Klotho expressions. Thus, our findings support the notion that even mild Na^+ restriction may extend cardiorenal health span.

Hypertension is a confirmed risk factor for cardiorenal morbidity and mortality[1,2]; BP elevation strongly correlates with aging, with an accelerated progression in middle adulthood[3,4], a timeline suggestive of prolonged exposure to environmental factors (e.g., diet/nutrition). The mechanisms underlying age-related BP progression are yet to be fully understood; the prevalent theory involves a cyclic relationship with imbalances in the RAAS, abnormalities in vascular resistance and renal dysfunction, with hypertension being both a significant contributor to – and the result of any combination of these[5–7,41–43]. Our

findings in animals document that renal dysfunction (as assessed by a rise in Alb/Cr) was an early event and likely driver of the development of BP progression, occurring at least one month (and as long as four months) prior to significant BP elevations – which translates to at least two human year equivalents[44]. Other groups have found low grade albuminuria as a predictor of hypertension, left ventricular hypertrophy, diastolic dysfunction, as well as cardiovascular and renal morbidity and mortality[45–51]; however, many of these studies were cross-sectional or focused on at-risk individuals. Our data in mice are consistent with human results that identified microalbuminuria – in short longitudinal studies in normotensives (follow up ~ 3 years) – as a potentially important precursor of essential hypertension and BP progression, even after adjustment for impaired glucose and antecedent blood pressure variables[52,53], supporting the translational potential of C57Bl6 mice in aging research. In contrast to these studies, our study herein also assessed the state of the RAAS and highlighted the importance of dietary Na^+ on longitudinal progression of albuminuria as a predictor of future development of hypertension.

Studies in humans and animal models have established a clear role for dietary Na^+ as a leading modifiable risk factor for several aging-associated chronic diseases, including hypertension, cardiovascular and kidney disease[10–14]. Indeed, cultures that do not consume salt do not display aging-associated BP increases, supporting the notion that dietary salt is a critical contributor to aging-induced BP elevation and hypertension[15–17]. However, these findings come from cross-sectional observational and acute dietary intervention studies; there is a paucity of information regarding the effects of Na^+ intake on longitudinal trajectories for cardiovascular and renal risk - in part due to inherent challenges in assessing the long-term effects of controlled diets on slowly progressing phenotypes over the lifetime. Because of these limitations, the use of animal models with shorter life span (e.g., mice) offers an attractive alternative for studying environmental effects on aging[54]. However, Palliyaguru et al.[55] recently underlined the lack of a comprehensive analysis of normal aging in mice, thus hindering their use as reliable models of human aging. Our data herein document that long-term dietary salt excess accelerates the age-induced increases in BP and Alb/Cr in C57BL6 mice, suggesting salt-sensitivity of these parameters over the long-term. Interestingly, the C57BL6 strain is used as a salt-resistant control in many studies, as we and others showed that high salt diet alone does not increase BP in this model[19,56–58]. The reasons for this discrepancy are not fully understood but may relate to the duration of Na^+ loading, as most of the above-mentioned studies are short-term. It has also been proposed that salt-sensitivity may be obscured in experiments using BP measurements by tail-cuff[59]; while our results reported herein did not assess BP responses to changes in dietary salt, tail-cuff measurements were able to detect significant longitudinal changes in BP, as well as differences between three levels of Na^+ intake. Our findings on the association of higher levels of Na^+ intake with earlier BP and Alb/Cr progression are consistent with the well-documented notion that dietary salt associates – in cross-sectional human studies – with hypertension and microalbuminuria[60–63]. However, to our knowledge our report is the first to describe the salt-induced acceleration of aging for cardiovascular and renal phenotypes. Consistent with our observations, HS consumption has been recently associated with shorter telomere length and accelerated aging processes at the cellular level[64], thus providing a potential mechanism underlying our data in mice.

To gain further understanding of mechanisms underlying the effects of long-term Na^+ loading on cardiovascular homeostasis, we performed end-of-study echocardiographic assessments. As compared to LS, 13-month-long exposure to high (but not moderate) salt intake was associated with significant changes in left ventricular mass, lumen, and cardiac output, consistent with an eccentric dilated cardiac hypertrophy phenotype. These data are in agreement with other studies that showed that aging associated with eccentric cardiac hypertrophy in C57BL6 mice[65–67]; however, in one study these changes occurred only at 24 months of age and were not accompanied by changes in BP[66]. The reasons for this discrepancy may lie in the Na^+ content of the standard chow used in these experiments, likely comparable to our NS diet (where differences in echocardiographic parameters were not significant at the end of the study, in 17 months old animals).

