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EPLERENONE PREVENTS ADVERSE CARDIAC REMODELLING INDUCED BY PRESSURE OVERLOAD IN ATRIAL NATRIURETIC PEPTIDE-NULL MICE

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SUMMARY

1. Atrial natriuretic peptide (ANP)-null mice (*Nppa*$^{-/-}$) exhibit cardiac hypertrophy at baseline and adverse cardiac remodelling in response to transverse aortic constriction (TAC)-induced pressure overload stress. Previous studies have suggested that natriuretic peptides could potentially oppose mineralocorticoid signalling at several levels, including suppression of adrenal aldosterone production, inhibition of mineralocorticoid receptor (MR) activation or suppression of MR-mediated production of pro-inflammatory factors. Thus, we hypothesized that the MR blocker eplerenone would prevent the exaggerated left ventricular (LV) remodelling/fibrosis and dysfunction after TAC in *Nppa*$^{-/-}$.

2. In the present study, *Nppa*$^{-/-}$ and wild-type *Nppa*$^{+/+}$ mice fed eplerenone- or vehicle (oatmeal)-supplemented chow since weaning were subjected to TAC or sham operation. The daily dose of eplerenone administered was approximately 200 mg/kg. At 1 week after TAC, LV size and function were evaluated by echocardiogram and LV cross-sections were stained with picrosirius red for collagen volume measurement. Total RNA was extracted from the LV for real-time polymerase chain reaction analysis of osteopontin.

3. Eplerenone had no effect on baseline hypertrophy observed in sham-operated *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ mice. Eplerenone attenuated the TAC-induced increase in LV weight in both genotypes and completely prevented LV dilation, systolic dysfunction and interstitial collagen deposition seen in *Nppa*$^{-/-}$ mice after TAC. However, serum aldosterone levels were lower in *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ wild types. No interaction between eplerenone and genotype in osteopontin mRNA levels was observed.

4. Eplerenone prevents adverse cardiac remodelling related to pressure overload in ANP-deficient mice, mainly due to an antifibrotic effect. The mechanism whereby ANP deficiency leads to excess hypertrophy, fibrosis and early failure following TAC is increased profibrotic signals resulting from excess or unopposed MR activation, rather than increased levels of aldosterone.

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INTRODUCTION

Atrial natriuretic peptide (ANP) modulates cardiac hypertrophy and remodelling in response to a variety of pathologic stimuli.[1–5] Mice with homozygous deletion of the pro-ANP gene (*Nppa*$^{-/-}$) or the natriuretic peptide receptor (NPR)-A gene exhibit cardiac hypertrophy under resting conditions[6–8] and develop exaggerated hypertrophy after volume or pressure overload[1–5] independent of blood pressure. Furthermore, we have demonstrated previously that the adverse cardiac hypertrophy and remodelling observed in ANP-null mice after haemodynamic stress is mainly due to changes in extracellular matrix (ECM) expression and increased interstitial and perivascular fibrosis.[3–5] These findings have led to the concept that natriuretic peptides act in a counter-regulatory fashion to oppose the effects of various trophic factors released in response to haemodynamic stress.

The mineralocorticoid receptor (MR) is highly expressed in the heart[9,10] and aldosterone has been postulated to act as a mediator of cardiac hypertrophy and resultant failure, predominantly by its effects on interstitial remodelling.[9,11–13] The addition of an MR antagonist to angiotensin-converting enzyme (ACE) inhibitors causes regression of left ventricular hypertrophy (LVH) in hypertensives and modulates adverse cardiac remodelling and improves survival in heart failure and in patients with left ventricular (LV) dysfunction after myocardial infarction (MI).[14–16] Relatively little is known about interactions between natriuretic peptides and mineralocorticoid signalling in the heart. Natriuretic peptides have been reported to inhibit adrenal production of aldosterone[17,18] and expression of aldosterone synthase in rat neonatal cardiomyocytes,[19] as well as to decrease nuclear translocation of the MR in rat colonic surface cells via a cGMP-dependent mechanism.[20] Characteristic features of hyperaldosteronism include increased expression of inflammatory factors in the wall of intramural coronary arteries, perivascular inflammation and, ultimately, interstitial and perivascular fibrosis.[21–23] Conversely, B-type natriuretic peptide (BNP) has been demonstrated to inhibit transforming growth factor (TGF)-β-stimulated expression of pro-inflammatory/trophic factors in human cardiofibroblasts via cGMP-mediated signalling.[24] Thus, natriuretic peptides could potentially oppose mineralocorticoid signalling at several levels, including suppression of adrenal aldosterone production, inhibition

of MR activation or suppression of MR-mediated production of pro-inflammatory/trophic factors downstream to MR activation.

