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Interactive roles of *NPR1* gene-dosage and salt diets on cardiac angiotensin II, aldosterone and pro-inflammatory cytokines levels inmutantmice

Di Zhao[*], **Subhankar Das**[*], and **Kailash N. Pandey**
Department of Physiology, Tulane University Health Sciences Center, School of Medicine, New Orleans, Louisiana, USA

Abstract

Objective—The objective of the present study was to elucidate the interactive roles of guanylyl cyclase/natriuretic peptide receptor-A (NPRA) gene (*Npr1*) and salt diets on cardiac angiotensin II (ANG II), aldosterone and proinflammatory cytokines levels in *Npr1* gene-targeted (1-copy, 2-copy, 3-copy, 4-copy) mice.

Methods—*Npr1* genotypes included 1-copy gene-disrupted heterozygous (+/−), 2-copy wild-type (+/+), 3-copy gene-duplicated heterozygous (++/+) and 4-copy gene-duplicated homozygous (++/++) mice. Animals were fed low, normal and high-salt diets. Plasma and cardiac levels of ANG II, aldosterone and pro-inflammatory cytokines were determined.

Results—With a high-salt diet, cardiac ANG II levels were increased (+) in 1-copy mice (13.7 ± 2.8 fmol/mg protein, 111%) compared with 2-copy mice (6.5 ± 0.6), but decreased (−) in 4-copy (4.0 ± 0.5, 38%) mice. Cardiac aldosterone levels were increased (+) in 1-copy mice (80 ± 4 fmol/mg protein, 79%) compared with 2-copy mice (38 ± 3). Plasma tumour necrosis factor alpha was increased (+) in 1-copy mice (30.27 ± 2.32 pg/ml, 38%), compared with 2-copy mice (19.36 ± 2.49, 24%), but decreased (−) in 3-copy (11.59 ± 1.51, 12%) and 4-copy (7.13 ± 0.52, 22%) mice. Plasma interleukin (IL)-6 and IL-1α levels were also significantly increased (+) in 1-copy compared with 2-copy mice but decreased (−) in 3-copy and 4-copy mice.

Conclusion—These results demonstrate that a high-salt diet aggravates cardiac ANG II, aldosterone and proinflammatory cytokine levels in *Npr1* gene-disrupted 1-copy mice, whereas, in *Npr1* gene-duplicated (3-copy and 4-copy) mice, high salt did not render such elevation, suggesting the potential roles of *Npr1* against salt loading.

Keywords

aldosterone; angiotensin II; arterial pressure; atrial natriuretic peptide; gene duplication; gene knockout; pro-inflammatory cytokines; salt diet

Correspondence to Kailash N. Pandey, PhD, Department of Physiology, SL-39 Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA. Tel: +1 504 988 1628; fax: +1 504 988 2675; kpandey@tulane.edu.
[*]Di Zhao and Subhankar Das contributed equally as first authors of this article.

Conflicts of interest
The authors have no conflicts of interest.

INTRODUCTION

The natriuretic peptides comprise at least three ligands: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide [1] (CNP) [2,3]. ANP and BNP are mainly released from the heart [3], whereas CNP is produced in endothelial cells [2,4]. Three distinct natriuretic peptide receptors have been identified and characterized by molecular cloning; these include natriuretic peptide receptor-A (NPRA), natriuretic peptide receptor-B (NPRB) and natriuretic peptide receptor-C (NPRC) [4–7]. ANP and BNP bind to NPRA, a membrane-bound form of guanylyl cyclase, which is termed as guanylyl cyclase-A (GC-A)/NPRA [5,6,8]. CNP binds to NPRB, which is also a membrane guanylyl cyclase and is known as GC-A/NPRB [8]. All three natriuretic peptides bind to NPRC, which lacks guanylyl cyclase activity [5,8]. The ligand binding to NPRA increases the intracellular second-messenger cyclic GMP (cGMP) concentrations by enhanced guanylyl cyclase activity [4,9–12]. The major functions of ANP-NPRA signalling include natriuretic, diuretic, vasodilatory [2,4,11,13,14], antifibrotic and antihypertrophic effects [8,15–17]. The *Npr1* (coding for GC-A/NPRA) has been found to lower arterial pressure and to increase guanylyl cyclase activity in a gene dose-dependent manner [13,18]. Previous studies [13,15,18–24] have suggested the important functions of NPRA signalling in cardiac hypertrophy and arterial blood pressure regulation. The interaction of salt sensitivity with ANP/NPRA system in the development of hypertension, cardiac hypertrophy and inflammatory responses is incompletely understood. A high-salt diet contributes to the pathogenesis of hypertension, which is recognized as a multifactorial trait resulting from the effects of a combination of both environmental and genetic factors [25–28]. Disruption of *Npr1* gene leads to hypertension and cardiac hypertrophy in null mutant mice [13,15]. Similarly, ANP homozygous null mutant (Nppa−/−) mice also show exaggerated cardiac hypertrophy and elevated blood pressures [15]. Sodium overload has been shown to be linked with essential hypertension, eliciting cardiac remodelling [29–32].

Aldosterone plays an important role in electrolyte transport and exerts direct effects on cardiac hypertrophy and heart failure by binding to mineralocorticoid receptors in the heart and blood vessels [33–35]. In addition, mineralocorticoid receptor antagonism has been shown to reverse myocardial and aortic fibrosis caused by aldosterone [36,37]. In humans, aldosterone has been shown to exert direct adverse effects on the heart that are independent of its effects on arterial pressure [38]. In patients with severe heart failure, the use of a mineralocorticoid receptor antagonist has been found to reduce morbidity and mortality by 30% [38]. Such studies clearly demonstrated the importance of aldosterone in cardiac hypertrophy and arterial blood pressure regulation. ANP inhibits aldosterone synthesis in cultured neonatal rat cardiomyocytes [39] and the adrenal glands [40,41]. The inhibitory effect of ANP on aldosterone synthesis should depend on the functionally active catalytic domain of NPRA [42]. As NPRA signalling counteracts the renin–angiotensin–aldosterone system (RAAS) [43–45], we tested the hypothesis that whether the cardiac angiotensin II (ANG II) and aldosterone levels are increased in *Npr1* gene-disrupted mice, but decreased in *Npr1* gene-duplicated mice, in a gene dose-dependent manner. We determined the effect of low-salt or high-salt diets on cardiac ANG II, aldosterone and pro-inflammatory cytokine levels in *Npr1* gene-targeted (gene-knockout and gene-duplicated) mice having varying gene

copy numbers. The part of our ongoing studies has previously reported the adrenal ANG II and aldosterone levels in *Npr1* gene-targeted mice [43]. In the present communication, we report the further results of additional findings on the status of cardiac ANG II, aldosterone and pro-inflammatory cytokines in these genetically altered *Npr1* mouse models.

MATERIALS AND METHODS

Generation of *Npr1* gene-targeted mice

Npr1 gene-targeted (gene-knockout and gene-duplication) mice were generated by homologous recombination as previously described [8,13]. Animals were generated from correctly targeted embryonic stem cells as previously described [46]. F1 gene-knockout heterozygous animals were identified by Southern blot and PCR analysis of DNA extracted from tail biopsies as previously reported [14,15]. Gene-duplicated homozygous and heterozygous mice were identified by Massachusetts Institute of Technology (MIT) markers analysis using primers D3MIT40 and D3MIT101 [47]. All mice were female littermate progenies of the C57/BL6 genetic background. Mice were bred in the Animal Care Facility at the Tulane University Health Sciences Center. The following are the mice genotypes: 1-copy (+/−) is a gene-disrupted heterozygous mice; 2-copy (+/+) is a wild-type mice; 3-copy (++/+) is a gene-duplicated heterozygous mice; and 4-copy (++/++) is a gene-duplicated homozygous mice as previously reported [38]. Animals were maintained in a 12 :12-h light–dark cycle (0600 to 1800 h) at 25°C. During the 3-week study period, 24- to 28-week-old female mice were given a low-salt (0.05% NaCl), a normal salt (0.3% NaCl) or a high-salt (8% NaCl) diet and tap water *ad libitum* as previously reported [43]. In the present studies, the survival of animals was as follow: low-salt diet (1-copy, 97%; 2-copy, 97%; 3-copy 100%; 4-copy, 100%), normal salt diet (1-copy, 94%; 2-copy, 91%; 3-copy 97%; 4-copy, 100%) and high-salt diet (1-copy, 84%; 2-copy, 94%; 3-copy 100; 4-copy, 100%). In the present studies, the major limitation was encountered to obtain the sufficient number of *Npr1* gene-disrupted homozygous null mutant (−/−; 0-copy) mice. Approximately, 25% *Npr1* 0-copy null mutant pups die after 1–2 days of birth. It has also been observed that some unborn pups die *in utero* just before the birth. Above all, approximately, 30% of the adult 0-copy mice die after 6 months of age due to congestive heart failure. Consequently, the adult 0-copy homozygous null mutant mice colonies are significantly reduced as compared with heterozygous (1-copy) and wild-type (2-copy) mice colonies. Thus, due to a low number of adult 0-copy mice colonies, we could not include these animals in the present studies. All protocols were approved by the Institutional Animal Care and Use Committee at Tulane University Health Sciences Center.

Arterial pressure measurement

The arterial pressure of *Npr1* mice was measured every other day by the noninvasive computerized tail-cuff method, using a Visitech BP2000 (Visitech Systems Inc., Apex, North Carolina, USA) as previously described [48]. After 7 days of training of the mice for arterial pressure measurement, an average arterial pressure level of five sessions per day was calculated for the analysis.

Urine and urinary sodium and potassium measurements

After the first 3 days adaptation in metabolic cage, mice were provided with a low-salt, a normal salt or a high-salt diet, respectively, for a 3-week periods. Urine was collected and urine volumes were recorded every other day and kept at −20°C until used. Urinary sodium and potassium were measured using Flame Photometer IL973 (Instrumentation Laboratory, Lexington, Massachusetts, USA). Urine outputs, urinary sodium and potassium excretions per day were normalized by kidney weight.

