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ORIGINAL CONTRIBUTION

Chronic cardiac pressure overload induces adrenal medulla hypertrophy and increased catecholamine synthesis

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Abstract Increased activity of the sympathetic system is an important feature contributing to the pathogenesis and progression of chronic heart failure. While the mechanisms and consequences of enhanced norepinephrine release from sympathetic nerves have been intensely studied, the role of the adrenal gland in the development of cardiac hypertrophy and progression of heart failure is less well known. Thus, the aim of the present study was to determine the effect of chronic cardiac pressure overload in mice on adrenal medulla structure and function. Cardiac hypertrophy was induced in wild-type mice by transverse aortic constriction (TAC) for 8 weeks. After TAC, the degree of cardiac hypertrophy correlated significantly with adrenal weight and adrenal catecholamine storage. In the medulla, TAC caused an increase in chromaffin cell size but did not result in chromaffin cell proliferation. Ablation of chromaffin α_{2C}-adrenoceptors did not affect adrenal weight or epinephrine synthesis. However, unilateral denervation of the adrenal gland completely prevented adrenal hypertrophy and increased catecholamine synthesis. Transcriptome analysis of microdissected adrenal medulla identified 483 up- and 231 downregulated, well-annotated genes after TAC. Among these genes, G protein-coupled receptor kinases 2 (*Grk2*) and 6 and phenylethanolamine *N*-methyltransferase (*Pnmt*) were significantly upregulated by TAC. In vitro, acetylcholine-induced *Pnmt* and *Grk2* expression as well as enhanced epinephrine content was prevented by inhibition of nicotinic acetylcholine receptors and Ca^{2+}/calmodulin-dependent signaling. Thus, activation of preganglionic sympathetic nerves innervating the adrenal medulla plays an essential role in inducing adrenal hypertrophy, enhanced catecholamine synthesis and induction of *Grk2* expression after cardiac pressure overload.

Keywords Adrenal medulla · Epinephrine · Sympathetic nervous system · Heart failure

Introduction

Overactivity of the sympathetic nervous system is a prominent characteristic of chronic heart diseases. Elevated sympathetic tone represents an essential compensatory mechanism to maintain cardiac output by increasing heart rate, cardiac contractility and peripheral vascular resistance [4, 8, 40]. As a long-term consequence, increased catecholamine levels facilitate the progression of cardiac hypertrophy and fibrosis [5]. Moreover, the incidence of arrhythmias and sudden cardiac death is increased [25–27]. In cardiac myocytes, chronic sympathetic stimulation results in depletion of sarcoplasmic calcium stores leading to impaired myocardial contractility [41]. In addition, chronically enhanced norepinephrine levels are associated with a worsened prognosis of chronic heart failure patients [10], thus providing an explanation for the beneficial therapeutic effects of β-blockers [9, 12, 31]. But also activation of myocyte α_1-adrenoceptors [15] as well as

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other factors worsens the development of chronic heart disease [23, 34, 39].

The level of circulating norepinephrine linearly correlates with adverse outcome of patients with chronic heart failure [10]. While plasma epinephrine concentrations may be normal during initial stages of heart failure, patients with cardiac cachexia showed elevated epinephrine plasma levels [1]. The contribution of adrenal catecholamines to the progression of heart failure has also been documented in experimental models. Mice lacking α_{2C}-adrenoceptors which control epinephrine secretion via an autocrine feedback loop in adrenal chromaffin cells show rapid deterioration of cardiac function and raised mortality after transverse aortic constriction [6, 7, 16]. Adrenal gland hypertrophy and increased plasma epinephrine levels have been discovered in rats after experimental myocardial infarction and in mice overexpressing the sarcoplasmic reticulum calcium-binding protein calsequestrin [28]. Interestingly, upregulation of the G protein-coupled receptor kinase 2 (*Grk2*) in the adrenal medulla was demonstrated to desensitize inhibitory feedback α_{2C}-receptors, thus allowing higher levels of adrenal epinephrine secretion and progression of heart failure [28, 29, 33].

Thus the aim of the present study was to determine the mechanisms of adrenal hypertrophy and increased epinephrine secretion in mice in response to the development of cardiac hypertrophy. Here, we demonstrate that preganglionic cholinergic nerves innervating the adrenal gland are essential to mediate adrenal hypertrophy, upregulation of *Grk2* and increased epinephrine synthesis during cardiac pressure overload. Increased expression of *Grk2* and *Pnmt* expression as well as epinephrine synthesis was induced by acetylcholine via a nicotinic receptor—Ca^{2+}/calmodulin-dependent pathway.

Methods

Transverse aortic constriction and unilateral adrenal denervation

Cardiac pressure overload was induced in 8- to 12-week-old male mice (C57BL/6J) [16]. For surgery, mice were anesthetized with isoflurane (2 vol% in O_2) and placed on a heating pad (37°C). After thoracotomy, the aortic arch was constricted to the width of a 27-G canula using a 6.0 nylon suture. All experiments were performed 8 weeks following TAC. Only one series of experiments was terminated 1 week after TAC (Fig. S2). To investigate the impact of sympathetic innervation of the adrenal gland, the left splanchnic nerve was transected at the left side within 48 h after aortic constriction. All experiments were approved by the responsible animal care committee of the University of Freiburg, Germany. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Measurement of left ventricular contractile function

A 1.4F pressure–volume catheter (Millar Instruments, Houston, TX, USA) was inserted into the right carotic artery and left ventricle (anesthesia with 2 vol% isoflurane) [3, 16]. Chart v5.4 software (AD Instruments, Castle Hill, Australia) was used for data analysis. Two-dimensional guided M-mode and Doppler echocardiography using a Vivid 7 Dimension echocardiograph (GE Healthcare, Munich, Germany) equipped with a 14-MHz transducer were used to determine left ventricular ejection fraction and blood flow across the aortic stenosis.

Histology

Hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and were embedded in paraffine [17]. Sections were stained with hematoxylin–eosin, Sirius red or wheat germ agglutinin (WGA, Alexa Fluor 488 conjugate, Invitrogen, Karlsruhe, Germany) [17]. Nuclei of WGA-stained sections were counterstained with propidium iodide. All adrenal weights given in this study refer to single adrenal glands. Adrenal tissues were fixed in paraformaldehyde solution for hematoxylin–eosin and WGA staining or frozen in liquid nitrogen for Ki67 staining. For Ki67 staining, cryosections were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and blocked with 3% bovine serum albumin and 3% normal goat serum. Following incubation with the Ki67 antibody (rabbit monoclonal 1:1,000, 4°C, overnight; ThermoScientific, Waltham, MA, USA), Alexa Fluor® 488 secondary antibody (goat anti-rabbit; Invitrogen, Karlsruhe, Germany) was used. Cell nuclei were counterstained with DAPI (Sigma-Aldrich, Munich, Germany), and histological analysis was performed using AxioVision Rel.4.5 software (Carl Zeiss AG, Heidenheim, Germany).