These observations led us to hypothesise that excess or unopposed MR activation may mediate the cardiac hypertrophy observed in ANP-deficient mice at baseline and, in the excess hypertrophy, ECM expansion, fibrosis and cardiac failure observed in response to haemodynamic stress. We administered the specific MR antagonist eplerenone, starting at weaning, to *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice and then performed either sham or transverse aortic constriction (TAC) surgery at 8–10 weeks of age, in order to determine whether: (i) MR activation mediates the excess cardiac hypertrophy observed in ANP-deficient mice at baseline; (ii) MR activation mediates cardiac hypertrophy in response to pressure overload in ANP-replete mice; (iii) MR activation mediates the excess hypertrophy, cardiac fibrosis and premature failure in ANP-deficient mice; (iv) aldosterone is elevated at baseline or following TAC in ANP-null mice relative to wild types; and (v) osteopontin mRNA levels are decreased by eplerenone treatment in ANP-null mice following TAC relative to vehicle controls.

METHODS

Animal preparation

Male *Nppa*$^{-/-}$ mice, 8–9 weeks of age, originally generated in the laboratory of Dr Oliver Smithies (Departments of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA),[1] and age-matched male controls (*Nppa*$^{+/+}$) of the C57BL/6 strain were investigated. Genotypes were identified by polymerase chain reaction (PCR). Mice were fed a standard diet (0.55% NaCl; Harlan-Teklad, Madison, WI, USA) supplemented with 2 mg/g eplerenone (produced in our laboratory) at a daily dose of approximately 200 mg/kg[10] beginning at weaning (age 3 weeks). Vehicle (oatmeal)-treated mice served as controls. Mice were housed three to four per cage and were maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C) and light cycle (lights on 0600–1800 h). All protocols were approved by the Institutional Animal Care and Use Committee at UAB and were consistent with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 96–01, revised 1996; http://www.nap.edu/readingroom/books/labrats/).

Surgical procedure

Eplerenone- and vehicle-treated *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice underwent either TAC or sham surgery under ketamine (8 mg/100 g, i.p.)–xylazine (1.2 mg/100 g, i.p.) anaesthesia, as described previously.[3,5] Using this methodology, we have previously demonstrated reproducible pressure gradients across the TAC of 50–65 mmHg.[3]

Mean arterial pressure

The effect of eplerenone on mean arterial pressure (MAP) was determined in a separate set of mice fed vehicle or eplerenone for 4 weeks starting at weaning. Under tribromoethanol (0.3 mL) anaesthesia, the left carotid artery was exposed and cannulated with a polyethylene cannula containing 20 U/mL heparinized saline. The catheter was exteriorized to the posterior neck and fixed with dental acrylic. After surgery, mice were housed in individual cages under a warm light to maintain body temperature and were allowed to recover for at least 6 h prior to measurement of MAP and heart rate (HR). We chose tribromoethanol as the anaesthetic agent because it has less of a depressant effect on MAP and HR postoperatively compared with phenobarbital or ketamine/xylazine. The MAP and HR of conscious, resting mice were recorded for 30 min on a Grass Model 7 Polygraph (Quincy, MA, USA).

Echocardiographic study

One week after surgery, echocardiography was performed, as described previously, using a 6–15 MHz transducer (Philips Andover, MA, USA) and a commercially available ultrasound system (Sonos 5500; Phillips).[2,5,25] The LV end-diastolic dimension (EDD) and LV end-systolic dimension (ESD) were measured by two-dimensional guided M-mode echo. Fractional shortening (FS) was calculated as (LVEDD – LVESD)/LVEDD. A single examiner, blinded to genotype and treatment, performed all studies.

Aldosterone measurement

Blood was collected via a retro-ocular approach from conscious mice on the day after echocardiographic study. Serum and cardiac aldosterone levels were measured using an aldosterone Coat-A-Count kit (Diagnostic Products, Los Angeles, CA, USA), which has a sensitivity to detect aldosterone of 5 pg/sample. For cardiac aldosterone measurement, 40–50 mg LV was homogenized in 500 μL cold phosphate-buffered saline (PBS), hydrolysed in 0.32 mol/L HCl for 24 h, mixed and extracted with ethyl acetate (2.5 mL) for 60 min. After centrifugation (1500 *g* for 5 min at room temperature), 250 μL ethyl acetate extract was dried with nitrogen gas in a polypropylene tube and used for aldosterone radioimmunoassay (RIA). Cardiac aldosterone was not measured in eplerenone-treated mice.

Tissue collection

Mice were killed, after blood collection, by cervical dislocation. Hearts and lungs were removed and LV, right ventricle (RV) and lungs were dissected carefully and weighed. The LV was fixed with 4% paraformaldehyde, paraffin embedded and sectioned for histological analysis.