Blood and tissue collection

After mice had been anaesthetized with CO_2, blood was collected by cardiac puncture in a tube containing 10μl 0.25 mol/l EDTA as previously reported [43]. The blood was centrifuged and the separated plasma was kept at −80°C until used. Hearts were removed and blood exuded from them, after which the hearts were weighed, frozen in liquid nitrogen and kept at −80°C until used. The body weight and tibia length of each mouse was measured for normalization of heart weight.

Cardiac angiotensin II assay

Frozen heart tissues were rinsed and homogenized in 10 mmol/l pyrophosphate buffer containing 100 mmol/l NaCl, 1 mmol/l phenylmelthylsulfonylfluoride (PMSF) and 1 mmol/l EDTA with a Polytron (Brinkmann Instruments, Westbury, New York, USA) at a setting of 10 (three times for 30 s) at 4°C as previously described [49]. The homogenate was centrifuged at 40 000*g* for 40 min at 4°C. The protein concentration of the heart extract was determined using a protein assay reagent (BioRad, Hercules, California, USA) for normalization and assay of cardiac ANG II levels. The extraction and assay of ANG II was performed as previously described [43].

Cardiac aldosterone assay

Frozen heart tissues were rinsed in phosphate buffer and homogenized as described above for ANG II assay. Aldosterone levels in tissue extract and plasma were assayed using a radioimmunoassay kit (Diagnostic System, Webster, Texas, USA) according to the manufacturer's protocol as previously described [21,43]. The assay of aldosterone indicated 100% cross-reactivity; however, corticosterone showed only 0.02% cross-reactivity and all other steroids were undetectable.

Plasma cyclic GMP assay

Blood samples were collected in tubes containing EDTA and immediately centrifuged at 2500 rpm for 10 min at 4°C. The plasma was separated and stored at −80°C until used. The plasma cGMP levels were assayed using a direct cGMP enzyme-linked immunoassay kit (Assay Designs, Ann Arbor, Michigan, USA), according to the manufacturer's protocol.

Assay of pro-inflammatory and cytokines in plasma and heart tissues

The concentration of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1β), was measured in plasma and heart tissues by multiplex bead array format (Millipore,

Billerica, Massachusetts, USA), using a Bio-Plex instrument (Bio-Rad) according to the manufacturer's guidelines. Spectrally addressed polystyrene beads coated with cytokine-specific monoclonal antibodies were used to capture the cytokines of interest. The instrument sorted out and measured the fluorescent signal from each bead by dual excitation sources.

Statistical analysis

Statistical analyses were performed by two-way analysis of variance (ANOVA) combined with Bonferroni's multiple comparison posthoc test, using the GraphPad PRISM program (version 4.0; GraphPad Software, San Diego, California, USA). Due to the nonnormality of some of the data, we also performed the nonparametric statistical analysis using Kruskal–Wallis test to confirm the ANOVA analysis. The results are presented as mean ± standard error. Significance was set at a P value of less than 0.05.

RESULTS

Plasma cyclic GMP levels in *Npr1* mice fed with different salt diets

With all three salt diets, the plasma cGMP levels were decreased (−) in 1-copy mice [normal salt: −53% (16.24 ± 1.82, $P<0.01$); low salt: −48% (8.71 ± 0.63, $P<0.05$); high salt: −56% (24.53 ± 2.22, $P<0.01$)] compared with 2-copy mice (Fig. 1). However, with all three salt diets, the plasma cGMP levels were increased (+) in 3-copy mice [normal salt: 40% (48.69 ± 3.02, $P<0.05$); low salt: 60% (26.75 ± 2.49, $P<0.05$); high salt: 28% (72.09 ± 7.99, $P<0.05$)] and 4-copy mice [normal salt: 80% (62.01 ± 5.15, $P<0.01$); low salt: 120% (36.96 ± 2.84, $P<0.01$); high salt: 64% (91.73 ± 7.08, $P<0.01$)] compared with 2-copy mice (Fig. 1). We also compared plasma cGMP levels in mice with the same *Npr1* gene copy number fed with the different salt diets. The low-salt diet decreased (−) plasma cGMP levels in 1-copy (−46%, $P<0.05$), 2-copy (−52%, $P<0.01$), 3-copy (−45%, $P<0.05$) and 4-copy mice (−40%, $P<0.01$) compared with plasma cGMP levels in mice given the normal salt diet (Fig. 1). However, with a high-salt diet, plasma cGMP levels were increased (+) in 1-copy (51%, $P<0.01$), 2-copy (62%, $P<0.01$), 3-copy (48%, $P<0.01$) and 4-copy mice (48%, $P<0.01$) (Fig. 1).

Effect of salt diets on cardiac angiotensin II and aldosterone levels in *Npr1* mice

On a normal salt diet, cardiac ANG II levels increased (+) in 1-copy mice (27%, 9.7 ± 0.5, $P<0.05$) compared with 2-copy mice (7.6 ± 0.7), but decreased (−) in 4-copy mice (31%, 5.3 ± 0.2, $P<0.05$) (Fig. 2). Similarly, cardiac ALDO levels decreased (−) in 3-copy (33%, 0.03 ± 0.004, $P<0.01$) and 4-copy mice (55%, 0.02 ± 0.002, $P<0.001$) fed a normal salt diet as compared with 2-copy mice (0.04 ± 0.003) (Fig. 3). With a low-salt diet, cardiac ANG II levels slightly increased (+) in 1-copy mice (35%, 8.6 ± 0.7) compared with 2-copy mice (6.4 ± 0.4), but slightly decreased (−) in 4-copy (37%, 4.0 ± 0.8) mice (Fig. 2). On the same diet, cardiac aldosterone levels decreased (−) in 3-copy (38%, 0.03 ± 0.004, $P<0.01$) and 4-copy mice (59%, 0.02 ± 0.003, $P<0.001$) compared with 2-copy mice (0.04 ± 0.005) (Fig. 3). With a high-salt diet, cardiac ANG II levels increased (+) in 1-copy mice (111%, 13.7 ± 2.8, $P<0.001$) compared with 2-copy mice (6.5 ± 0.6), but decreased (−) in 4-copy (38%, 4.0 ± 0.5, $P>0.05$) mice (Fig. 2). Similarly, cardiac aldosterone levels also increased (+) in 1-

copy mice (79%, 0.08 ± 0.004, $P<0.001$) at a high-salt diet compared with 2-copy mice (0.04 ± 0.003), but decreased (−) in 3-copy (43%, 0.02 ± 0.005, $P<0.001$) and 4-copy mice (61%, 0.02 ± 0.002, $P<0.001$) (Fig. 3). We also compared cardiac ANG II and aldosterone levels in mice with the same *Npr1* gene copy number given different diets. The high-salt diet increased (+) cardiac ANG II (42%, $P<0.001$) (Fig. 2) and aldosterone levels (40%, $P<0.001$) (Fig. 3) only in 1-copy mice as compared with mice given a normal salt diet.

With a normal salt diet, plasma ANG II levels decreased (−) in 1-copy (45.1 ± 3.5, $P<0.001$), 3-copy (62.1 ± 3.8, $P<0.01$) and 4-copy mice (54.6 ± 3.2, $P<0.01$) compared with 2-copy mice (86.0 ± 3.5) (Fig. 4). In contrast, plasma aldosterone levels slightly increased (+) in 1-copy mice (2.5 ± 0.3) compared with 2-copy mice (1.8 ± 0.2), but slightly decreased (−) in 3-copy (1.3 ± 0.1) and 4-copy mice (1.1 ± 0.1) (Fig. 5). With a low-salt diet, plasma ANG II levels decreased (−) in 1-copy (86.8 ± 4.9, $P<0.01$), 3-copy (91.2 ± 4.8, $P<0.05$) and 4-copy mice (79.3 ± 5.3, $P<0.001$) compared with 2-copy mice (106.9 ± 5.0) (Fig. 4). On the contrary, plasma aldosterone levels increased (+) in 1-copy (5.9 ± 0.5, $P<0.001$) compared with 2-copy mice (4.1 ± 0.4), but decreased (−) in 3-copy (3.0 ± 0.2, $P<0.01$) and 4-copy mice (3.1 ± 0.3, $P<0.001$) (Fig. 5). With a high-salt diet, plasma ANG II levels decreased (−) in 1-copy (25.0 ± 3.9, $P<0.001$) compared with 2-copy mice (51.9 ± 5.3), but there were no statistical differences in 3-copy (51.4 ± 3.6) or 4-copy mice (50.0 ± 4.2) compared with 2-copy mice (Fig. 4). Similarly, there were no statistical differences among plasma aldosterone levels in 1-copy (1.0 ± 0.2), 3-copy (0.9 ± 0.1) or 4-copy mice (0.8.1) compared with 2-copy mice (1.1 ± 0.2) (Fig. 5). We also compared plasma ANG II and aldosterone levels in mice with the same *Npr1* gene copy number fed the different diets. The low-salt diet increased (+) plasma ANG II levels in 1-copy ($P<0.01$), 2-copy ($P<0.05$), 3-copy ($P<0.05$) and 4-copy mice ($P<0.05$) compared with mice given the normal salt diet (Fig. 4). However, the high-salt diet decreased (−) plasma ANG II levels in 1-copy ($P<0.05$) and 2-copy mice ($P<0.01$) (Fig. 4). The low-salt diet significantly increased (+) plasma aldosterone levels in 1-copy ($P<0.01$), 2-copy ($P<0.01$), 3-copy ($P<0.001$) and 4-copy mice ($P<0.001$) compared with mice fed a normal salt diet (Fig. 5). However, the high-salt diet significantly decreased (−) plasma aldosterone levels in 1-copy ($P<0.001$) mice (Fig. 5).