Catecholamine determination

For measuring the catecholamine content, an isocratic HPLC with electrochemical detection was used consisting of an HPLC pump (UltiMate® 3000 Quaternary Analytical, Dionex), an autosampler (WISP 717plus, Waters, Eschborn) and an amperometric detector (Antec Intro, Antec-Leyden) [16, 17]. Data were registered and analyzed using Chromeleon 6 software (Dionex, Sunnyvale, CA, USA). Catecholamines were separated on a Prontosil 120-3-C18 AQ column (3 µm; 120 × 2 mm; Bischoff chromatography, Leonberg, Germany) [16, 17].

Microdissection and microarray analysis

Adrenal glands were frozen in liquid nitrogen. 12 µm cryosections were dehydrated in xylene. Adrenal medullary tissue was isolated by microdissection with a MicroChisel (Eppendorf, Hamburg, Germany) using a Leica AM6000 inverted microscope (Leica Microsystems, Wetzlar, Germany). Dissected tissue was aspirated in xylene, and RNA was isolated with the RNeasy Micro-Kit (Qiagen, Hilden, Germany). RNA expression was determined with a Murine Genome Chip 430 2.0 (Affymetrix, Santa Clara, CA, USA) and analyzed with ArrayAssist 5.0 software (Stratagene, Amsterdam, The Netherlands) [17]. Microarray data have been deposited in NCBI's Gene Expression Omnibus (accession number GSE21829).

For determination of cardiac gene expression, RNA was isolated and analyzed as described previously [16, 17].

Adrenal medulla in vitro culture

Adrenal glands were prepared, collected in Locke's buffer on ice, and the cortex was removed. The adrenal medulla was incubated at 37°C and 7.5% CO_2 for 24 h using incubation medium (M199 Medium Earle (Biochrom AG, Berlin, Germany), 1% bovine serum albumin, 10% fetal calf serum, 100 U/mL penicillin/streptomycin). Samples were incubated with 200 µmol/L acetylcholine, 100 µmol/L hexamethonium, 1 µmol/L calmidazolium or 10 µmol/L KN-62 (Sigma-Aldrich, Munich, Germany). After 24 h, RNA of the adrenal medulla was isolated using the RNeasy Micro-Kit (Qiagen, Hilden, Germany), reverse transcribed (QuantiTect Rev. Transcription Kit, Qiagen, Hilden, Germany) and quantitative real-time PCR was performed as described previously [17] (Table 1).

Statistical analysis

Unpaired two-tailed Students *t* test or two-way ANOVA followed by Bonferroni post-hoc tests were performed for statistical analysis using GraphPad Prism software. A p value of <0.05 was considered statistically significant. Data are expressed as mean ± SEM.

Results

Adrenal hypertrophy in response to chronic cardiac pressure overload

After 8 weeks of TAC, mice developed significant cardiac and adrenal hypertrophy (Fig. 1a–g). Cardiac ventricle weight/tibia length ratios were increased from 6.7 ± 0.14 to 10.7 ± 0.50 mg/mm. 24% of the TAC-operated mice developed heart failure as indicated by severe lung edema. The hypertrophic response of the ventricle was accompanied by a 40% increase of the adrenal weight:tibia length ratios compared to levels observed in sham-operated mice (Fig. 1a, b). Adrenal weight showed a significant linear correlation with cardiac weight (Fig. 1c). This correlation was similar when the parameters were corrected for tibia length (Fig. S1), while the correlation coefficients for the individual sham-operated and TAC groups did not reach statistical significance (sham $p = 0.244$; TAC $p = 0.54$). After 8 weeks of TAC, the adrenal content of epinephrine and norepinephrine was increased as compared with sham-operated mice (Fig. 1h, i). Further experiments revealed that the concordant increase of adrenal weight and ventricle weight was already present after 1 week of TAC (Fig. S2), indicating a fast onset of the hypertrophic response in both tissues.

Table 1 Primer sequences used for quantitative real-time PCR analysis

Gene	Primer sequence [5′–3′]
Grk2	s: GAT CTT TTC GCA GAA GTT AGG G
	as: TGG CCT CTT CCA GAT GGT T
Gapdh	s: TGC ACC ACC AAC TGC TTA GC
	as: GGC ATG GAC TGT GGT CAT GAG
Grk6	s: CGA GAA CAT CGT AGC GAA CA
	as: AGC TCC TCA CAC TGG CTG AT
Nppa	s: GCT TCC AGG CCA TAT TGG AG
	as: GGG GGC ATG ACC TCA TCT T
Pnmt	s: CCT ATC TCC GCA ACA ACT AC
	as: TAT CAA TGA GAA CCC GTC CC
Postn	s: ACT TCA GCT CCT GTA AGA ACT G
	as: AGG GCA GCA TTC ATA TAG CAC A
Rps29	s: ATG GGT CAC CAG CAG CTC TA
	as: GCT AGC CAA AGA CTT GTG CCA TGC AG

Grk2 G protein-coupled receptor kinase 2, *Gapdh* glyceraldehyde-3-phosphate dehydrogenase, *Grk6* G protein-coupled receptor kinases 6, *Nppa* atrial natriuretic peptide, *Pnmt* phenylethanolamin *N*-methyltransferase, *Postn* periostin, *Rps29* ribosomal protein S29

Effect of ablation of adrenal α_{2C}-adrenoceptor expression

Previous studies have demonstrated that α_{2C}-adrenoceptors are important feedback regulators of catecholamine secretion from the adrenal gland [6, 7, 16, 28]. Genetic ablation of α_{2C}-receptor expression led to increased circulating epinephrine levels and accelerated progression of cardiac hypertrophy [7]. Thus, we sought to determine whether loss of α_{2C}-adrenoceptors affected adrenal weight and catecholamine storage. When compared with wild-type mice, α_{2C}-deficient mice did not show differences in adrenal,