Collagen volume

Left ventricular collagen (interstitial) volume percentage, at the level below the mitral valve, was measured in picrosirius red (0.1%)-stained cross-sections using a microscopic system with a green (540 nm) filter to enhance contrast for computer imaging analysis (Image-1 Software). A minimum of 10 randomly selected images was counted from each LV. A single examiner, blinded to the experimental group, performed all histological analyses.

Real-time reverse transcription–PCR analysis of osteopontin

Total RNA was extracted from left ventricles using the TRIZOL total RNA isolation reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (2 μg) from LV was reverse transcribed (RT) using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers as primers. The cDNA (2 μL) was amplified in an iCycler (Bio-Rad, Hercules, CA, USA) for 40 cycles using the SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA, USA). Levels of osteopontin mRNA were normalized to GAPDH mRNA. The primers used for the real-time quantitative RT-PCR were as follows: (i) osteopontin: sense 5′-CACTCCAATCGTCCCTAC-3′ and antisense 5′-AGACTCACCGCTCTTCAT-3′; and (ii) GAPDH: sense 5′-GTTGTCTC-CTGCGACTTCA-3′ and antisense 5′-GTGGTCCAGGGTTTCTTACT-3′.

Left ventricular cardiomyocyte diameter

Left ventricular cardiomyocyte diameter was measured as described previously.[2,4] Briefly, hearts were stained with haematoxylin–eosin (HE) and examined by light microscopy. Morphometric analysis of each heart section was performed with a computer-based morphometric system. At least eight cross-sections of each LV were examined and the measurements were averaged for statistical analysis. All morphometric analyses were performed by a single examiner, who was blinded with respect to the experimental group to which each sample belonged. To evaluate the mean diameter of LV

Table 1 Normalized heart weights, lung weight and echocardiographic parameters after sham or transverse aortic constriction

Treatment (n)	Tissue weight			Echocardiographic parameters		
	LV (mg)	RV (mg)	Lung (mg)	LVEDD (mm)	FS (%)	AWT (mm)
Sham						
Vehicle						
Nppa$^{+/+}$ (7)	81 ± 3	18 ± 0	153 ± 5	35 ± 1	60 ± 1	0.53 ± 0.02
Nppa$^{-/-}$ (7)	115 ± 5†	39 ± 2†	158 ± 5	36 ± 1	58 ± 2	0.67 ± 0.02†
Eplerenone						
Nppa$^{+/+}$ (6)	78 ± 2	17 ± 1	154 ± 4	34 ± 1	60 ± 2	0.52 ± 0.02
Nppa$^{-/-}$ (9)	112 ± 3†	23 ± 2*†	155 ± 3	33 ± 1*	62 ± 16	0.72 ± 0.01†
Transverse aortic constriction						
Vehicle						
Nppa$^{+/+}$ (7)	114 ± 3	18 ± 1	137 ± 4	33 ± 1	59 ± 1	0.64 ± 0.02
Nppa$^{-/-}$ (8)	208 ± 6†	34 ± 2†	218 ± 14†	39 ± 1†	47 ± 1†	0.86 ± 0.03†
Eplerenone						
Nppa$^{+/+}$ (5)	100 ± 2*	14 ± 1*	129 ± 2	31 ± 1	64 ± 1	0.62 ± 0.01
Nppa$^{-/-}$ (5)	152 ± 13*†	20 ± 1*†	168 ± 14*†	30 ± 1*	60 ± 1*	0.85 ± 0.06†
Genotype × eplerenone (sham group), two-way ANOVA, P values						
Genotype	< 0.01	< 0.01	0.46	0.75	0.88	< 0.01
Eplerenone	0.40	< 0.01	0.80	0.01	0.15	0.36
Interaction	0.99	< 0.01	0.62	0.06	0.16	0.12
Genotype × eplerenone (TAC group), two-way ANOVA, P values						
Genotype	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Eplerenone	< 0.01	< 0.01	< 0.05	< 0.01	< 0.01	0.64
Interaction	< 0.01	< 0.01	0.07	< 0.01	< 0.01	0.88

Results are the mean ± SEM. *$P < 0.05$ compared with respective vehicle; $^{\dagger}P < 0.05$ compared with respective *Nppa*$^{+/+}$.
The normalized tissue weights and chamber sizes were determined by ANCOVA with bodyweight as a covariate.
LV, left ventricle; RV, right ventricle; LVEDD, left ventricular end-diastolic dimension; FS, fractional shortening; AWT, average wall thickness.

cardiomyocytes, the shortest diameter of each cardiomyocyte was measured only in nucleated transverse sections. Eighty cardiomyocytes in each LV were measured using an ocular micrometre disc with a linear scale at a magnification of × 40 and the average cardiomyocyte diameter of each specimen was calculated.