Our current report also provides additional insight into mechanisms underlying the effects of long-term Na^+ loading on renal homeostasis. As compared to LS, long-term exposure to high (but not moderate) dietary Na^+ was associated with significant changes in renal outcomes – including kidney hypertrophy with higher glomerular volumes, increased renovascular resistance, as well as higher MR and AT_1R but decreased Klotho expressions. These results are consistent with previous reports, that support an association between aging and kidney hypertrophy, increased glomerular volumes and activation of the intrarenal RAAS[68–71] in animals and humans. Short-term dietary Na^+ interventions have also been shown to induce similar changes in the kidney[72–76]. In our studies, increased renal MR and AT_1R levels in response to long-term Na^+ loading may mediate both BP and Alb/Cr progression, given their critical roles in vascular and Na^+ homeostasis, and despite the expected suppression of circulating RAAS in response to both aging and Na^+. Furthermore, 13-month exposure to HS (but not NS) diet significantly suppressed Klotho, a critical anti-aging molecule expressed predominantly in the kidney[77,78]. In humans, renal Klotho levels were markedly reduced in patients with chronic kidney disease, while decreases in renal Klotho expression in aging mice have been associated with enhanced kidney fibrosis and oxidative stress[79,80]. Our data is also in agreement with a recent report involving Klotho in dietary salt- and aging-related kidney damage and hypertension[81].

Several limitations need to be considered. 1) First, our longitudinal study did not include female mice, based on our pilot data reported herein. However, it is possible that female protection against aging- and dietary Na^+-induced dysfunction may cease at later ages, as the anestrus phase has been described to occur in older animals than those included in our pilot study[82,83]. Thus, future studies are warranted to include similar assessments in female mice. 2) Second, given the duration of our study (13 months) and the limited warranty on the probes, our BP measurements were performed by tail-cuff rather than telemetry. However, we previously demonstrated excellent correlation between SBPs assessed simultaneously by tail-cuff plethysmography and telemetry in mice[19,20]. 3) Our study does not provide a longitudinal ultrasound assessment of cardiac and renal parameters in response to aging and salt. Recent data suggest that aging does not induce significant variations except for the expected increase in left ventricular mass[84]; however, future ultrasound studies are needed to investigate the role of extended exposure to dietary Na^+ on longitudinal assessments of cardiac and renal parameters. 4) Given the longitudinal study design, molecular assessments

are provided only for the end-of-study timepoint (**Mo 13**). In absence of cross-sectional data at earlier time points (for functional, molecular and histological studies), it is difficult to predict the specific changes that may occur in the aging kidney to result in BP progression. However, based on our end-of-study results, we speculate that AT1R, MR and Klotho are likely to play a role in renal damage initiation, at vascular, tubular and/or glomerular levels. Thus, targeting these mechanisms early on, before overt BP increases, may improve cardiovascular and renal outcomes associated with aging. 5) Although many of our findings appear to match the trajectories of change driving the aging phenotypes in humans, additional studies are needed to establish the C57BL6 mouse as a translatable animal model for aging research[55]. While the findings in this report support even a moderate reduction of salt intake, as well as the use of angiotensin receptor blockers and MR antagonists as preventive measures of aging- and salt-induced cardiorenal damage, future studies are needed to assess the longitudinal effectiveness of such interventions.

5. CONCLUSIONS

The present study suggests that earlier elevated urinary albumin excretion is associated with the development of hypertension in animals fed by normal and high salt content. Further, our data suggest that renal mechanisms at the vascular, tubular and/or glomerular levels may lead to albuminuria and thus precede the progression to higher blood pressure stages. Additional studies are needed to identify such mechanisms and to determine whether measurement of Alb/Cr alone or in combination with other markers is enough for adequate prognosis of BP progression, thus opening a window of opportunity for early intervention to prevent hypertension and associated conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations.