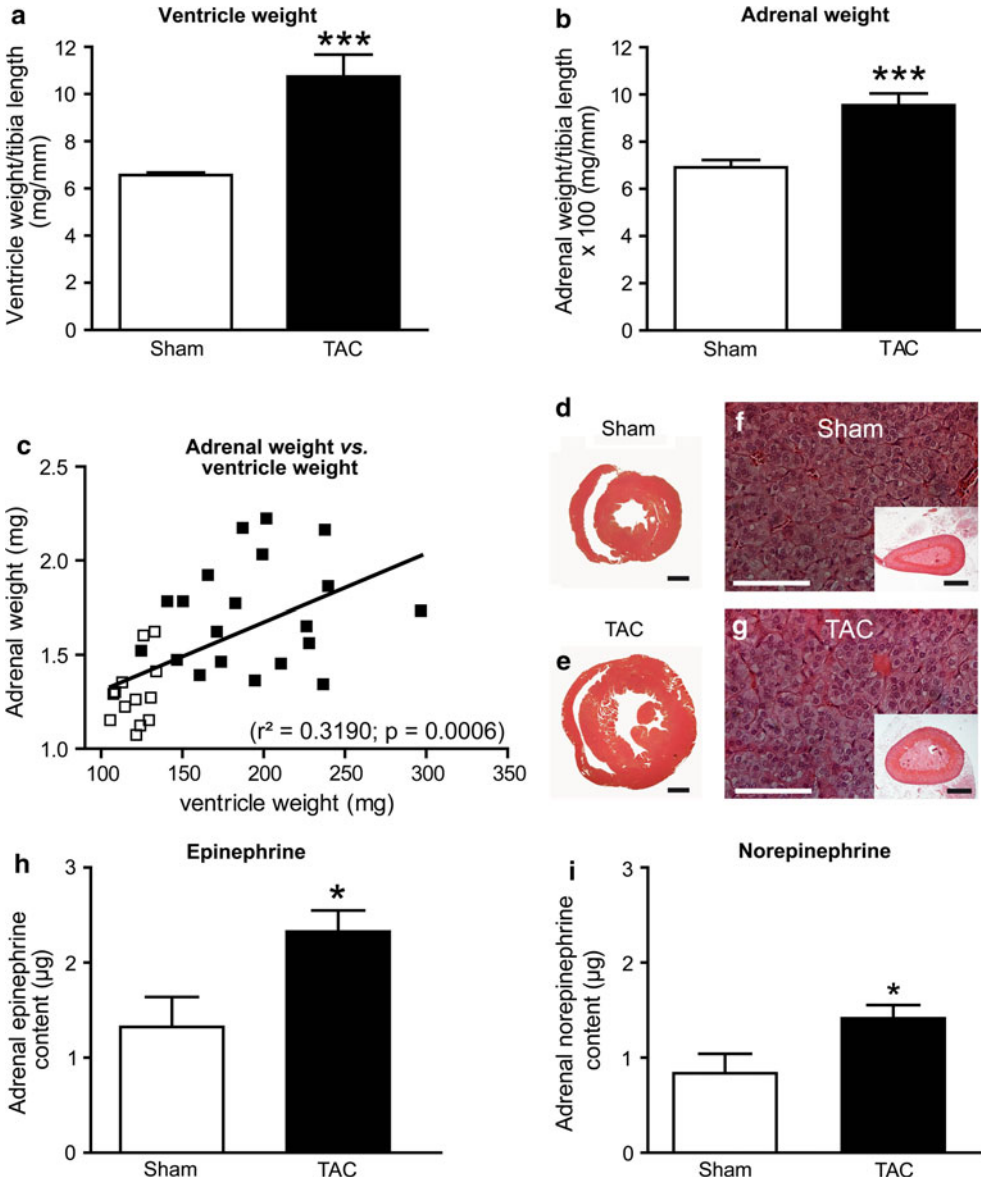

Fig. 1 Increased weight and catecholamine content of adrenal glands following transverse aortic constriction (TAC). Cardiac ventricle weight/tibia length ratio (**a**) or adrenal weight/tibia length ratio (**b**) of wild-type C57BL/6 mice 8 weeks after TAC or sham operation ($n = 10$–19 per group). **c** Correlation of adrenal weight to ventricle weight in sham (*open squares*) and TAC (*filled squares*) operated mice ($r^2 = 0.3190$; $p = 0.0006$). **d**–**g** Hematoxylin–eosin staining of mid-ventricular cardiac (**d**, **e**, *scale bars* 1 mm) or adrenal gland (**f**, **g**, *insert*, *scale bars* 500 µm) and adrenal medulla (**f**, **g**, *large picture*, *scale bars* 100 µm) sections following sham and TAC surgery. **h**, **i** Adrenal epinephrine (**h**) and norepinephrine (**i**) content as determined by HPLC ($n = 6$–12 per group; $^*p < 0.05$, $^{***}p < 0.001$ TAC vs. sham)

heart or body weight and did not store higher amounts of epinephrine in the adrenal gland (Table 2). This finding indicates that increased catecholamine release due to loss or desensitization of α_2-adrenoceptors per se does not lead to adrenal hypertrophy.

Cardiac function and morphology after unilateral adrenal denervation

Thus, we investigated whether circulating factors or sympathetic innervation of the adrenal gland were responsible for adrenal hypertrophy after cardiac pressure overload. To distinguish between these two possibilities, preganglionic nerves innervating the adrenal gland were unilaterally transected in sham- and TAC-operated mice. 8 weeks of TAC resulted in impaired cardiac function as evidenced by a reduction in left ventricular ejection fraction (EF) and fractional shortening (FS) (EF −43.8%, FS −59.7% vs. sham, $p < 0.05$; Fig. 2a–c). Heart rate and systolic blood pressure in the ascending aorta were increased after TAC compared to controls (+56.4 and +42.9%, respectively vs. sham, $p < 0.05$; Fig. 2d, e). Unilateral adrenal denervation within the first 48 h after transverse aortic constriction had no effect on cardiovascular parameters, both under basal conditions and following TAC compared with the corresponding control groups (Fig. 2).

After 8 weeks of pressure overload, cardiac myocyte cross-sectional area and interstitial fibrosis were significantly increased compared to controls (+62.9 and +752%, respectively vs. sham, $p < 0.05$; Fig. 3a–d). Similarly, cardiac atrial natriuretic peptide and periostin mRNA expression were increased after TAC (Fig. 3e, f). Again,

Table 2 Adrenal, cardiac and body weight in wild-type (*Adra2c*$^{+/+}$) and α_{2C}-adrenoceptor-deficient mice (*Adra2c*$^{-/-}$)

	Adra2c$^{+/+}$ ($n = 8$)	*Adra2c*$^{-/-}$ ($n = 6$)	p value
Body weight (g)	25.0 ± 0.8	26.7 ± 0.8	0.36
Tibia length (mm)	18.1 ± 0.1	17.9 ± 0.2	0.35
Ventricle weight (mg)	116.5 ± 4.0	123.1 ± 7.5	0.42
Ventricle weight/body weight (mg/)	4.7 ± 0.2	4.6 ± 0.2	0.74
Ventricle weight/tibia length (mg/mm)	6.4 ± 0.2	6.9 ± 0.5	0.32
Adrenal weight (mg/gland)	1.42 ± 0.1	1.27 ± 0.1	0.32
Adrenal epinephrine content (μg/gland)	1.67 ± 0.3	1.45 ± 0.1	0.45

unilateral adrenal gland denervation had no effect on these parameters as compared with the corresponding control groups (Fig. 3a–f).