Statistical analysis

Results are expressed as the mean±SEM. Tissue weights and echocardiographic measurements were normalized by analysis of covariance (ANCOVA) with bodyweight as the covariate. Our primary statistical test was analysis of variance (ANOVA). Two-way ANOVA was performed to examine interactions between eplerenone therapy and TAC, as well as to confirm significant interaction between genotype and TAC on the different tissue and echocardiographic parameters evaluated. One-way ANOVA and *t*-test were performed to evaluate differences in mean values due to main effects (*Nppa*$^{-/-}$ *vs Nppa*$^{+/+}$ or vehicle *vs* eplerenone). A repeated-measures ANOVA design was performed to compare cardiomyocyte diameters. $P < 0.05$ was considered significant.

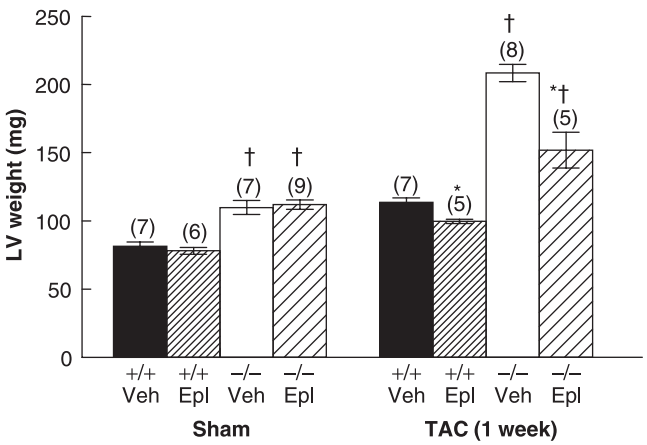

Fig. 1 Normalized left ventricular (LV) weight after sham or transverse aortic constriction (TAC) in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice. Normalized tissue weight was determined by ANCOVA with bodyweight as a covariate. Results are shown as the mean±SEM, with the number of mice given in parentheses. *$P < 0.05$ compared with respective vehicle; $^{\dagger}P < 0.05$ compared with respective *Nppa*$^{+/+}$. Veh, vehicle; Epl, eplerenone.

RESULTS

Effect of eplerenone on cardiac structure and MAP in sham-operated mice

Left ventricular and RV weights were increased in vehicle-treated, sham-operated *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ mice (Table 1; Fig. 1). Eplerenone treatment from weaning had no significant effect on LV or RV weight in wild types and prevented RV hypertrophy, but not LVH, in ANP-null mice. Lung weight did not differ among the four groups.

The LVEDD determined by echocardiography did not differ between genotypes in vehicle-treated mice, but average wall thickness (AWT) was increased in *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ (Table 1). Eplerenone treatment resulted in a small but significant decrease in LVEDD in *Nppa*$^{-/-}$, but not *Nppa*$^{+/+}$, mice compared with respective vehicle

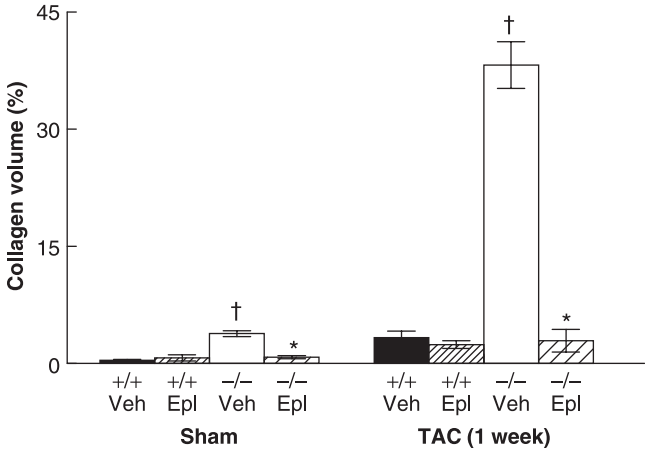

Fig. 2 Interstitial collagen volume calculated in picrosirius red-stained cross-sections of the left ventricle at the level below the mitral valve in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice after sham or transverse aortic constriction (TAC). Results are shown as the mean ± SEM ($n = 4$–6 mice per group). *$P < 0.05$ compared with respective vehicle; †$P < 0.05$ compared with respective *Nppa*$^{+/+}$. Veh, vehicle; Epl, eplerenone.

controls. The AWT and FS were unaffected by eplerenone treatment in both genotypes.