Effect of different salt diets on urine output and urinary sodium and potassium excretion in *Npr1* mice

As shown in Fig. 6, with a normal salt diet, urine outputs decreased (−) in 1-copy mice (32%, 3.06 ± 0.38, $P<0.05$) compared with 2-copy mice (4.49 ± 0.35), but increased (+) in both 3-copy (33%, 5.97 ± 0.47, $P<0.05$) and 4-copy (68%, 7.56 ± 0.86, $P<0.05$) mice. Similarly, with a low-salt diet, urine outputs decreased (−) in 1-copy mice (28%, 2.38 ± 0.23, $P<0.05$) compared with 2-copy mice (3.29 ± 0.19), but increased (+) in 3-copy (36%, 4.48 ± 0.42, $P<0.05$) and 4-copy (51%, 4.96 ± 0.44, $P<0.01$) mice (Fig. 6a). Also, with a high-salt diet, urine outputs decreased (−) in 1-copy mice (12%, 29.78 ± 0.95, $P<0.01$) compared with 2-copy mice (33.79 ± 0.65), but slightly increased (+) in 3-copy (20%, 40.48 ± 1.38, $P<0.001$) and 4-copy (27%, 42.97 ± 1.74, $P<0.001$) mice (Fig. 6b). Normal salt diet decreased (−) urinary sodium excretions in 1-copy mice (32%, 0.68 ± 0.06, $P<0.001$) compared with 2-copy mice (0.99 ± 0.04), but increased (+) in 3-copy (20%, 1.19 ± 0.06, $P<0.05$) and 4-copy (35%, 1.33 ± 0.09, $P<0.05$) mice (Fig. 7a). At a low-salt diet, urinary

sodium excretions decreased (−) in 1-copy mice (27%, 0.024 ± 0.002, $P<0.05$) compared with 2-copy mice (0.032 ± 0.003), but increased (+) in 3-copy (48%, 0.048 ± 0.005, $P<0.05$) and 4-copy (60%, 0.052 ± 0.006, $P<0.05$) mice (Fig. 7b). Also, with a high-salt diet, urinary sodium excretions decreased (−) in 1-copy mice (18%, 12.75 ± 0.48, $P<0.01$) compared with 2-copy mice (15.54 ± 0.54), but increased (+) in 3-copy (27%, 19.68 ± 1.18, $P<0.01$) and 4-copy mice (31%, 20.35 ± 1.55, $P<0.01$, Fig. 7c). There were no statistical differences for urinary potassium excretions in 1-copy, 3-copy and 4-copy mice compared with 2-copy mice with normal salt, low-salt or high-salt diets, respectively (Fig. 8).

Effect of different salt diets on proinflammatory cytokines levels in *Npr1* mice

Plasma pro-inflammatory cytokines including TNF-α, IL-6 and IL-1α in salt-treated *Npr1* mice are shown in Fig. 9a–c. TNF-α levels were increased (+) in low-salt treated 1-copy mice (80%, 18.25 ± 2.12, $P<0.001$) compared with 2-copy (10.14 ± 0.92) mice, but decreased (−) in 3-copy (30%, 7.08 ± 0.65, $P<0.05$) and 4-copy (43%, 5.78 ± 1.35, $P<0.01$) mice. Similarly, with a normal salt diet, TNF-α levels were increased (+) in 1-copy mice (40%, 21.98 ± 2.01, $P<0.01$) compared with 2-copy mice (15.66 ± 1.84), but decreased (−) in 3-copy (34%, 10.33 ± 0.71, $P<0.05$) and 4-copy (63%, 5.83 ± 0.72, $P<0.05$) mice. High-salt diet increased (+) TNF-α levels in 1-copy mice (56%, 30.27 ± 2.32, $P<0.01$) but decreased (−) in 3-copy (40%, 11.59 ± 1.51, $P<0.05$) and 4-copy (63%, 7.13 ± 0.52, $P<0.05$) mice compared with 2-copy mice (19.36 ± 2.49). Likewise, low-salt, normal salt and high-salt diets increased (+) plasma IL-6 levels in 1-copy mice (145%, 34.93 ± 1.73, $P<0.01$; 80%, 48.69 ± 1.99, $P<0.01$; 95%, 63.07 ± 4.02, $P<0.01$, respectively) but decreased (−) in 3-copy (37%, 8.92 ± 1.72, $P<0.05$; 36%, 17.22 ± 2.23, $P<0.05$; 39%, 19.57 ± 1.94, $P<0.05$) and 4-copy (47%, 7.59 ± 1.91, $P<0.05$; 62%, 10.32 ± 1.47, $P<0.01$; 63%, 11.87 ± 1.71, $P<0.05$) mice, respectively, compared with 2-copy controls (14.23 ± 1.77, 27.01 ± 3.02, 32.27 ± 1.50). Plasma IL-1α levels were also increased in low-salt treated 1-copy mice (152%, 14.84 ± 2.31), whereas 3-copy (43%, 3.37 ± 0.43) and 4-copy (62%, 2.26 ± 0.38) mice showed a decrease (−) compared with 2-copy mice. Normal and high-salt diets showed a significant increase (+) in IL-1α levels in 1-copy mice (115%, 20.21 ± 0.72; 70%, 27.83 ± 1.43) but decreased in 3-copy (36%, 6.04 ± 0.63; 44%, 9.10 ± 1.78) and 4-copy (56%, 4.12 ± 0.54; 63%, 6.01 ± 1.08) mice. Cardiac TNF-α, IL-6 and IL-1α levels were significantly increased (+) in 1-copy mice at low-salt (3.5-fold, 9.58 ± 0.78; 37%, 36.55 ± 3.93; two-fold, 50.25 ± 5.14, respectively), normal salt (five-fold, 17.08 ± 1.65; 43%, 56.69 ± 4.62; 2.3-fold, 42.82 ± 4.06, respectively) and high-salt diets (three-fold, 20.86 ± 2.03; 29%, 78.90 ± 9.62; 2.7-fold, 68.12 ± 7.04, respectively) compared with 2-copy mice (Fig. 9d and e). On the contrary, low-salt, normal salt and high-salt diets showed a reduction (−) in cardiac TNF-α levels in 3-copy (37%, 1.71 ± 0.27; 36%, 2.30 ± 0.39; 46%, 3.65 ± 0.26) and 4-copy (52%, 1.30 ± 0.21; 56%, 1.58 ± 0.32; 72%, 1.88 ± 0.35) mice. Further, cardiac IL-6 (36%, 17.06 ± 0.68; 24%, 30.40 ± 3.48; 38%, 37.95 ± 4.97) and IL-1α levels (53%, 10.32 ± 1.21; 44%, 10.40 ± 0.90; 41%, 14.76 ± 1.04) were also decreased (−) in 3-copy (67%, 8.78 ± 1.10; 62%, 15.08 ± 2.07; 52%, 29.36 ± 3.20) and 4-copy (68%, 6.97 ± 0.51; 63%, 6.83 ± 0.91; 63%, 9.28 ± 0.51) mice receiving low-salt, normal salt and high-salt diets (Fig. 9d and e).

Effect of different salt diets on the heart weight to tibia length and heart weight to body weight ratios in *Npr1* mice

On a high-salt diet, arterial pressures were increased (+) in 1-copy (11%, $P<0.01$) and 2-copy (10%, $P<0.05$,) mice, whereas a low-salt diet did not alter arterial pressures in 1-copy, 2-copy, 3-copy or 4-copy mice (Table 1). With a normal salt diet, the heart weight to tibia length ratio increased (+) in 1-copy mice (9.3%, 16.5 ± 0.2, $P<0.05$) compared with 2-copy mice (15.1 ± 0.1), but decreased (−) in 4-copy mice (8.6%, 13.8 ± 0.1, $P<0.01$) (Table 1). On a low-salt diet, the ratio of heart weight to tibia length decreased (−) in 4-copy mice (10.8%, 13.2 ± 0.4, $P<0.05$) compared with 2-copy mice (14.8 ± 0.2). However, on a high-salt diet, this ratio increased (+) in 1-copy mice (10.7%, 17.6 ± 0.6, $P<0.01$) compared with 2-copy mice (15.9 ± 0.4), but decreased (−) in 3-copy (11.3%, 14.1 ± 0.3, $P<0.05$) and 4-copy (14.5%, 13.6 ± 0.7, $P<0.01$) mice. As shown in Table 1, with a normal salt diet, the heart weight to body weight ratio decreased (−) in 4-copy mice (5.0 ± 0.1, $P<0.01$) compared with 2-copy mice (5.6 ± 0.2). Similarly on a low-salt diet, the heart weight to body weight ratio also decreased (−) in 4-copy mice (5.1 ± 0.1, $P<0.05$) compared with 2-copy mice (5.7 ± 0.1). With a high-salt diet, this ratio decreased (−) in both 3-copy (5.5 ± 0.2, $P<0.01$) and 4-copy mice (5.2 ± 0.1, $P<0.001$) mice compared with 2-copy mice (6.3 ± 0.1).