Adrenal morphology and epinephrine secretion after unilateral adrenal denervation

After 8 weeks of TAC, unilateral denervation within 48 h after transverse aortic constriction completely prevented the increase in adrenal gland weight after pressure overload (Fig. 4a, b). Both adrenal medulla and cortex contributed to the enlargement of the adrenal gland after TAC (Fig. 4c, d). Both adrenal compartments were unchanged after TAC and unilateral denervation, indicating an essential role of sympathetic innervation for adrenal gland hypertrophy. After TAC the cross-sectional area of chromaffin cells in the adrenal medulla was significantly increased (Fig. 4e). The cellular hypertrophy was in addition reflected by a reduced density of nuclei in the adrenal medulla (Fig. S3), while the number of chromaffin cells which were positive for the proliferation marker Ki67 did not differ between sham and TAC specimens (Fig. 4f).

To measure the impact of TAC and unilateral denervation on epinephrine secretion, 24 h urine was collected. 8 weeks after TAC-operation, the epinephrine content was 2.5-fold higher than after sham-operation. Unilateral denervation did not affect the amount of secreted epinephrine, but no further raise in epinephrine secretion was observed after TAC (Fig. S4).

Transcriptome analysis of the adrenal medulla after cardiac pressure overload

In order to identify which changes in gene expression accompany the development of adrenal hypertrophy, the adrenal medulla from sham- and TAC-operated mice was microdissected, and mRNA was subjected to microarray expression analysis. After TAC, expression of 714 well annotated and 108 probe sets of hypothetical genes was changed more than 1.5-fold in the adrenal medulla (Fig. 5a; Suppl. Table 1). Among the well-annotated genes, 483 genes were increased and 231 genes were decreased in their expression levels. Pathway analysis revealed that adrenal medullary MAPK signaling, notch signaling, focal adhesion and actin cytoskeleton pathways were significantly affected by cardiac pressure overload (Suppl. Table 1). Among the adrenergic target genes, the G protein-coupled receptor kinases 2 and 6 (*Grk2*, *Grk6*) and the phenylethanolamine *N*-methyltransferase (*Pnmt*) were significantly upregulated after TAC (Fig. 5b, c). Increased expression of *Grk2*, *Grk6* and *Pnmt* after TAC was validated by quantitative real-time PCR in independently microdissected medullary specimens (Fig. 5c). Unilateral denervation prevented the increased expression of *Grk2* and *Pnmt* which was induced by cardiac pressure overload (Fig. 5d, e). In addition, the β4-subunit of the nicotinic acetylcholine receptor (*Chrnb4*) was increased in its mRNA expression after TAC to 2.04-fold of control (Suppl. Table 1).

In vitro regulation of gene expression and adrenal epinephrine content

In order to confirm that preganglionic cholinergic innervation is essential for gene expression of *Pnmt*, *Grk2* and *Grk6*, mRNA expression of these genes was measured after in vitro stimulation of adrenal medulla biopsies with acetylcholine (Fig. 6a). Similar to the in vivo situation, in vitro incubation with acetylcholine increased *Pnmt* mRNA expression to 2.3-fold and *Grk2* mRNA expression to 1.6-fold of unstimulated control (Fig. 6a, b; $p < 0.05$). Hexamethonium, an inhibitor of nicotinic acetylcholine receptors, prevented the acetylcholine-induced rise in *Pnmt* and *Grk2* expression (Fig. 6a, b). Similarly, acetylcholine-induced *Pnmt* and *Grk2* expression could be inhibited by the calmodulin inhibitor calmidazolium and the CaM kinase II inhibitor KN-62 (Fig. 6a, b). No alterations in *Grk6* mRNA expression were observed under the various conditions (Fig. 6c).

Concordant with the induction of *Pnmt* mRNA expression, adrenal epinephrine content was elevated after in vitro stimulation with acetylcholine (Fig. S5a). This effect was also prevented by application of hexamethonium, calmidazolium or KN-62. These results underpin the