Vehicle-treated *Nppa*$^{-/-}$ mice had higher collagen volume than vehicle-treated *Nppa*$^{+/+}$ mice under non-stress conditions (Figs 2, 3), without evidence of replacement fibrosis. The increased collagen volume in *Nppa*$^{-/-}$ mice was normalized by eplerenone treatment started at time of weaning ($P < 0.01$, two-way ANOVA; $P < 0.05$ *Nppa*$^{-/-}$ vehicle *vs* *Nppa*$^{+/+}$ vehicle and *Nppa*$^{-/-}$ eplerenone *vs* *Nppa*$^{-/-}$ vehicle). Osteopontin mRNA levels were higher in the *Nppa*$^{-/-}$ group compared with wild types (2.3 ± 0.6, 1.0 ± 0.1, 3.2 ± 0.9 and 1.5 ± 0.7 in *Nppa*$^{-/-}$ vehicle, *Nppa*$^{+/+}$ vehicle, *Nppa*$^{-/-}$ eplerenone and *Nppa*$^{+/+}$ eplerenone, respectively), as reported previously;[3] however, there was no effect of eplerenone on osteopontin mRNA and no interaction between treatment and genotype ($P = 0.77$, two-way ANOVA). Myocyte diameter did not differ significantly between genotypes (17.5 ± 0.1, 16.4 ± 0.1, 15.9± 0.2 and 15.7 ± 0.1 in *Nppa*$^{-/-}$ vehicle, *Nppa*$^{+/+}$ vehicle, *Nppa*$^{-/-}$ eplerenone and *Nppa*$^{+/+}$ eplerenone, respectively), but did tend to be lower in eplerenone-compared with vehicle-treated *Nppa*$^{-/-}$ mice ($P = 0.06$).

Mean arterial pressure was higher in *Nppa*$^{-/-}$ than *Nppa*$^{+/+}$ mice, as expected. However, there was no significant effect of eplerenone on MAP in either genotype (Table 2).