Alb/Cr	albumin/creatinine ratio
Aldo	Aldosterone
BP	Blood pressure
BW	Body weight

HS	High salt
LS	Low salt
MR	Mineralocorticoid receptor
Na$^+$	sodium
NS	Normal salt
PRA	Plasma renin activity
LRA RI	Left renal artery resistive index

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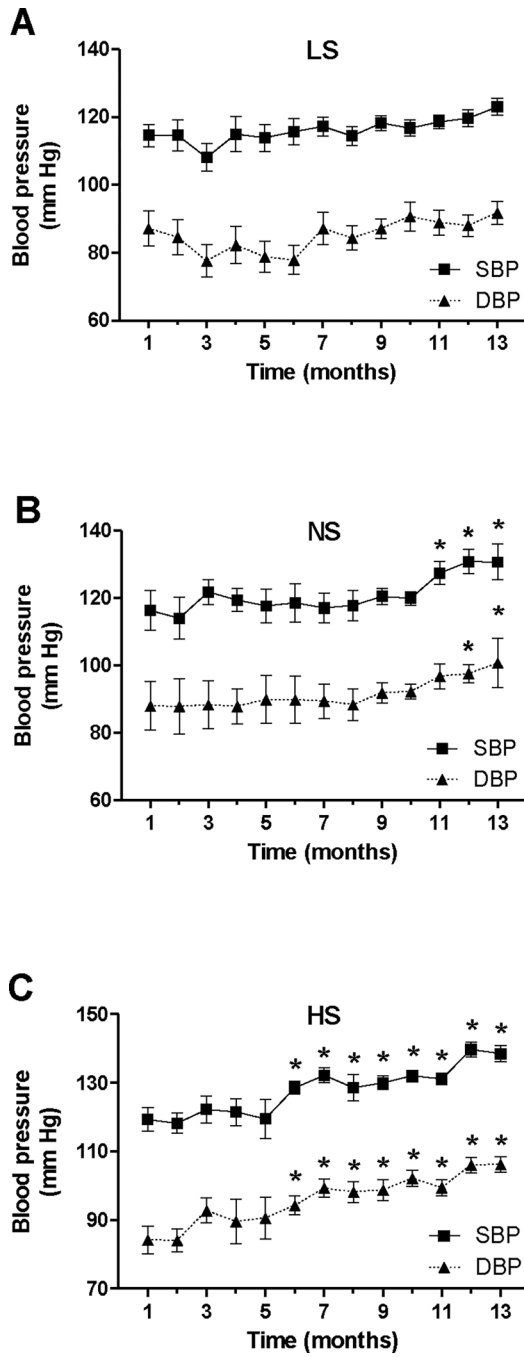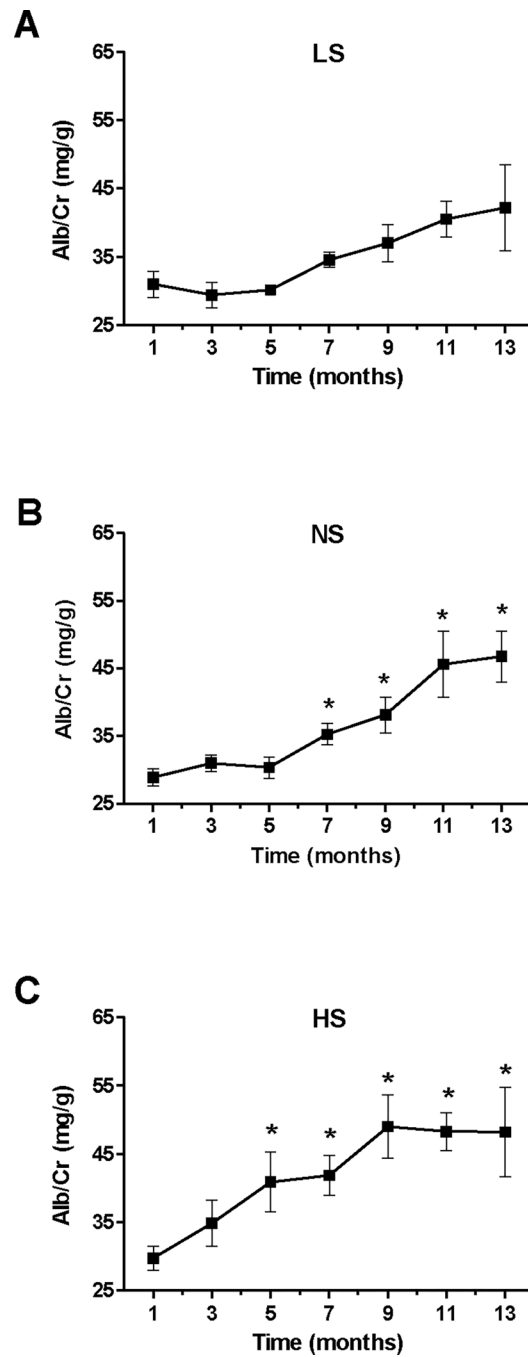
Figure 1. Longitudinal effect of aging and dietary sodium on blood pressure.
Mice were maintained on a LS (**A**), NS (**B**) or HS diet (**C**) for 13 months; SBP and DBP were assessed monthly in each