DISCUSSION

Increased cardiac angiotensin II levels in *Npr1* mice with a high-salt diet

The present findings provide the evidence that cardiac ANG II levels are increased (+) in *Npr1* gene-disrupted mice but decreased (−) in *Npr1* gene-duplicated mice in a gene dose-dependent manner. As cardiac ANG II plays roles in cardiac remodelling and function [50,51], an increased cardiac ANG II level may participate in the process of cardiac hypertrophy and heart failure in *Npr1* null mutant mice. Previous studies have suggested that most of the cardiac ANG II appears to be produced at tissue sites by the conversion of in-situ synthesized ANG I rather than blood-derived ANG I [52]. Factors such as the circulating renin levels and ANG II binding sites in the heart affect cardiac ANG II production [53]. Enzymatic degradation of ANG II and ANG II-type 1 (AT1) receptor-mediated endocytosis also affect the cardiac ANG II levels [53]. Earlier studies have shown that AT1 receptor signalling in cardiac myocytes and fibroblasts elicits growth and fibrosis [54]. It has been reported that ANP inhibits Ang II-stimulated proliferation in foetal cardiomyocytes, indicating the inhibitory role of ANP-NPRA signalling in cardiac hypertrophy [55]. In comparison with a normal salt diet, a high-salt diet increased (+) cardiac ANG II levels only in 1-copy mice, suggesting that an increased (+) cardiac ANG II may promote cardiac hypertrophy in *Npr1* gene-disrupted mice. It has also been reported that a high-salt diet causes cardiac hypertrophy in Dahl salt-sensitive rats [56].

Cardiac aldosterone levels are elevated in *Npr1* mice fed with a high-salt diet

It has been previously reported that a low-salt diet stimulates plasma aldosterone levels, whereas a high-salt diet suppresses its production [57]. In the present study, a low-salt diet increased (+) plasma aldosterone levels in 1-copy, 2-copy, 3-copy and 4-copy mice compared with mice given a normal salt diet. However, a high-salt diet suppressed (−)

plasma aldosterone levels in 1-copy mice, but not in 3-copy and 4-copy mice. It is possible that an increased sodium excretion and urine output with a decreased arterial pressure in 3-copy and 4-copy mice attenuate the inhibitory effect of a high-salt diet on plasma aldosterone levels [58]. Our findings suggest that NPRA signalling exerts protective function with regard to blood volume homeostasis and arterial pressure regulation in *Npr1* gene-duplicated mice as compared with *Npr1* gene-disrupted mice. Interestingly, it has been reported that a high-salt diet did not affect plasma renin activity in ANP gene-knockout mice as compared with wild-type mice [59]. The authors considered that ANP gene-knockout mice develop a salt-sensitive component of hypertension in association with a failure to downregulate plasma renin activity adequately. There were interactions of salt with *Npr1* gene copy numbers for both cardiac and plasma aldosterone levels. However, the data on cardiac ALDO synthesis are still controversial [52,53]. The present results suggest that ANP/NPRA/cGMP signalling reduces the cardiac aldosterone levels and protects the heart from cardiac hypertrophy and remodelling process in disease states.

Effect of salt diets on urine volume and urinary sodium, and potassium levels in *Npr1* mice

Variations in dietary sodium chloride intake are closely associated with changes in renal renin and Ang II content [60]. Increased sodium excretion and urine output with relatively lower blood pressure in *Npr1* gene-duplicated mice may attenuate the inhibitory effect of a high-salt diet on Ang II levels. ANP-NPRA signalling is critical in mediating the natriuresis and diuresis after acute volume expansion [61,62]. In the present study, urinary sodium excretion and urine output decreased (−) in *Npr1* 1-copy mice as compared with wild-type mice, but increased (+) in *Npr1* gene-duplicated (3-copy and 4-copy) mice with a normal salt, a low-salt or a high-salt diet, respectively. Our result indicates that decreased ANP-NPRA signalling can lead to sodium retention that causes blood pressure elevation in *Npr1* gene-disrupted mice. On the contrary, increased (+) ANP-NPRA signalling effectively attenuates sodium retention and blood pressure elevation in *Npr1* gene-duplicated mice. These present results further support the concept that ANP-NPRA signalling plays a critical role in mediating natriuresis and diuresis in a gene dose-dependent manner.

Elevated levels of cardiac pro-inflammatory cytokines in *Npr1* mice fed a high-salt diet

In the present study, the plasma and cardiac cytokine levels were decreased (−) in all the groups receiving a low-salt diet compared with mice receiving a normal salt diet, suggesting that salt restriction could reduce the pro-inflammatory cytokine levels. Similar results have also been reported in rats receiving ANG II for 10 days [63]. The present results showed that TNF-α, IL-6 and IL-1α levels were elevated (+) in both plasma and heart tissues of *Npr1* mice on a high-salt diet compared with a normal salt diet. Our previous studies have shown that pro-inflammatory cytokines promote ventricular remodelling and contractile dysfunction in *Npr1* mice [15]. Although a high-salt diet showed a significant baseline elevation (+) for all cytokines in 1-copy mice, yet only a small increase occurred in 3-copy and 4-copy mice. The data suggest that increasing *Npr1* gene dosage may play a regulatory role in maintaining the pro-inflammatory cytokine levels in sodium-overloaded mice.

Role of a high-salt diet on heart weight/tibia length and heart weight/body weight ratios in *Npr1* mice

In the present study, the ratios of heart weight/tibia length and heart weight/body weight increased (+) in *Npr1* gene-disrupted 1-copy mice compared with wild-type mice, but decreased (−) in *Npr1* gene-duplicated 4-copy mice, implicating that NPRA signalling protects the heart. The high-salt diet increased (+) arterial pressure only in 1-copy and 2-copy mice, but not in 3-copy and 4-copy mice. It has been reported that arterial pressure was decreased (−) in 4-copy mice fed a high-salt diet as compared with 4-copy mice kept on a low-salt diet [13]. Those previous findings suggested that the *Npr1* gene, similar to the gene coding for ANP, may directly affect the sensitivity of arterial pressure to salt loading [13]. Although our data provide the evidence that NPRA signalling exerts a protective effect on arterial pressure regulation in mice fed a high-salt diet, in another *Npr1* gene-knockout mouse model, a minimal or a high-salt diet did not affect systemic arterial pressure [62]. Both of these models focus on the *Npr1* gene disruption, but they have used gene-targeting methods that differ in their details [8,13,62]. However, a similar degree of hypertension has been confirmed in both mouse models. The plasma cGMP levels were decreased (−) in 1-copy mice and increased (+) in 3-copy and 4-copy mice compared with 2-copy mice. The low-salt diet suppressed (−) plasma cGMP levels in all three genotypes of *Npr1* mice, whereas the high-salt diet increased (+) plasma cGMP levels in all *Npr1* mice. The present results suggest that reduced cGMP signalling increases the heart weight/body weight and heart weight/tibia length ratios impacting cardiac remodelling in *Npr1* 1-copy gene-disrupted mice.

In conclusion, the present results provide the evidence that cardiac ANG II and aldosterone concentrations are increased (+) in*Npr1* gene-disrupted heterozygous (1-copy) mice fed a high-salt diet, however, greatly reduced (−) in *Npr1* gene-duplicated (3-copy and 4-copy) mice. The urinary sodium excretion and urine output decreased (−) in *Npr1* 1-copy mice as compared with wild-type 2-copy mice, but increased (+) in *Npr1* gene-duplicated mice. The pro-inflammatory cytokines levels are elevated (+) in both plasma and heart tissues of *Npr1* mice kept on a high-salt diet. Furthermore, the high-salt diet showed a significant baseline elevation (+) of pro-inflammatory cytokines in 1-copy and 2-copy mice; yet, the magnitudes of elevation (+) were only small in 3-copy and 4-copy mice. The present results demonstrate that a high-salt diet elevated (+) cardiac ANG II, aldosterone and pro-inflammatory cytokines in 1-copy mice; however, *Npr1* gene-duplicated mice did not render such elevated (+) effect indicating the potential role of NPRA against salt-loading and remodelling process in a *Npr1* gene dose-dependent manner. The low-salt diet suppressed (−) plasma cGMP levels in all three genotypes of *Npr1* mice, whereas the high-salt diet increased (+) plasma cGMP levels in these *Npr1* gene-targeted mice. The present results suggest that ANP/NPRA/cGMP signalling decreases (−) the cardiac ANG II, aldosterone and pro-inflammatory cytokine levels and protects the heart from salt-loading and cardiac remodelling process in the disease state.

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Abbreviations

ACE	angiotensin-converting enzyme
ANG II	angiotensin II
ANP	atrial natriuretic peptide
BSA	bovine serum albumin
GC-A/NPRA	guanylyl cyclase/natriuretic peptide receptor-A
HW/BW	heart weight/body weight
HW/TL	heart weight/tibia length
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-6	interleukin-6
TFA	trifluoroacetic acid
TNF-α	tumour necrosis factor-alpha