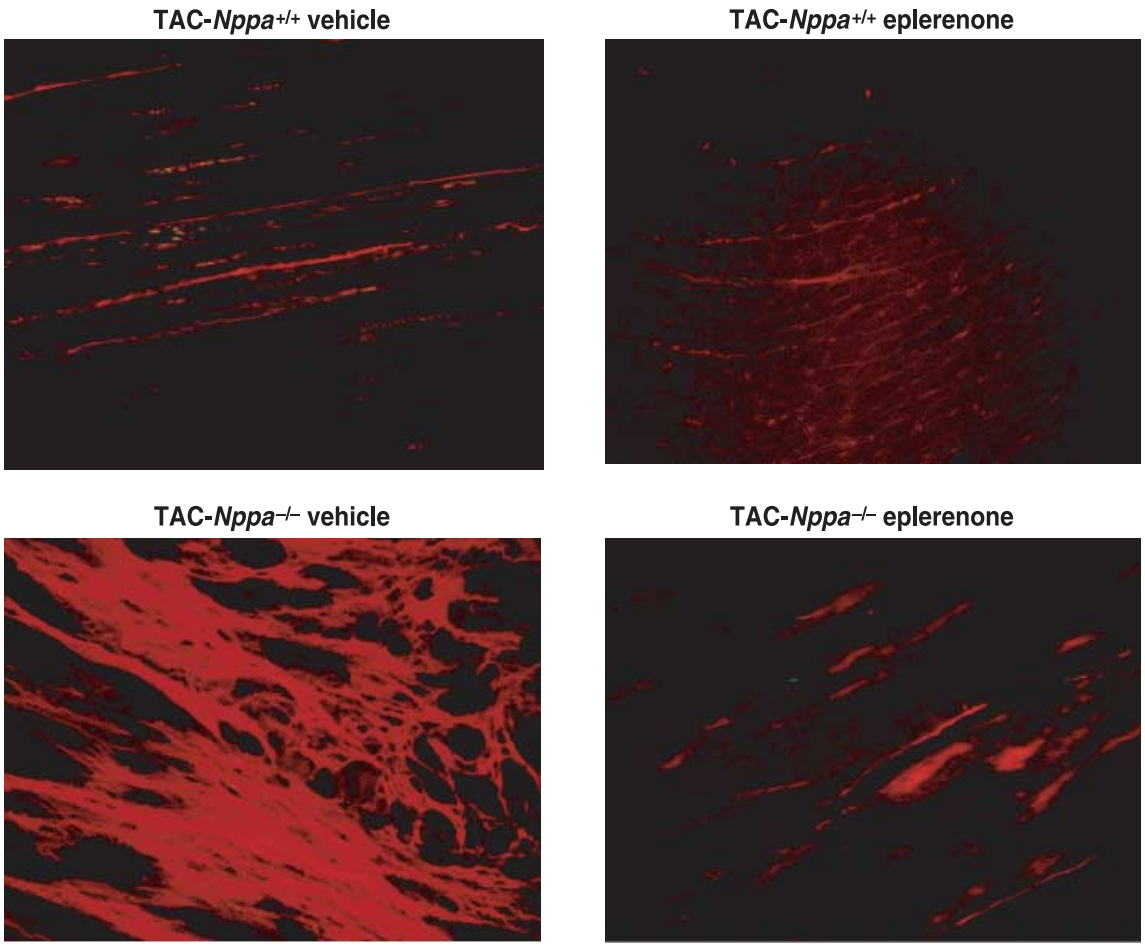

Fig. 3 Representative left ventricular sections (interstitial) stained for collagen by picrosirius red 0.1% in vehicle- and eplerenone-treated *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice following transverse aortic constriction (TAC). The excess fibrosis in *Nppa*$^{-/-}$ mice was completely prevented by eplerenone treatment.

Table 2 Mean arterial blood pressure and heart rate in unoperated mice

Treatment (*n*)	MAP (mmHg)	HR (b.p.m.)
Vehicle		
Nppa$^{+/+}$ (5)	129 ± 7	696 ± 15
Nppa$^{-/-}$ (6)	149 ± 8†	708 ± 44
Eplerenone		
Nppa$^{+/+}$ (5)	133 ± 3	690 ± 21
Nppa$^{-/-}$ (8)	154 ± 8†	720 ± 20
Genotype × eplerenone, two-way ANOVA, *P* values		
Genotype	0.007	0.426
Eplerenone	0.518	0.909
Interaction	0.913	0.731

Results are the mean±SEM. $^{\dagger}P < 0.05$ compared with respective *Nppa*$^{+/+}$. MAP, mean arterial pressure; HR, heart rate.

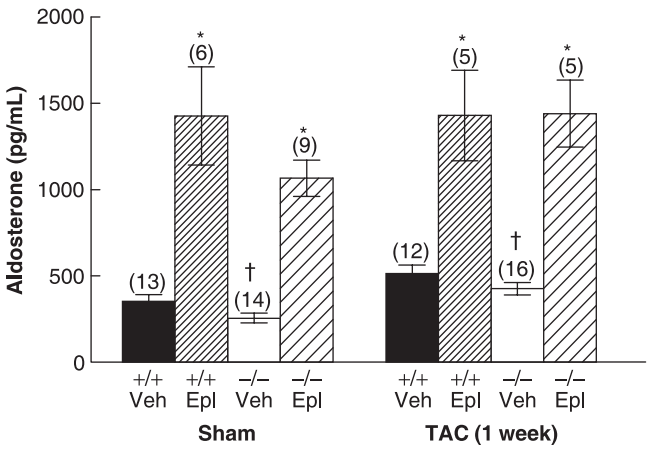

Fig. 4 Serum aldosterone levels before and after transverse aortic constriction (TAC). Results are shown as the mean±SEM, with the number of mice given in parentheses. Aldosterone was lower in atrial natriuretic peptide (ANP)-null mice compared with wild types at baseline and increased to a similar extent in both genotypes following TAC ($P < 0.0001$ for sham *vs* TAC operation; $P < 0.003$ for *Nppa*$^{+/+}$ *vs* *Nppa*$^{-/-}$; $P = 0.52$ for operation × genotype). $^{*}P < 0.05$ compared with respective vehicle; $^{\dagger}P < 0.05$ compared with respective *Nppa*$^{+/+}$. Veh, vehicle; Epl, eplerenone.

Effect of eplerenone on TAC-induced Cardiac hypertrophy and remodelling in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice

Eplerenone treatment significantly reduced the TAC-induced increases in RV and LV weight in both genotypes (Table 1; Fig. 1). There was a significant eplerenone–genotype interaction on both LV and RV weight in TAC animals, indicating a greater effect of eplerenone on preventing cardiac hypertrophy in *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ mice. Lung weight was increased in vehicle-treated *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ mice following TAC, suggesting early pulmonary oedema. This increase was greatly attenuated by eplerenone treatment.

A significant genotype–eplerenone interaction in LVEDD and LVFS was noted after pressure overload ($P < 0.05$, two-way ANOVA; Table 1). The LVEDD was greater after pressure overload stress in *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ vehicle-treated mice. This adverse LV dilatation and remodelling was completely prevented by eplerenone treatment. Fractional shortening was significantly reduced only in vehicle-treated *Nppa*$^{-/-}$ mice (Table 1). Importantly, eplerenone prevented FS depression in *Nppa*$^{-/-}$ mice after pressure overload.