animal as described under Methods. Data are presented as mean ± SEM. *P<0.05 vs. measurements in month 1.
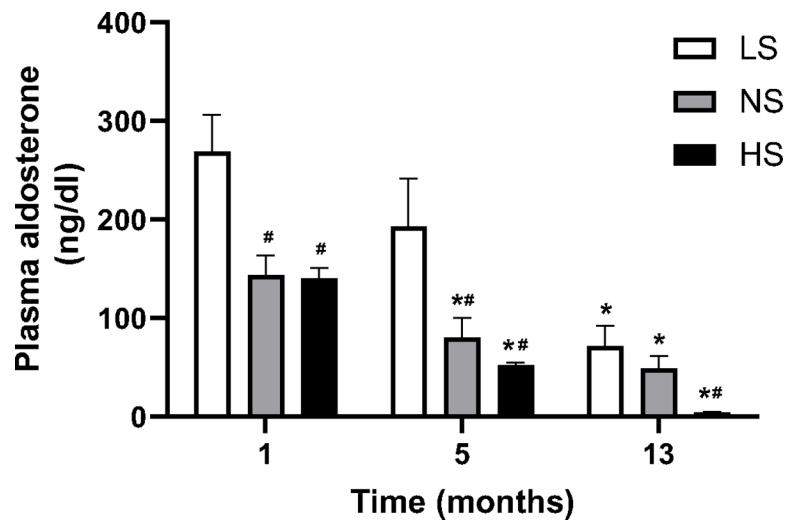
Figure 2. Longitudinal effect of aging and dietary sodium on plasma aldosterone levels.
Mice were maintained on three levels of dietary sodium intake (LS, NS, HS) for 13 months; blood samples were collected at indicated times and assessed for Aldo levels as described under Methods. Data are presented as mean ± SEM. *P<0.05 vs. **Mo 1** measurements on the same diet. #P<0.05 vs. LS diet in the same month.