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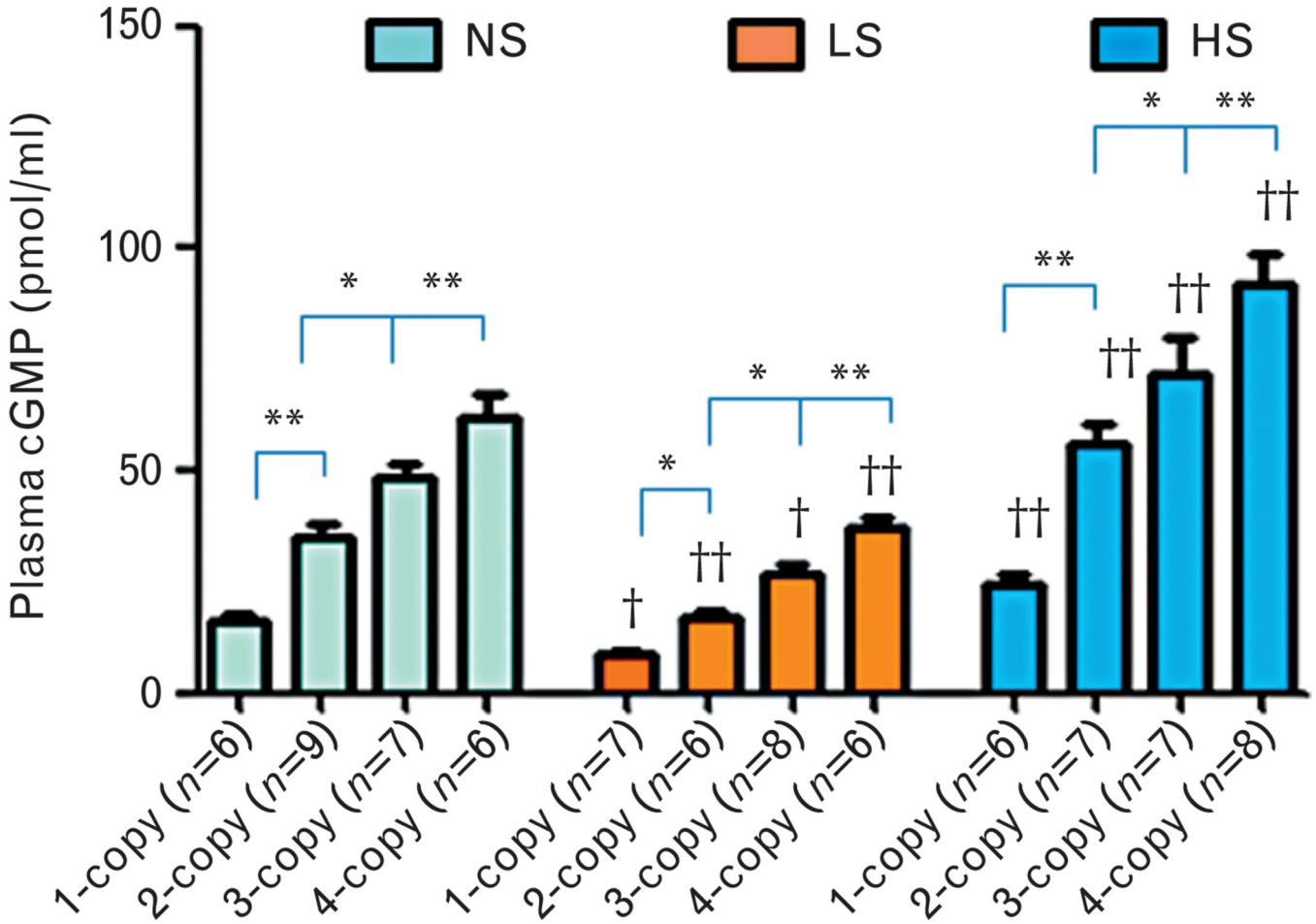

FIGURE 1.

Plasma cyclic GMP levels in *Npr1* gene-targeted mice with a normal salt, a low-salt or a high-salt diet: Comparisons were made among mice having different *Npr1* genotypes fed the same salt diet: $^{*}P<0.05$; $^{**}P<0.01$. Comparisons were also made among mice having the same *Npr1* gene copy number fed the different salt diets: $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$. Bars indicate the mean ± SE values for the representative genotypes. *n* = number of mice; cGMP, cyclic GMP; HS, high-salt diet; LS, low-salt diet; NS, normal salt diet.

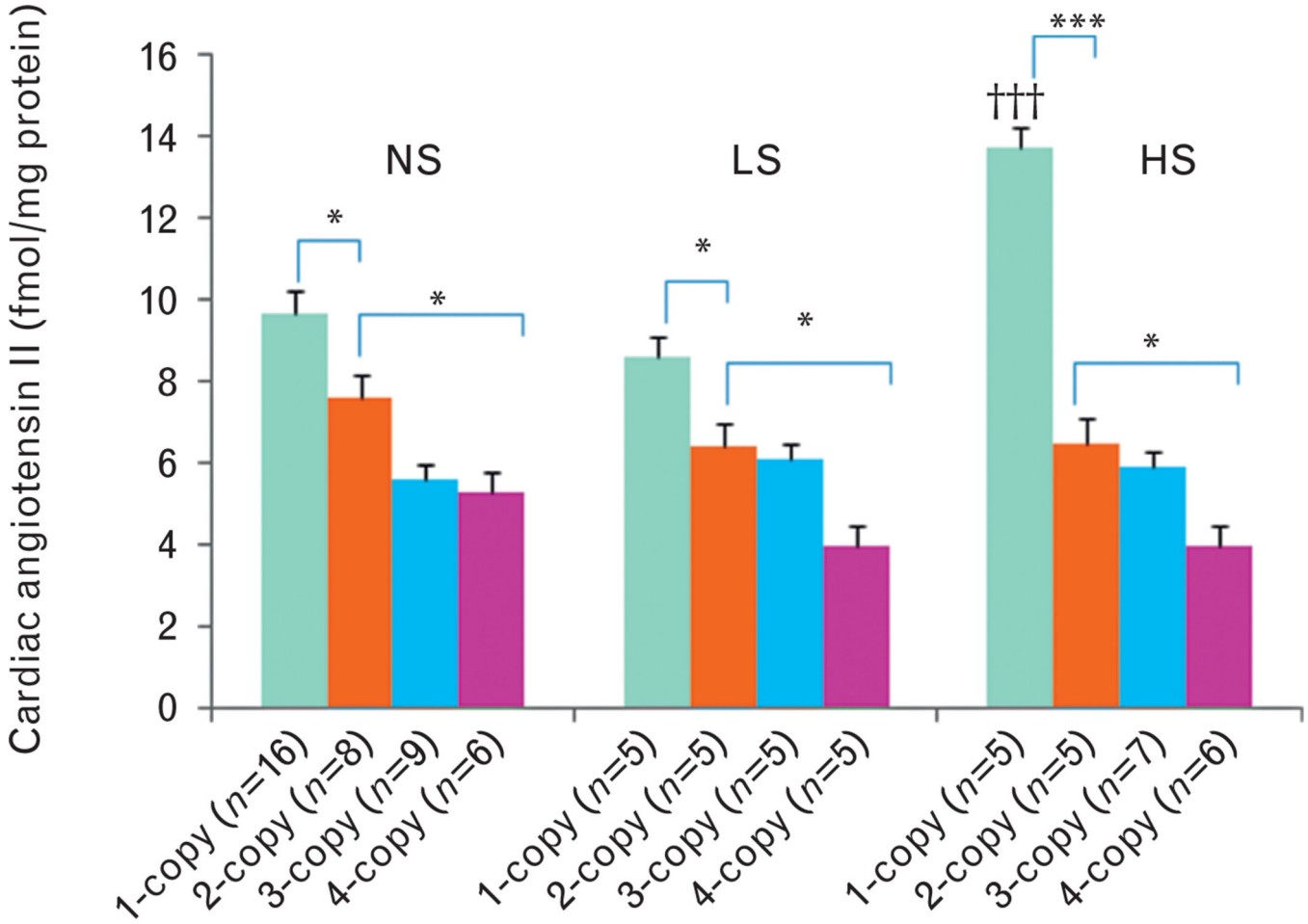

FIGURE 2.

Cardiac angiotensin II levels in *Npr1* gene-targeted mice with a normal salt, a low-salt or a high-salt diet: Comparisons were made among mice having different *Npr1* genotypes and fed the same salt diet: $^{*}P<0.05$; $^{***}P<0.001$ and having the same *Npr1* gene copy number with different salt diets: $^{\dagger\dagger\dagger}P<0.001$. Bars indicate the mean ± SE values for the representative genotypes. *n* = number of mice; HS, high-salt diet; LS, low-salt diet; NS, normal salt diet.

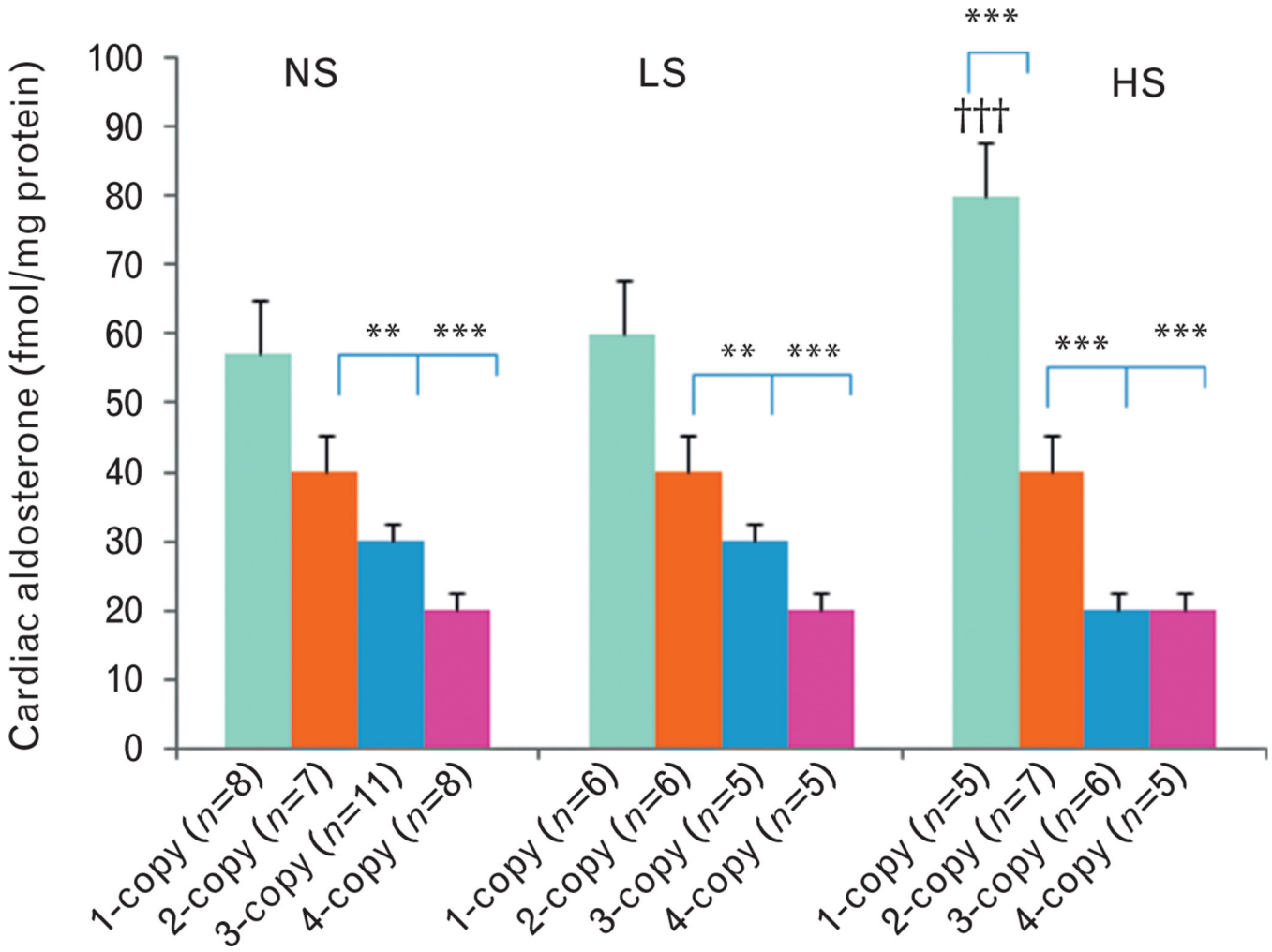

FIGURE 3.