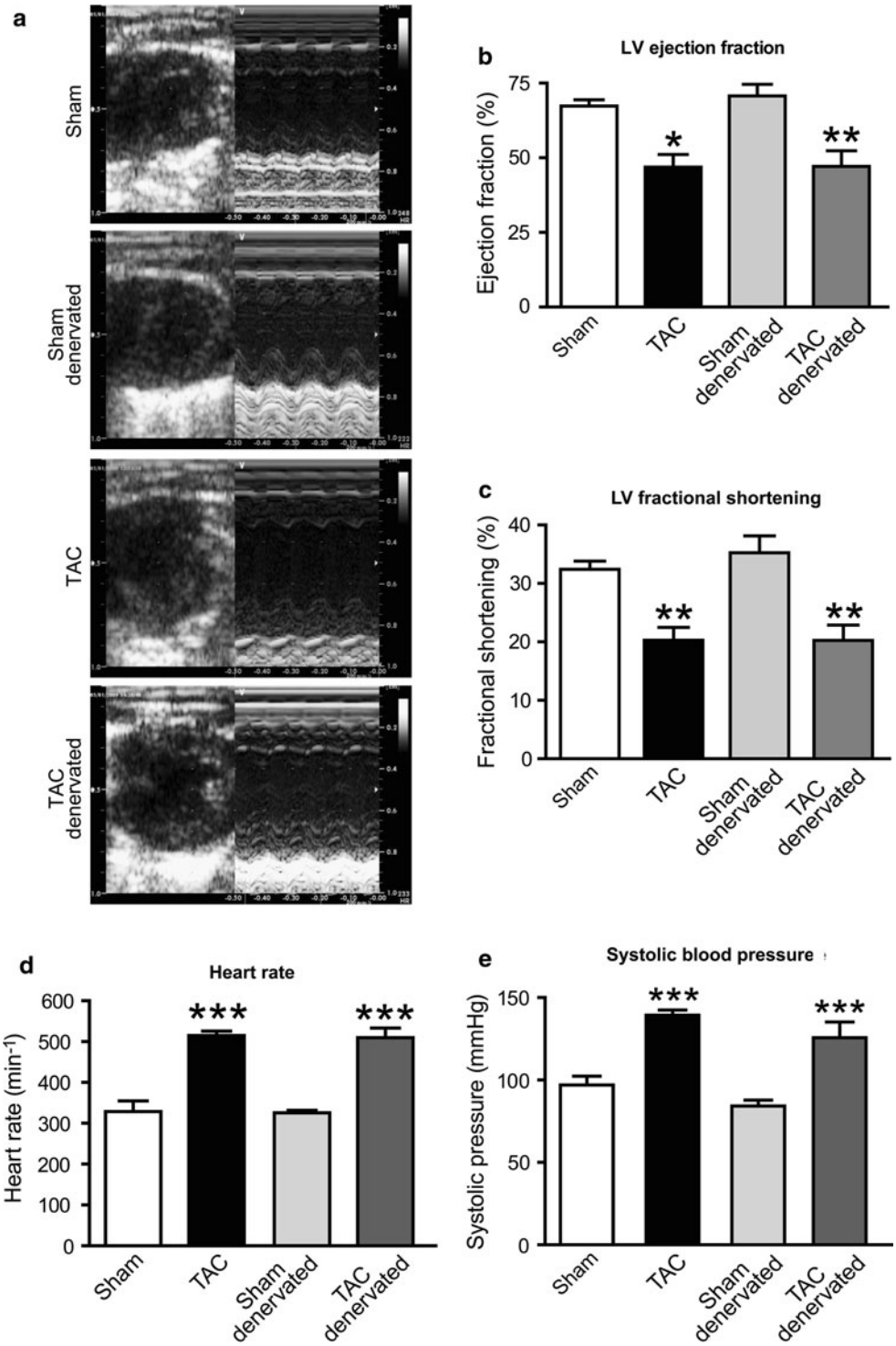

Fig. 2 Alterations in left ventricular contractile function after transverse aortic constriction and unilateral adrenal denervation. **a**–**c** Representative 2D-guided M-mode echocardiography from parasternal short axis view (**a**), left ventricular ejection fraction (**b**) and fractional shortening (**c**) in sham- or TAC-operated mice with or without unilateral adrenal denervation. **d**, **e** Heart rate and systolic aortic pressure as determined by direct catheterization during isoflurane anesthesia ($n = 4$–12, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ TAC vs. sham)

relevance of this pathway for the regulation of epinephrine synthesis in the adrenal medulla. However, pretreatment with acetylcholine did not affect the amount of epinephrine released into media upon acute acetylcholine stimulation (Fig. S5b). Furthermore, incorporation of [^{14}C]phenylalanine and cell size were not altered by 24 h of acetylcholine stimulation (Fig. S5c, d).

Discussion

The main finding of the present study is that chronic cardiac pressure overload in mice induces adrenal medulla hypertrophy, increased epinephrine synthesis and induction of *Grk2* via a nicotinic acetylcholine receptor-dependent pathway in chromaffin cells.

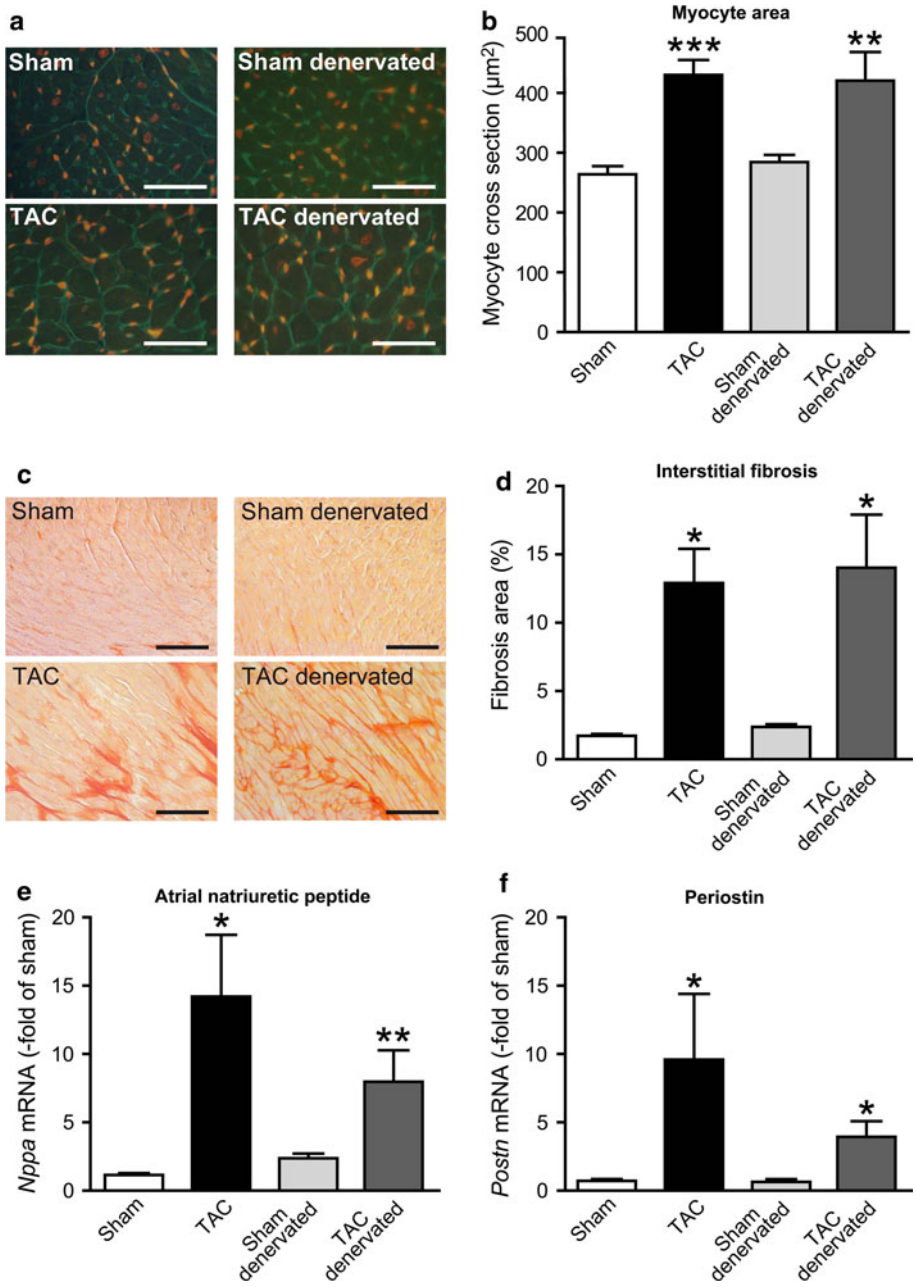

Fig. 3 Cardiac hypertrophy and fibrosis following TAC and unilateral adrenal denervation. **a**, **b** Cardiac myocyte cross-sectional area as determined by wheat germ agglutinin staining of mid-ventricular cardiac sections (**a**, *scale bars* 50 µm). **c**, **d** Left ventricular interstitial fibrosis detected by Sirius red staining of mid-ventricular cardiac sections (**c**, *scale bars* 100 µm). **e**, **f** Cardiac mRNA expression of atrial natriuretic peptide (*Nppa*, **e**) and periostin (*Postn*, **f**) ($n = 4$–7; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ TAC vs. sham)

Adrenal hypertrophy has been observed in several animal models of cardiac dysfunction or stress. Induction of heart failure by experimental myocardial infarction in rats or by transgenic cardiac expression of calsequestrin in mice resulted in hypertrophy of the adrenal gland [28]. Adrenal hypertrophy has also been shown in several stress models in rodents, e.g. chronic immobilization [18], normobaric hypoxia [42] and exposure to a hyperbaric environment [19]. Similar to the observation in the present study, hyperbaric conditions led to increased size of both adrenal cortex and medulla [19]. Adrenocortical hypertrophy has been shown to be reversible and is likely to be a stress response [22]. This may be explained at least partly by the observation that heart failure causes not only an activation of the sympathetic nervous system, but also an enhanced activity of the renin–angiotensin–aldosterone system [21, 24, 30, 32, 35]. Elevated levels of epinephrine and norepinephrine and also cortisol and aldosterone have been described for patients with cardiac cachexia [2]. There are strong interactions between the adrenal medulla and the cortex. In isolated adrenal glands with intact splanchnic nerves, activation of the splanchnic nerve as well as perfusion with epinephrine caused release of cortisol and aldosterone [13]. After incubation with epinephrine, increased steroidogenesis and enhanced levels of P450-mRNAs were observed. Interactions have also been