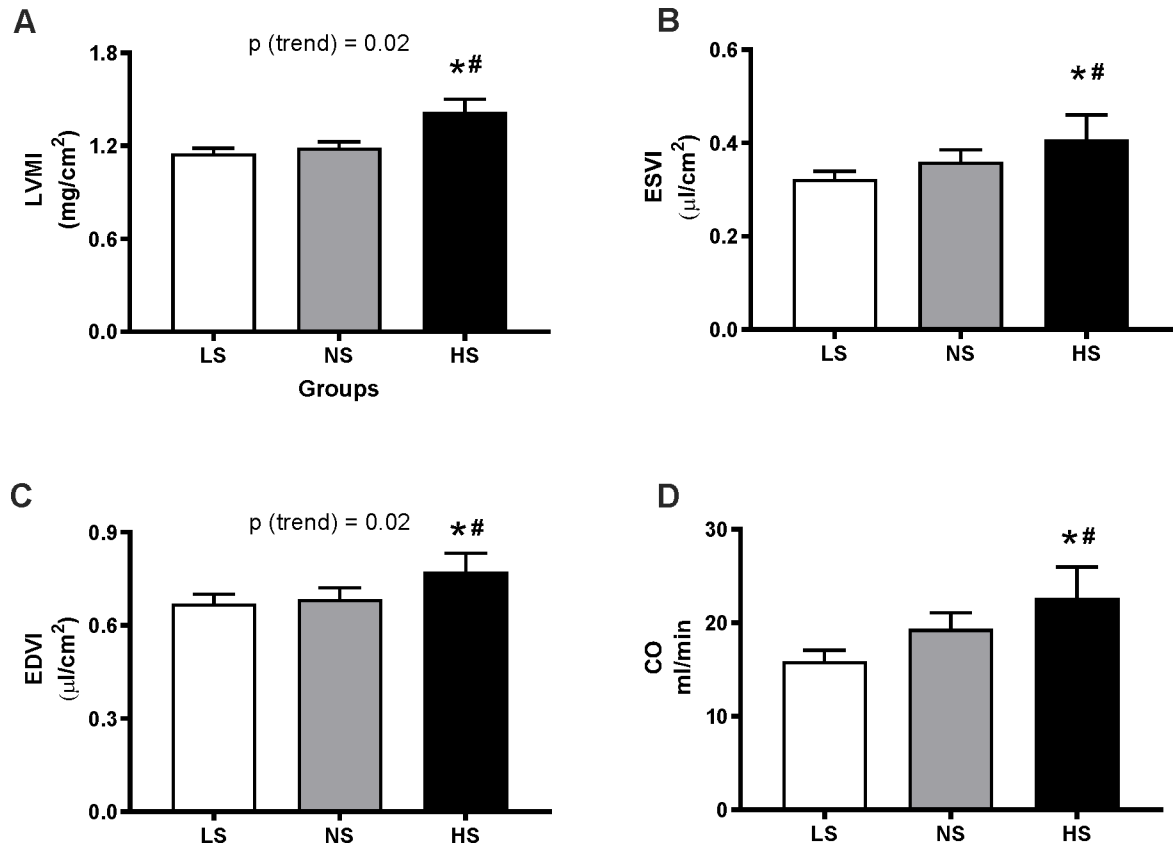
Figure 3. Effect of long-term dietary sodium on echocardiographic measures.
Mice underwent cardiac ultrasonography after 13 months of LS, NS or HS diet. Panels show the left ventricular mass index (LVMI, **A**), end-systolic volume index (ESVI, **B**), end-diastolic volume index (EDVI, **C**) and cardiac output (CO, **D**). Data are presented as mean ± SEM. *P<0.05 vs LS, #P<0.05 vs NS. P(trend) was obtained from simple linear regression analyses, using the level of sodium in the diets as independent variable, as described in the Methods.