Cardiac aldosterone levels in *Npr1* gene-targeted mice with a normal salt, a low-salt or a high-salt diet: Comparisons were made among mice having different *Npr1* genotypes fed the same salt diet: $^{**}P<0.01$; $^{***}P<0.001$. Similarly, comparisons were also made among mice having the same *Npr1* gene copy number and fed the different salt diets: $^{\dagger\dagger\dagger}P<0.001$. Bars indicate the mean ± SE values for the representative genotypes. *n* = number of mice; HS, high-salt diet; LS, low-salt diet; NS, normal salt diet.

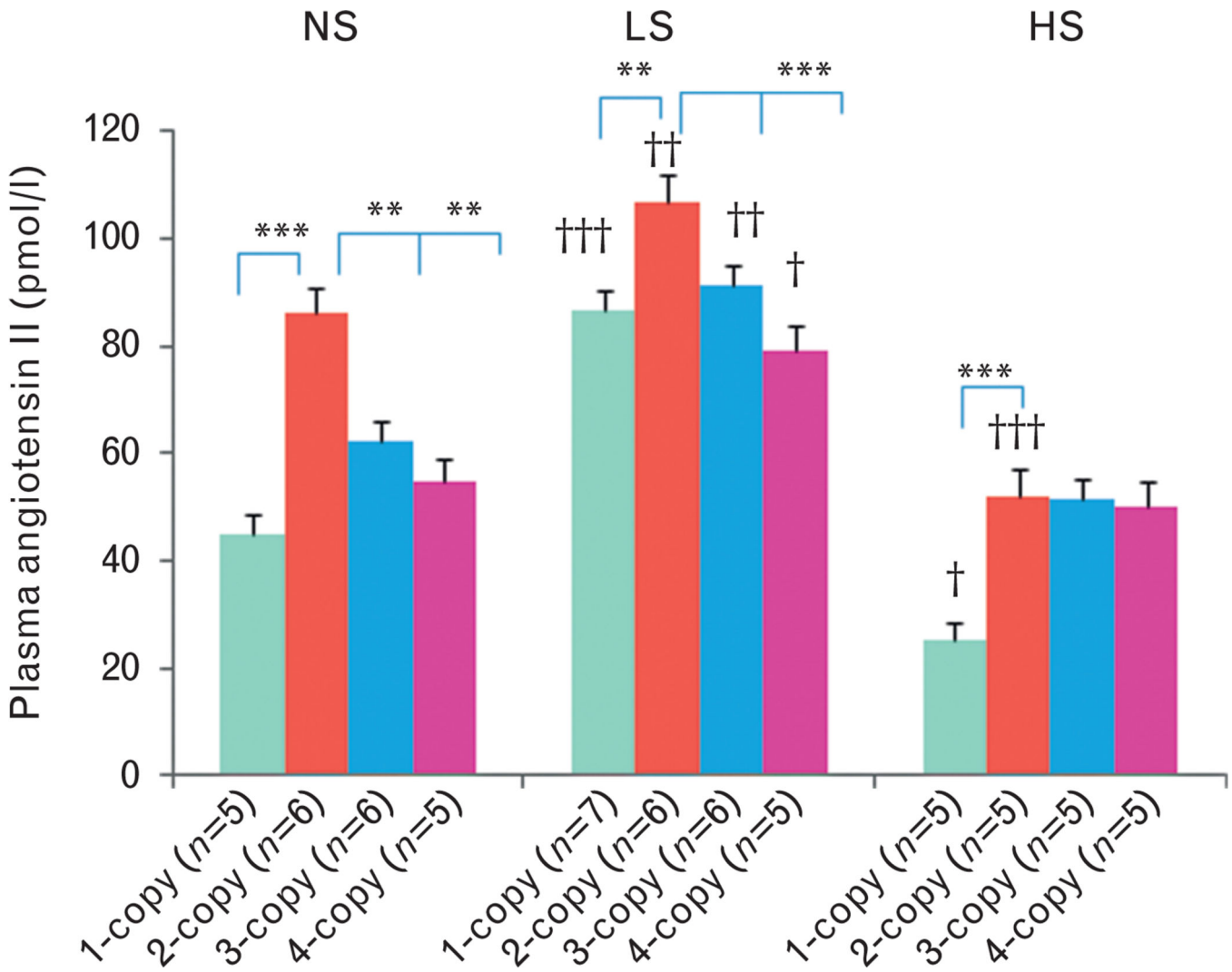

FIGURE 4.

Plasma angiotensin II levels in *Npr1* gene-targeted mice with a normal salt, a low-salt or a high-salt diet: Comparisons were made among mice having different *Npr1* genotypes fed the same salt diet: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Comparisons were also made among mice having the same *Npr1* gene copy number and given the different salt diets: $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$. Bars indicate the mean ± SE values for the representative genotypes. *n* = number of mice; HS, high-salt diet; LS, low-salt diet; NS, normal salt diet.

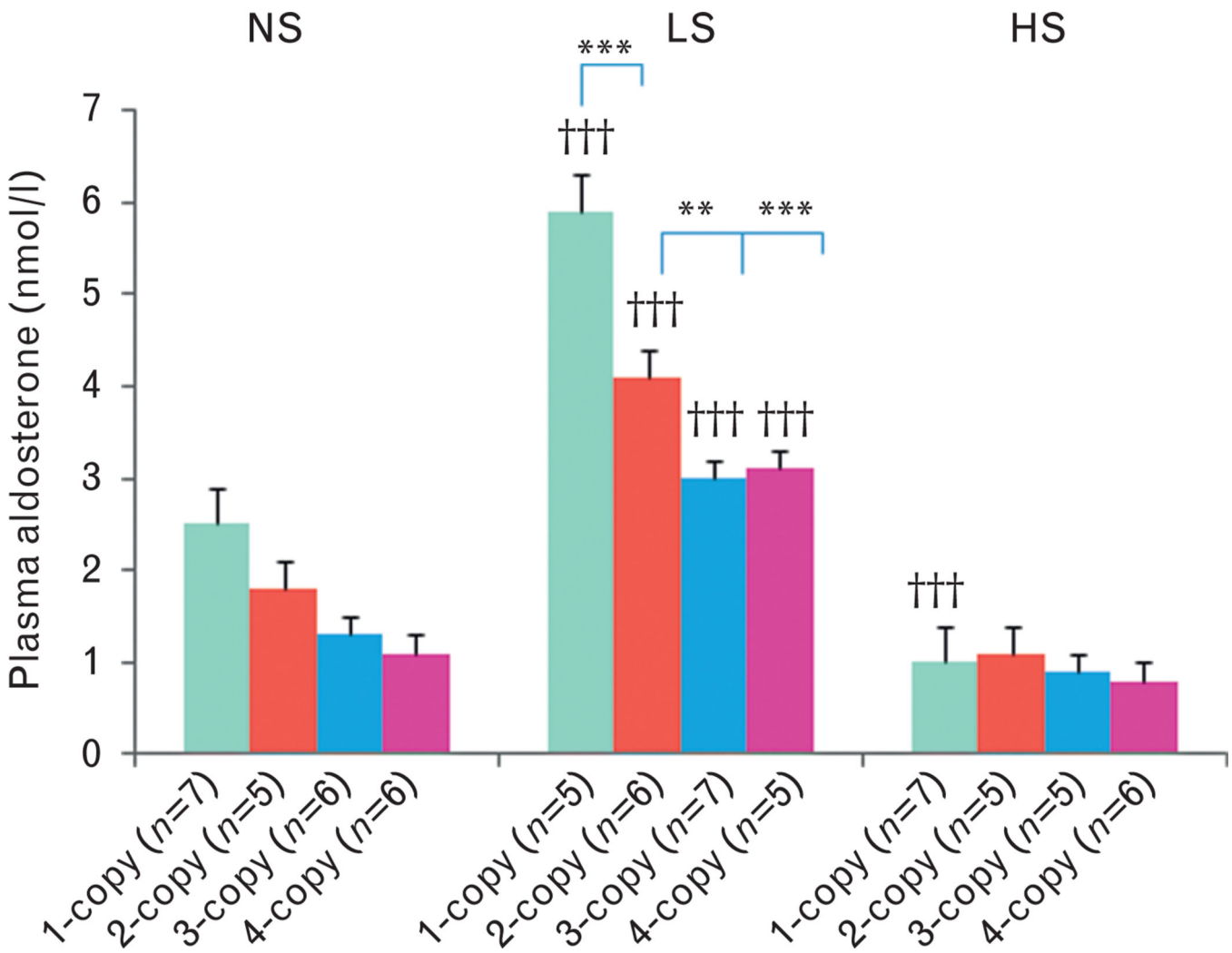

FIGURE 5.