Collagen volume increased significantly in both genotypes following TAC, but the increase was much greater in *Nppa*$^{-/-}$ mice, which developed robust interstitial fibrosis (Figs 2,3). Eplerenone treatment prevented excess fibrosis in the *Nppa*$^{-/-}$ TAC mice (Fig. 3). Eplerenone-treated TAC *Nppa*$^{-/-}$ mice had collagen volumes similar to those observed in the *Nppa*$^{+/+}$ TAC group ($P < 0.01$, two-way ANOVA; genotype × eplerenone). Thus, eplerenone was effective in modulating fibrosis in the setting of ANP deficiency under either basal or pressure overload conditions, but had no significant effect on fibrosis in ANP-replete mice. Osteopontin mRNA levels did not differ between TAC groups (21.2 ± 7.2, 10.7 ± 3.1, 12.6 ± 5.1 and 8.9 ± 4.5 in *Nppa*$^{-/-}$ vehicle, *Nppa*$^{+/+}$ vehicle, *Nppa*$^{-/-}$ eplerenone and *Nppa*$^{+/+}$ eplerenone, respectively), nor was an interaction between genotype and eplerenone observed ($P = 0.59$, two-way ANOVA). Myocyte diameter did not differ significantly between *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$, or between vehicle- and eplerenone-treated groups (17.6 ± 0.4, 17.4 ± 0.1, 17.8 ± 0.2 and 16.6 ± 0.3 in *Nppa*$^{-/-}$ vehicle, *Nppa*$^{+/+}$ vehicle, *Nppa*$^{-/-}$ eplerenone and *Nppa*$^{+/+}$ eplerenone, respectively). The results indicate that the excess hypertrophy in *Nppa*$^{-/-}$ following TAC reflects expansion of the ECM, rather than myocyte hypertrophy, and that this excess ECM expansion can be modulated by eplerenone.

Serum and cardiac aldosterone levels

Serum aldosterone levels were higher in sham-operated, vehicle-treated wild-type animals than in sham-operated ANP-null mice and increased to a similar extent following TAC in both genotypes ($P < 0.0001$ for sham *vs* TAC; $P < 0.003$ for *Nppa*$^{+/+}$ *vs* *Nppa*$^{-/-}$; $P = 0.52$ for operation × genotype; Fig. 4). Serum aldosterone increased approximately threefold in all groups on eplerenone treatment. Cardiac aldosterone levels were undetectable in vehicle-treated *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ following both sham and TAC surgery.

DISCUSSION

The major finding of the present study is that eplerenone attenuates cardiac hypertrophy in both *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice following TAC and completely prevents the fibrosis and the early development of systolic dysfunction in *Nppa*$^{-/-}$ mice following TAC. The modulation of LVH after TAC was more marked in the ANP-deficient mice compared with wild-type controls. Interestingly, however, eplerenone started at the time of weaning had no effect on baseline cardiac hypertrophy observed in sham-operated *Nppa*$^{-/-}$ mice compared with *Nppa*$^{+/+}$ controls. The results indicate that MR activation is an important mediator of hypertrophy in response to pressure overload in both ANP-deficient and -replete mice. Furthermore, the present study indicates that unopposed MR activation underlies the excess fibrosis and premature cardiac failure seen in response to pressure overload in ANP-knockout mice.

The most striking finding of the present study is that eplerenone prevents fibrosis while preserving ventricular function in ANP-null mice. These findings are consistent with a growing body of evidence that remodelling of the cardiac interstitium is a major determinant of pathological hypertrophy, leading to cardiac dysfunction and failure.[26–28] Previous studies have implicated both ANP and aldosterone as having important roles with regard to cardiac remodelling

in response to haemodynamic stress, particularly with regard to the cardiac interstitium.[3–5,11,29] However, the present study is the first to demonstrate that MR blockade can prevent the adverse interstitial remodelling seen in ANP deficiency in response to pressure overload.

Previously, we have demonstrated increased synthesis of ECM components following pressure overload in ANP-null mice.[3] In the present study, the excess hypertrophy following TAC in *Nppa*$^{-/-}$ mice was due to excess expansion of the ECM, rather than increased myocyte hypertrophy. Furthermore, the beneficial effect of eplerenone following TAC was due to modulation of this excess ECM expansion. This observation is consistent with the suggestion from previous studies that MR activation is a crucial factor in mediating adverse cardiac remodelling via expansion of the ECM.[11,12,16] In the Randomized ALdactone Evaluation Study (RALES) study, the beneficial effect of spironolactone on survival was confined to patients with elevated serum levels of procollagen III aminoterminal peptide (PIIIINP), a marker of increased turnover of ECM.[16] More recently, Kuster *et al*. demonstrated that 8 weeks treatment with eplerenone, started 1 week after ascending aortic constriction in mice, ameliorates myocardial fibrosis, activation of myocardial matrix metalloproteinase, LV dilatation and decreased FS.[29] Based on our previous report that osteopontin levels are higher 1 week after TAC and that there was an exaggerated response in ANP-null mice,[3] in the present study we evaluated the effects of eplerenone on osteopontin mRNA expression. We found no significant effect of eplerenone on osteopontin expression in either genotype.