Figure 4. Longitudinal effect of aging and dietary sodium on albumin/creatinine ratios.
Mice were maintained on a LS (**A**), NS (**B**) or HS diet (**C**) for 13 months; urine Alb/Cr ratios were assessed bimonthly in each animal as described under Methods. Data are presented as mean ± SEM. *P<0.05 vs. measurements in **Mo 1**.
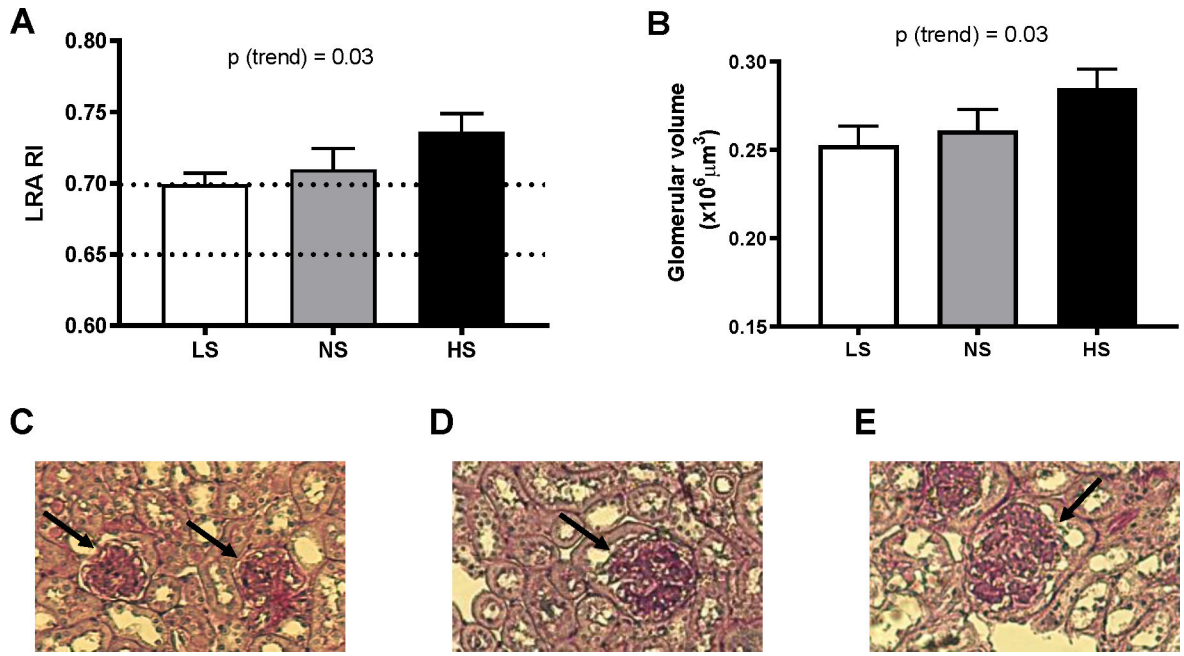
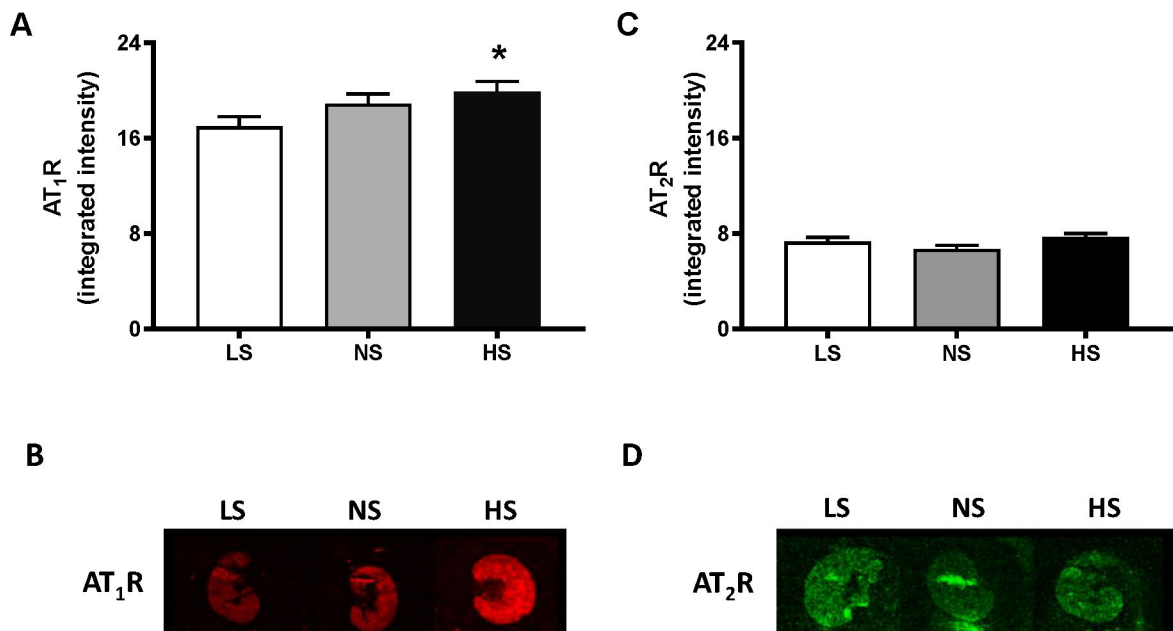**Figure 5. Long-term dietary sodium effect on renal dysfunction.**
Left renal artery resistive index (LRA RI, **A**) and glomerular volume (**B**) was assessed in mice fed a LS, NS, or HS diet for 13 months, as described under Methods. Representative images of glomeruli (PAS stained sections) are shown in **C**, **D** and **E**, for LS, NS and HS respectively. Dotted lines in panel **A** represent the cutoffs for normal (RI< 0.65), high-normal (0.65 RI < 0.7) and high resistive index (RI 0.7). Data are presented as mean ± SEM. *P<0.05 vs LS. P(trend) was obtained from simple linear regression analyses, using the level of sodium in the diets as independent variable, as described in the Methods.