Plasma aldosterone levels in *Npr1* gene-targeted mice with a normal salt, a low-salt or a high-salt diet: Comparisons were made among mice having different *Npr1* genotypes fed the same salt diet: $^{**}P<0.01$; $^{***}P<0.001$. Comparisons were also made among mice having the same *Npr1* gene copy number fed the different salt diets: $^{\dagger\dagger\dagger}P<0.001$. Bars indicate the mean ± SE values for the representative genotypes. *n* = number of mice; HS, high-salt diet; LS, low-salt diet; NS, normal salt diet.

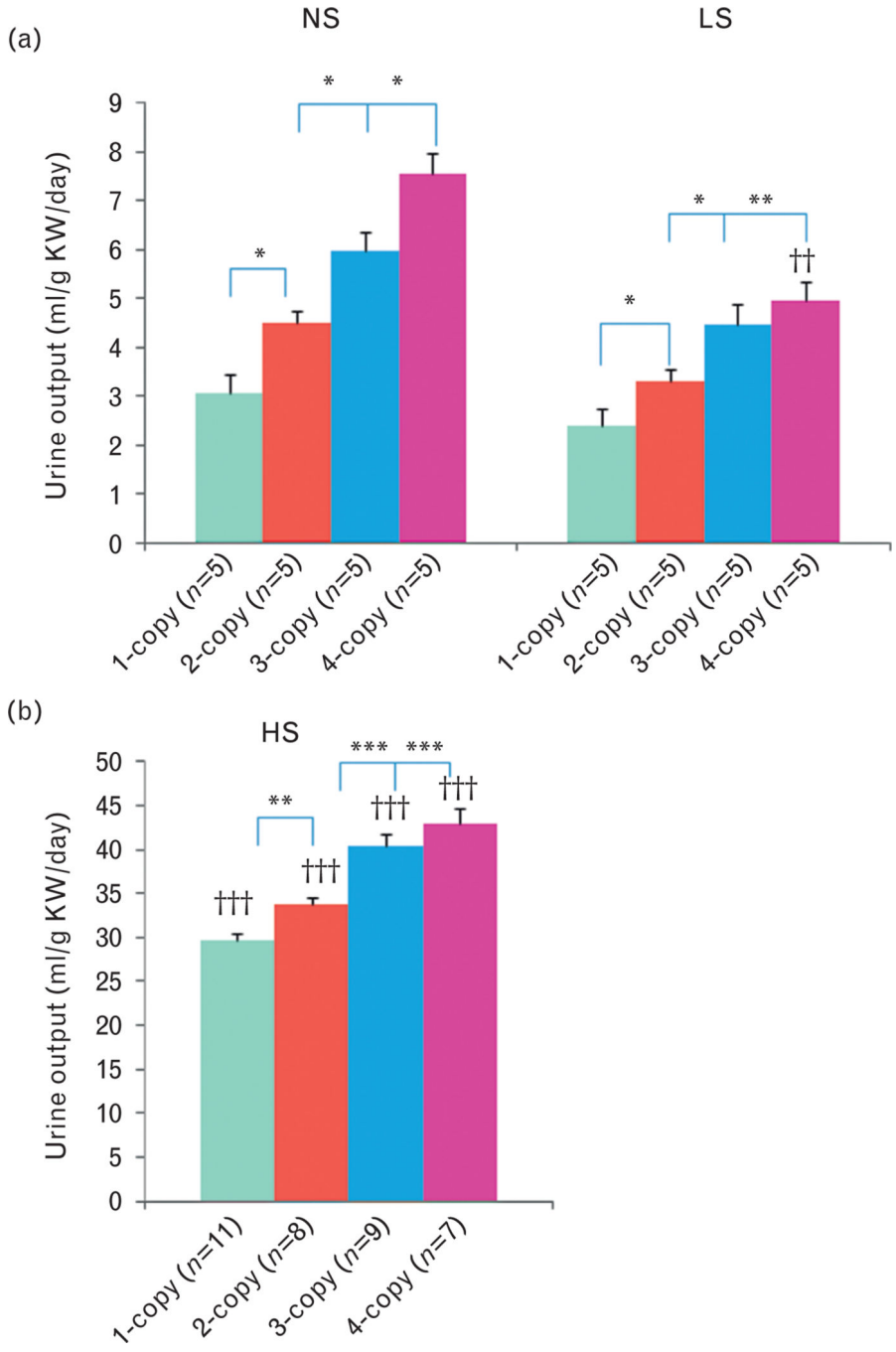

FIGURE 6.

Urine outputs in *Npr1* gene-targeted mice with (A) normal salt, low-salt or (B) high-salt diet: Comparisons were made among different *Npr1* mice genotypes with same salt diet: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Bars indicate the mean ± SE values for the representative genotypes. Comparisons were also made among mice having same *Npr1* gene copy number fed the different salt diets: $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$. *n* = number of mice; HS, high-salt diet; KW, kidney weight; LS, low-salt diet; NS, normal salt diet.

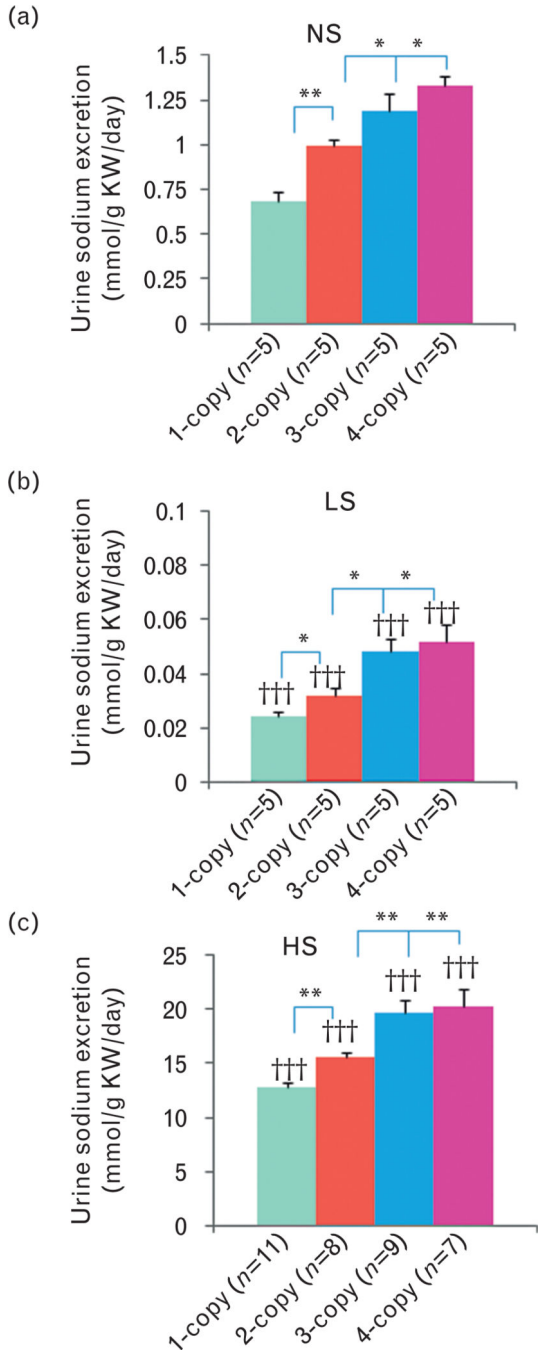

FIGURE 7.

Urinary sodium execrations in *Npr1* gene-targeted mice with (A) low-salt, (B) normal salt or (C) high-salt diet: Comparisons were made among different *Npr1* mice genotypes with the same salt diet: $^{*}P<0.05$; $^{**}P<0.01$. Bars indicate the mean ± SE values for the representative genotypes. Comparisons were also made among mice having same *Npr1* gene copy number fed the different salt diets: $^{\dagger\dagger\dagger}P<0.001$. *n*=number of mice; HS, high-salt diet; KW, kidney weight; LS, low-salt diet; NS, normal salt diet.