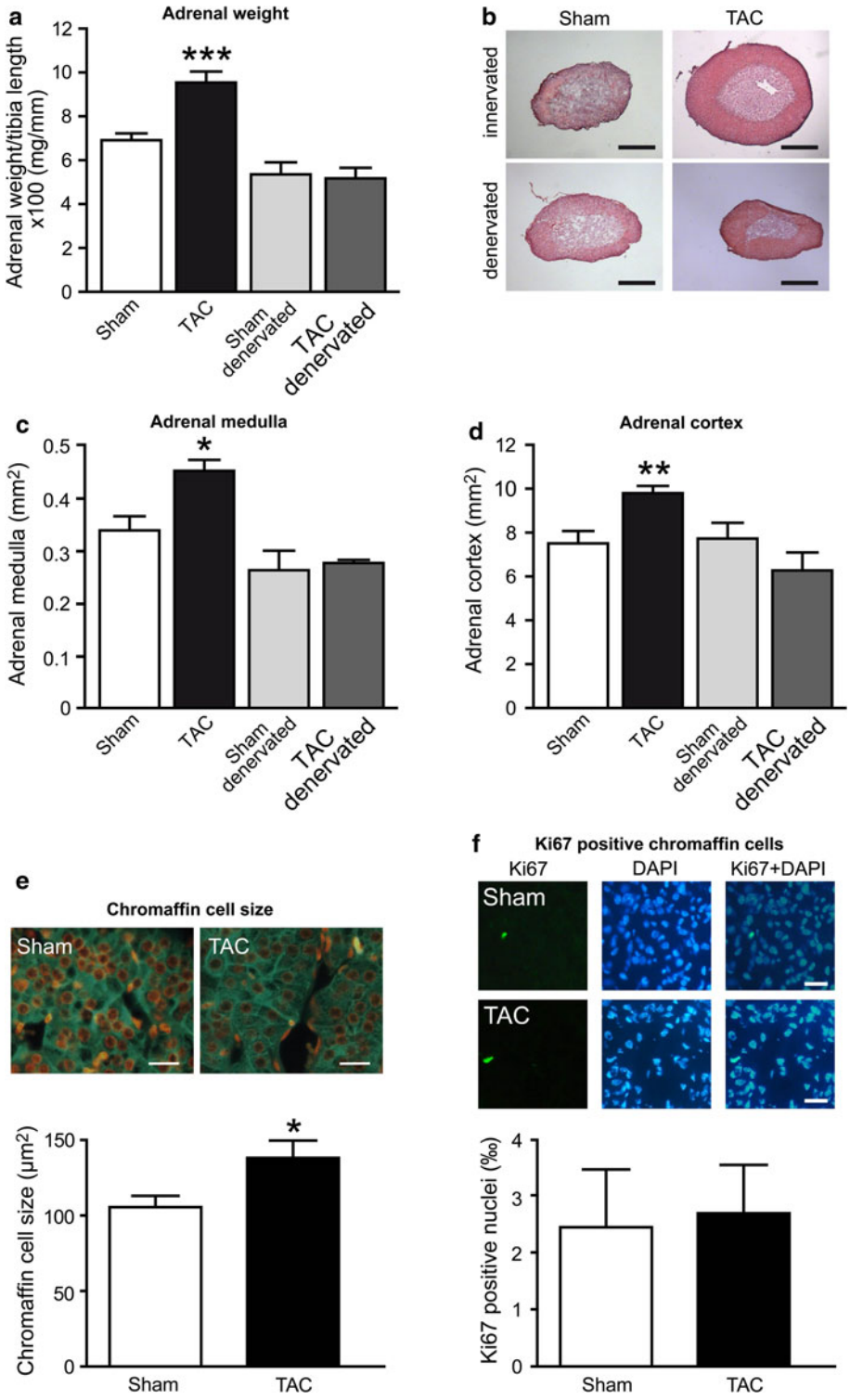

Fig. 4 Influence of unilateral adrenal denervation on adrenal hypertrophy following chronic cardiac pressure overload. **a**, **b** Adrenal weight/tibia length ratio and histology after transverse aortic constriction and unilateral adrenal denervation (**b**, hematoxylin–eosin staining, *scale bars* 500 µm). **c**, **d** Morphometric analysis of adrenal medulla (**c**) and cortex (**d**) cross-sectional areas. **e** Wheat germ agglutinin staining and morphometry of adrenal medullary sections to determine chromaffin cell size (*scale bars* 20 µm). **f** Ki67 and DAPI staining of the adrenal medulla (*scale bars* 20 µm) ($n = 3$–15, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ TAC vs. sham)

described vice versa: venous blood from the adrenal cortex goes to the adrenal medulla by an intra-adrenal portal vascular system. This causes extremely high glucocorticoid concentrations in the adrenal medulla inducing *PNMT* expression. Suppression of glucocorticoid production is described to determine a decrease of epinephrine level [46]. The *PNMT* promoter region contains a glucocorticoid response element, where the activated glucocorticoid receptor can bind and induce *PNMT* expression [47]. Mice lacking the glucocorticoid receptor do not show a central adrenal medulla and are not able to produce epinephrine [11]. These interactions show that an isolated hypertrophy