The present findings of excess hypertrophy and fibrosis in the *Nppa*$^{-/-}$ mice could not be explained by increased serum aldosterone. Serum aldosterone levels increased in both genotypes following TAC, but were slightly depressed in *Nppa*$^{-/-}$ compared with *Nppa*$^{+/+}$ controls following either sham or TAC surgery. The slightly lower levels of aldosterone seen in the *Nppa*$^{-/-}$ mouse likely reflects volume overload. The finding of lower aldosterone levels in the ANP-null mouse is in contradistinction to the NPR-A-knockout mouse, in which serum aldosterone levels are elevated.[17] This may be due to preserved action of BNP in our model,[3] as opposed to the NPR-A-knockout model, in which the actions of both ANP and BNP are lost.[6]

Increased cardiac aldosterone levels have been observed post-MI in the rat, as well as in patients with hypertrophic cardiomyopathy,[12,30] and have been proposed as an explanation for the beneficial effects of MR inhibition in the absence of elevated serum aldosterone levels under those conditions. Furthermore, local synthesis of aldosterone has been reported in both rat isolated heart and in the failing human heart.[13,31,32] In the present study, we were unable to detect aldosterone in the hearts of our mice either at baseline or following TAC. It is possible that this reflects the insensitivity of our assay methodology for the very small amounts of aldosterone in the mouse heart. However, based on recent reports, we consider it unlikely that alterations in cardiac aldosterone levels account for the findings of the present study.[33,34] The former investigators, using a highly selective aldosterone antibody, detected only very low levels of aldosterone in homogenized cardiac tissue of adrenalectomized rats. Furthermore, levels of cardiac aldosterone synthase mRNA levels were one-millionth of levels in the adrenal gland. In the adrenal-intact rat, cardiac aldosterone levels mirrored plasma levels under conditions of low, normal or high dietary salt. These authors,[33,34] as well as an accompanying editorial,[35] concluded that the adrenal gland is the primary source of plasma and cardiac aldosterone and that cardiac synthesis is unlikely to be of any physiological or pathological significance. Similarly, Ye *et al*. were unable to detect mRNA for aldosterone synthase (CYP11β2) or 11β-hydroxylase (CYP11β1) in the hearts of several strains of rat using real-time quantitative RT-PCR.[34]

Eplerenone did not lower MAP or prevent baseline LVH in the sham-operated *Nppa*$^{-/-}$ mice, despite initiation of treatment at time of weaning, indicating that neither hypertension nor LVH requires MR activation in the *Nppa*$^{-/-}$ mouse. The ineffectiveness of eplerenone in preventing baseline hypertrophy in the ANP-null mouse could be due to irreversible developmental changes *in utero* as a result of ANP deficiency (e.g. an increased number of myocytes). Eplerenone has been shown to reverse LVH in human essential hypertension,[14] but its mechanism of action in this setting is uncertain. Mineralocorticoid receptor blockade with spironolactone has been shown to ameliorate fibrosis, but does not prevent myocyte hypertrophy in the free wall of the rat post-MI.[36] Eplerenone did not decrease myocyte cross-sectional area following aortic constriction in the present study, similar to the findings of Kuster *et al*.[29] Interestingly, eplerenone treatment prevented RV hypertrophy and had an effect on LV size in ANP-null mice, perhaps reflecting decreased volume due to a diuretic/natriuretic effect.

In summary, eplerenone treatment ameliorated hypertrophy following pressure overload from TAC in both ANP-replete and -deficient mice. However, the beneficial effect of eplerenone was much more marked in ANP-deficient mice following TAC, preventing both the excess fibrosis and the deterioration of LV function observed in untreated ANP-null mice. The findings suggest that the balance between ANP and aldosterone plays an important role in mediating adverse cardiac remodelling in response to haemodynamic stress and that the early excess fibrosis and LV dysfunction seen in ANP-deficient mice is mediated via the MR.

The present findings have important clinical implications, because functionally significant variations in ANP levels have been reported in humans. Blunted secretion of ANP has been observed in African-American salt-sensitive hypertensives in response to high salt and a polymorphism of the ANP gene has been observed more frequently in African-American salt-sensitive hypertensives compared with normotensives or Caucasian hypertensives.[37–39] Obese individuals demonstrate decreased levels of ANP and BNP, possibly related to more rapid clearance by adipocyte NPR-C receptors.[40] The results of the present study suggest that consideration should be given to conducting clinical trials of MR antagonists as therapy for these ANP-deficient states.

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