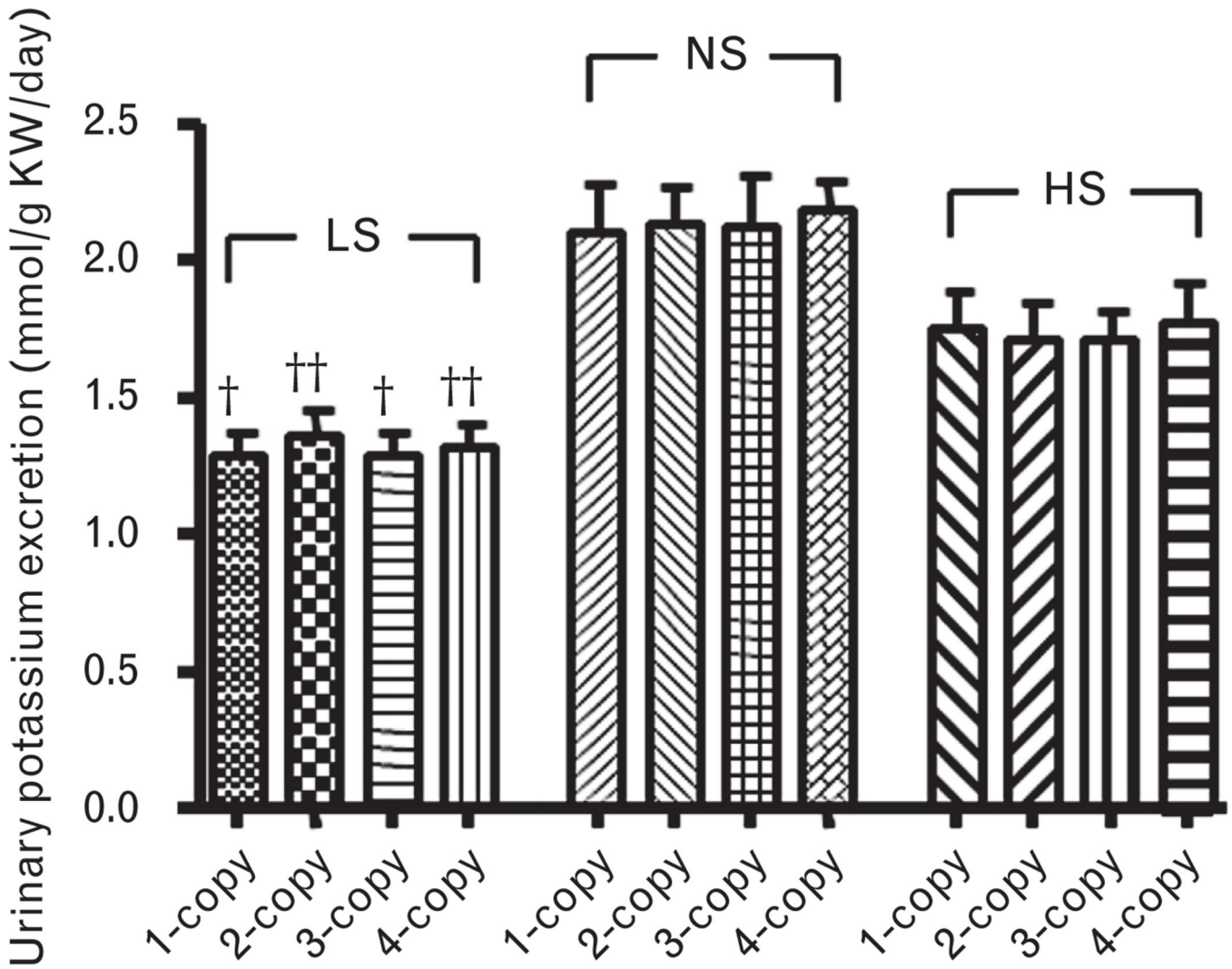

FIGURE 8.

Urinary potassium execrations in *Npr1* gene-targeted mice kept on a low-salt, a normal salt or a high-salt diet: Bars indicate the mean ± SE values for the representative genotypes. Comparisons were made among mice having same *Npr1* gene copy number fed the different salt diets also *Npr1* mice genotypes with same salt diet: $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$. *n* = number of mice; HS, high-salt diet; KW, kidney weight; LS, low-salt diet; NS, normal salt diet.

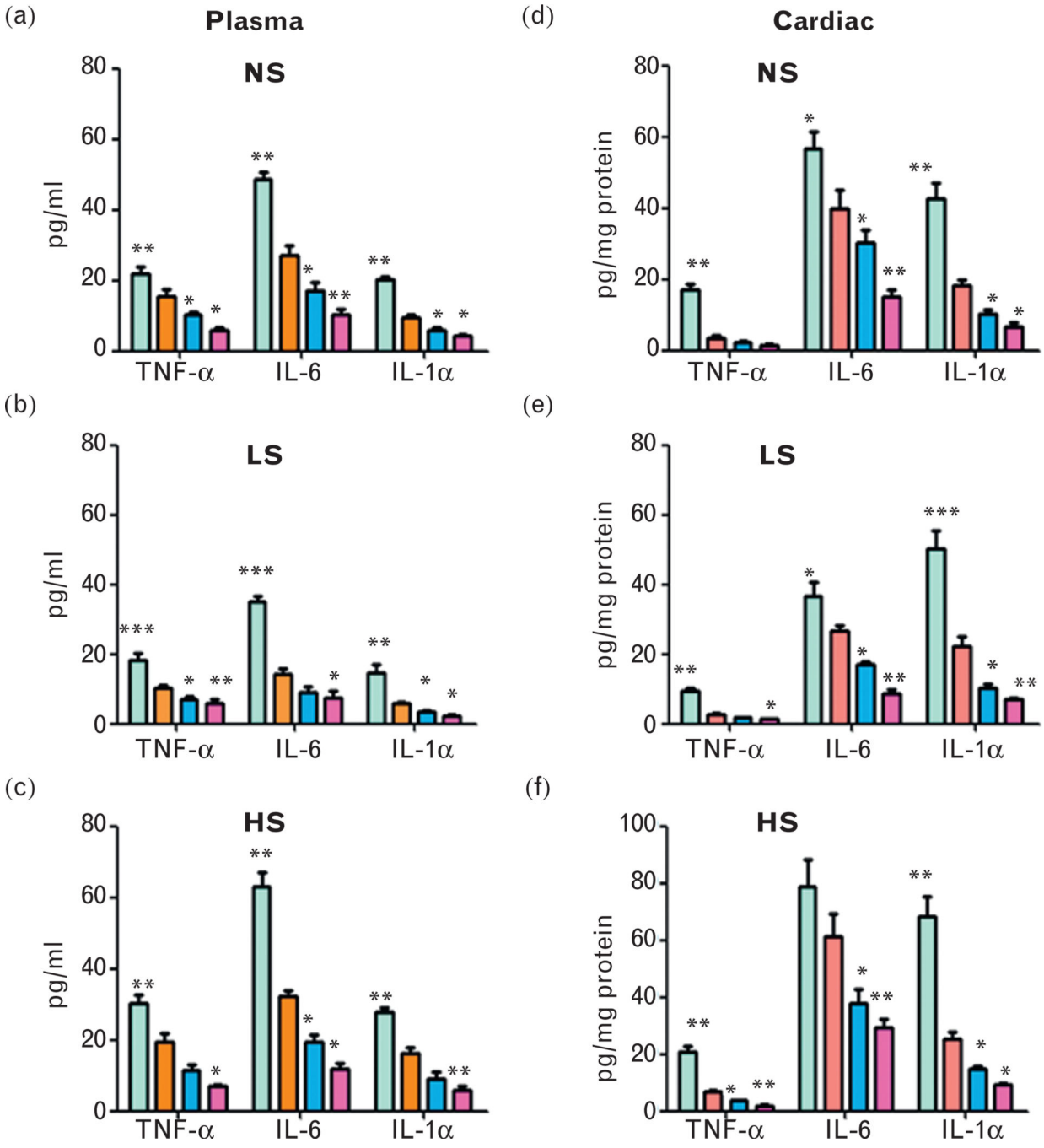

FIGURE 9.

Quantitative analysis of plasma and cardiac pro-inflammatory cytokines in *Npr1* mice fed with low-salt, normal salt and high-salt diets: Analysis in (a)–(c) shows plasma cytokine levels and the one in (d)–(f) shows the cardiac cytokine levels in 1-copy, 2-copy, 3-copy and 4-copy mice. Comparisons were made among different *Npr1* mice genotypes with the same salt diet: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $n = 5$ number of mice; HS, high-salt diet; IL-6,

interleukin-6; IL-1α, interleukin-1 alpha; LS, low-salt diet; NS, normal salt diet; TNF-α, tumour necrosis factor alpha.

TABLE 1

Comparisons of blood pressure and the heart weight/tibia length and heart weight/body weight ratios among *Npr1* genotypes fed the different salt diets

	***Npr1* genotype**			
Parameters	**1-copy**	**2-copy**	**3-copy**	**4-copy**
Normal salt diet				
Blood pressure (mmHg)	114 ± 1.9	102 ± 2.0	92 ± 1.8	86 ± 2.2
Heart weight (mg)	138 ± 2.9	126 ± 1.7	118 ± 2.5	114 ± 3.6
Body weight (g)	22 ± 0.8	21 ± 0.7	22 ± 0.5	22 ± 0.6
Tibia length (mm)	8.4 ± 0.2	8.4 ± 0.1	8.5 ± 0.2	8.3 ± 0.3
HW/BW	6.4 ± 0.1*	5.6 ± 0.2	5.1 ± 0.1	5.0 ± 0.1†
HW/TL	16.5 ± 0.2*	15.1 ± 0.1	13.9 ± 0.3	13.8 ± 0.1†
Low-salt diet				
Blood pressure (mmHg)	116 ± 4.4	104. ± 2.1	93 ± 1.8	85 ± 2.6
Heart weight (mg)	136 ± 3.0	127 ± 2.2	118 ± 3.2	114 ± 4.7
Body weight (g)	22.7 ± 0.4	22.4 ± 0.7	22.3 ± 0.4	22.1 ± 0.5
Tibia length (mm)	8.6 ± 0.2	8.6 ± 0.1	8.7 ± 0.1	8.7 ± 0.1
HW/BW	6.0 ± 0.1	5.7 ± 0.1	5.2 ± 0.1	5.1 ± 0.1*
HW/TL	15.9 ± 0.3	14.8 ± 0.2	13.6 ± 0.2	13.2 ± 0.4*
High-salt diet				
Blood pressure (mmHg)	126 ± 2.3	112 ± 3.2	94 ± 21.8	85 ± 2.4
Heart weight (mg)	149 ± 5.8	140 ± 3.8	122 ± 3.0	114 ± 6.6*
Body weight (g)	22.6 ± 0.4	22.3 ± 0.3	22.2 ± 0.4	22.1 ± 0.5
Tibia length (mm)	8.5 ± 0.2	8.7 ± 0.1	8.7 ± 0.1	8.4 ± 0.1
HW/BW	6.7 ± 0.1*	6.3 ± 0.1	5.5 ± 0.2†	5.2 ± 0.1‡
HW/TL	17.6 ± 0.6†	15.1 ± 0.4	14.1 ± 0.3*	13.6 ± 0.7†

Comparisons were made among mice having different *Npr1* genotypes fed the same diet. $n = 6$ mice per group. BW, body weight; HW, heart weight; TL, tibia length.

*$P<0.05$.

†$P<0.01$.

‡$P<0.001$.