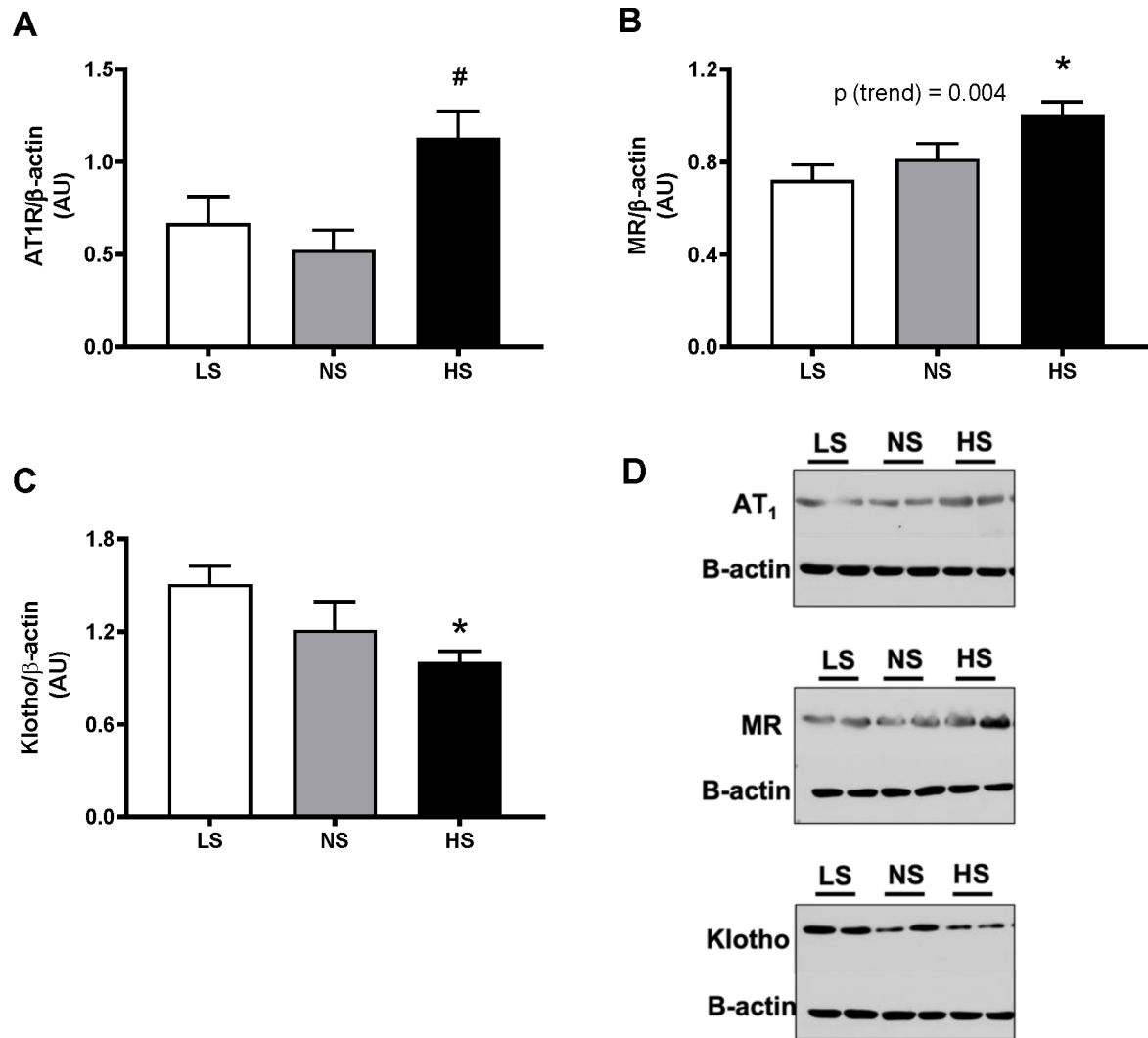
Figure 6. Effect of long-term dietary sodium on renal molecular marker expressions.
Mouse kidney cortex tissues were collected after 13 months of LS, NS or HS diet, and assessed for levels of AT_1R (**A**), AT_2R (**B**), Klotho (**C**) and MR (**D**) by Western blot, as described in Methods. *$P<0.05$ vs LS, #$P<0.05$ vs NS. P(trend) was obtained from simple linear regression analyses, using the level of sodium in the diets as independent variable, as described in the Methods.

Figure 7. Effect of long-term dietary sodium on renal AT$_1$R and AT$_2$R conformational status.
Mouse kidney cortex tissues were collected after 13 months of LS, NS or HS diet. Activation status-related changes in conformation were assessed for the AT$_1$R (**A**) and AT$_2$R (**C**), as described in Methods. Representative images are shown in panels **B** (for AT$_1$R) and **D** (for AT$_2$R). *P<0.05 vs LS.