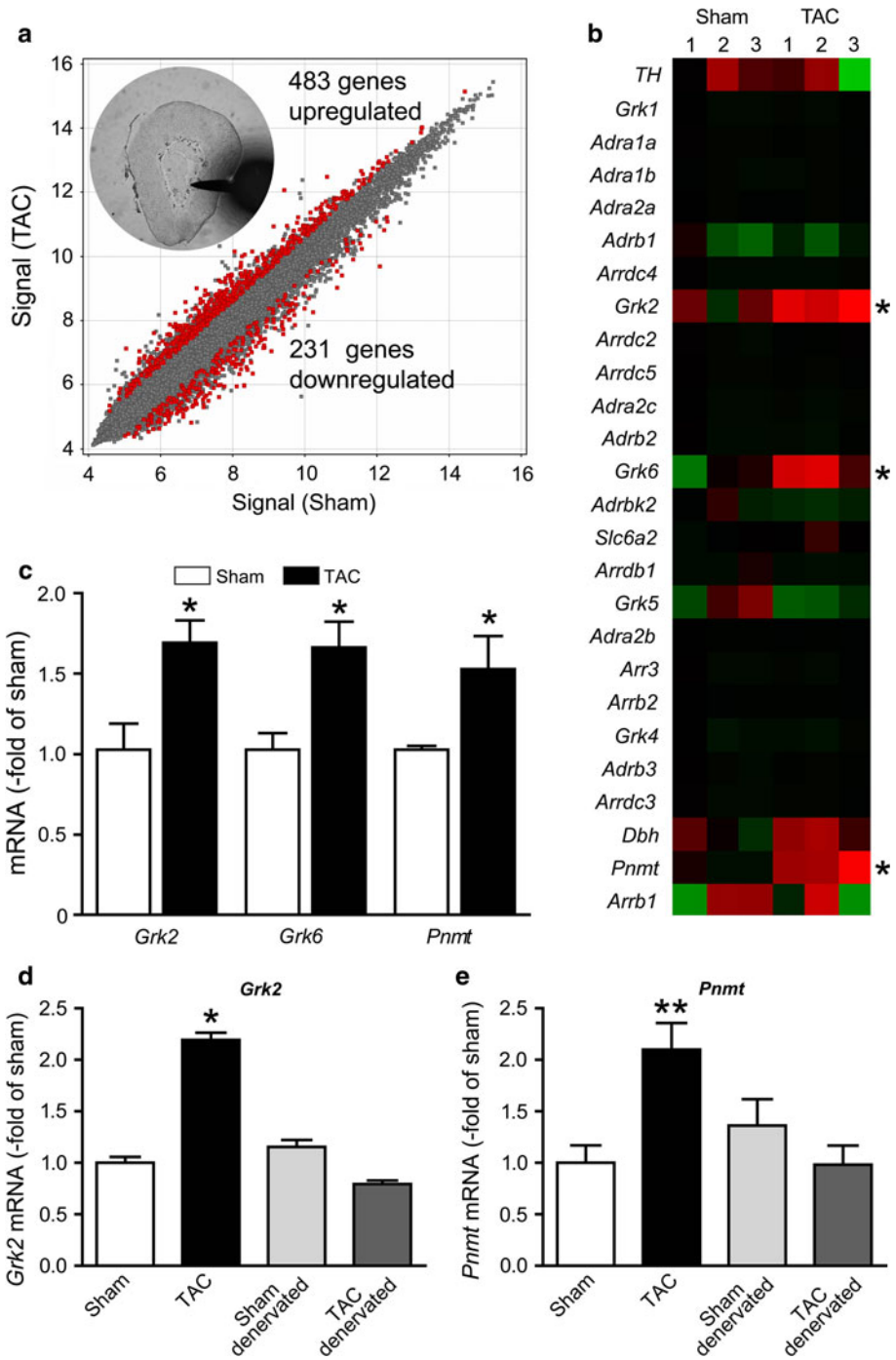

Fig. 5 Microarray analysis of gene expression in the adrenal medulla after chronic cardiac pressure overload. **a** Adrenal medulla was isolated by microdissection of cryostat sections using a piezo-driven microneedle (**a**, *insert*). Microarray analysis of total adrenal medulla mRNA from sham- versus TAC-operated wild-type C57BL/6 mice revealed 714 well-annotated genes which were differentially expressed (*red*, >1.5-fold, $p < 0.05$). **b** Expression of adrenergic target genes in adrenal medulla ($n = 3$ sham, 3 TAC samples). *Red color* depicts higher expression than mean of control; *green* indicates lower expression. **c** Expression of G protein-coupled receptor kinases 2 (*Grk2*) and 6 (*Grk6*) and phenylethanolamine *N*-methyltransferase (*Pnmt*) derived from microarray analysis in the adrenal medulla of sham- or TAC-operated mice ($n = 3$ per group). **d**, **e** Expression of *Grk2* and *Pnmt* in microdissected adrenal medulla as measured by qPCR ($n = 4$–6; $^{*}p < 0.05$, $^{**}p < 0.01$ TAC vs. sham)

of one compartment is unlikely. Further studies are required to clarify the involved pathways and the relevance for the pathophysiology of cardiac hypertrophy and heart failure.

The underlying mechanisms of adrenal hypertrophy were dissected in an elegant series of recent studies demonstrating that cardiac failure led to upregulation of the G protein-coupled receptor kinase 2 (*Grk2*) in adrenal chromaffin cells [28, 29, 33]. In the heart, increased *Grk2* levels contribute significantly to β-adrenoceptor desensitization and left ventricular dysfunction [36]. In the adrenal medulla, *Grk2* potentially downregulates and desensitizes α_{2C}-adrenoceptors, which play an essential role as inhibitory feedback regulators of adrenal catecholamine secretion (Fig. 6b) [6, 28]. Genetic deletion of the α_{2C}-receptor gene in mice led to increased circulating epinephrine levels and facilitated the development of heart failure after cardiac pressure overload [6, 7, 16].

Here, we demonstrate that loss of α_{2C}-receptor expression per se was not sufficient to induce adrenal hypertrophy

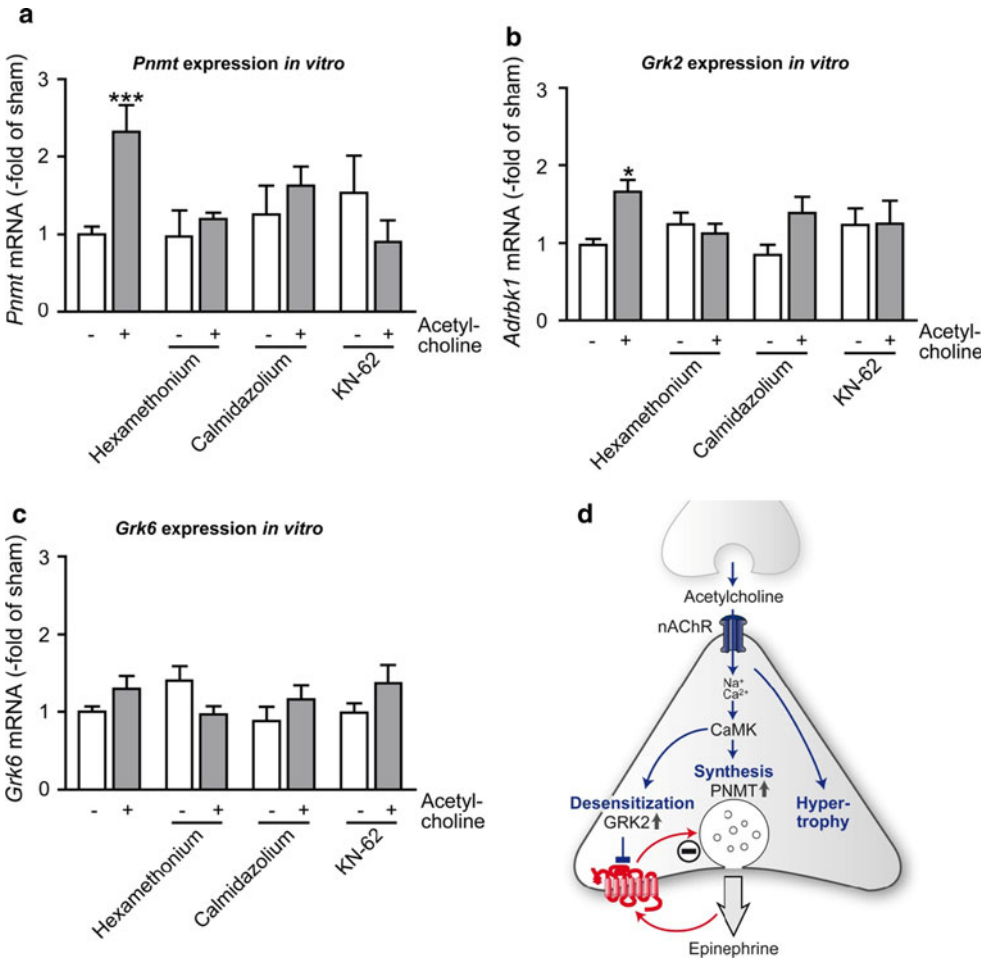

Fig. 6 Regulation of *Pnmt*, *Grk2* and *GRK6* expression in adrenal medulla in vitro. **a–c** *Pnmt*, *Grk2* and *Grk6* mRNA levels in adrenal medulla after 24-h incubation in vitro. Medulla specimens were stimulated with 200 µmol/L acetylcholine in the absence or presence of the nicotinic acetylcholine receptor antagonist hexamethonium (100 µmol/L), calmidazolium (1 µmol/L, calmodulin antagonist) or KN-62 (10 µmol/L, CaM kinase II inhibitor) ($n = 4$–6; $^{*}p < 0.05$, $^{***}p < 0.001$ acetylcholine vs. unstimulated). **d** Model of the effect of preganglionic cholinergic nerves on adrenal chromaffin structure and function after chronic cardiac pressure overload. For details, see "Discussion"

or increased catecholamine storage. Thus, in addition to α_{2C}-adrenoceptors, *Grk2* may have additional targets which contribute to adrenal hypertrophy and enhanced catecholamine secretion during heart failure. Indeed, a number of G protein-coupled receptors were found to be dysregulated in the adrenal medulla after cardiac pressure overload, and the transcriptome analysis performed in the present study may help to identify these additional pathways engaged by *Grk2* in chromaffin cell hypertrophy. The central role of *Grk2* was proposed in a recent study by selective ablation of *Grk2* expression in chromaffin cells by using a *Pnmt*-driven Cre recombinase mouse line [29]. Loss of *Grk2* in the adrenal medulla resulted in reduced adrenal gland weight; it prevented α_2-receptor downregulation and functional desensitization and caused improvement of circulating catecholamine levels and cardiac function after experimental myocardial infarction [29]. However, *Grk2* deletion did not completely prevent adrenal hypertrophy after infarction indicating that additional pathways beside *Grk2* may contribute to adrenal dysfunction in this situation [29].

In order to distinguish between circulating versus neuronal factors activating adrenal hypertrophy and catecholamine synthesis, adrenal glands were unilaterally denervated from the preganglionic cholinergic nerves. Previous studies in dogs have demonstrated that bilateral adrenal denervation greatly reduced cardiac remodeling and dysfunction after cardiac pressure overload [43]. In order to avoid potential problems with adrenal cortical function after bilateral adrenal denervation, the present study was not designed to further document the effects of adrenal medulla catecholamines on cardiac remodeling. However, we chose the unilateral adrenal denervation to determine the effect of cardiac pressure overload on one adrenal with intact sympathetic innervation and one adrenal with disrupted innervation in the same animal. Interestingly, unilateral adrenal denervation prevented adrenal hypertrophy, *Grk2* upregulation and increased expression of *Pnmt* and associated catecholamine storage.

Urine epinephrine levels indicate that the remaining innervated adrenal gland functionally replaces the denervation of one adrenal in sham-operated animals, but fails to elevate epinephrine levels further in response to cardiac pressure overload.

In vitro experiments confirmed the central role of acetylcholine released from preganglionic sympathetic nerves for the induction of *Pnmt* and *Grk2* via a nicotinic

acetylcholine—Ca^{2+}/calmodulin dependent pathway. The relevance of the *Pnmt* regulation is supported by experiments showing that epinephrine content of the adrenal gland is modulated by this pathway in vitro. The anticipated hypertrophic effect elicited by induction of *Grk2* expression was not observed after in vitro stimulation with acetylcholine. Maybe the onset of the hypertrophic effect is too slow to be detectable after 24 h, but at least in vivo only 1 week of cardiac pressure overload induced adrenal hypertrophy. This discrepancy may suggest the implication of additional hypertrophic pathways.

The main class of nicotinic receptors in chromaffin cells consists of $\alpha 3$- and $\beta 4$-subunits [37]. Their effect is to increase the permeability of sodium, potassium and calcium ions upon activation by acetylcholine. Expression of $\alpha 3\beta 4$-nicotinic receptors has been shown to increase after chronic stimulation with agonists [37]. In the present study, mRNA levels of the $\beta 4$-nicotinic receptor subunit in chromaffin cells were twofold elevated after TAC as compared with sham-operated mice. Thus, a positive feedback loop activated by acetylcholine may facilitate further intracellular signaling events in chromaffin cells.

Activation of nicotinic receptors is able to modulate the expression of genes within minutes [20]. The regulation of *Pnmt* expression by calcium has been shown in bovine chromaffin cell culture after stimulation with angiotensin II [38]. *Pnmt* expression was increased after treatment with calcium ionophores and could be inhibited by nifedipine. This suggested a dependency on calcium. Enhanced *Pnmt* expression by angiotensin II stimulation could be inhibited by calmidazolium. Calmidazolium was also used in our study and was able to inhibit enhanced *Pnmt* expression following acetylcholine stimulation. Our findings are consistent with several reports, demonstrating an involvement of nicotinic receptors in vivo and in vitro in *Pnmt* regulation [14, 44, 45]. Antagonists of nicotinic receptors prevented an increase of *Pnmt* expression in bovine chromaffin cell culture [14].

In conclusion, the present study demonstrates that activation of preganglionic sympathetic nerves innervating the adrenal medulla plays an essential role in induction of adrenal hypertrophy, enhanced catecholamine synthesis and induction of *Grk2* expression, which is thought to desensitize feedback control of catecholamine release in chromaffin cells. Future studies are required to unravel the precise molecular mechanisms of adrenal medulla hypertrophy and the interplay between nicotinic receptors, *Grk2* and α_{2C}-adrenoceptors in chromaffin cells. Uncovering the role of these target proteins in the adrenal medulla may not only lead to novel therapeutic strategies to halt progression of heart failure but also to prevent adverse adrenal effects of chronic stress.

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