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Heterozygous α_{2C}-adrenoceptor-deficient mice develop heart failure after transverse aortic constriction

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Abstract

Objective: Feedback regulation of norepinephrine release from sympathetic nerves is essential to control blood pressure, heart rate and contractility. Recent experiments in gene-targeted mice have suggested that α_{2C}-adrenoceptors may operate in a similar feedback mechanism to control the release of epinephrine from the adrenal medulla. As heterozygous polymorphisms in the human α_{2C}-adrenoceptor gene have been associated with cardiovascular disease including hypertension and chronic heart failure, we have sought to characterize the relevance of α_{2C}-gene copy number for feedback control of epinephrine release in gene-targeted mice.
Methods: Adrenal catecholamine release, basal hemodynamics and susceptibility to develop heart failure after transverse aortic constriction were tested in mice with two copies (+/+), one copy (+/−) or no functional α_{2C}-adrenoceptor gene (α_{2C}−/−).
Results: Heterozygous α_{2C}-receptor deletion (α_{2C}+/−) resulted in a 43% reduction of adrenal α_{2C} mRNA copy number and in a similar decrease in α_2-receptor-mediated inhibition of catecholamine release from isolated adrenal glands in vitro. Urinary excretion of epinephrine was increased by 74±15% in α_{2C}+/− and by 142±23% in α_{2C}−/− mice as compared with wild-type control mice. Telemetric determination of cardiovascular function revealed significant tachycardia but no hypertension in α_{2C}-adrenoceptor-deficient mice. α_{2C}+/− mice were more susceptible to develop cardiac hypertrophy, failure and mortality after left-ventricular pressure overload than α_{2C}+/+ mice.
Conclusion: Adrenal α_2-mediated feedback regulation of epinephrine secretion differs fundamentally from sympathetic feedback control. A single adrenoceptor subtype, α_{2C}, operates without a significant receptor reserve to prevent elevation of circulating epinephrine levels. This genetic model may provide an experimental basis to study the pathophysiology of α_{2C}-adrenoceptor dysfunction in humans.

Keywords: Neurotransmitters; Transgenic animal models; Hypertrophy; Autonomic nervous system; Adrenergic system

This article is referred to in the Editorial by Petrashevskaya and Liggett (pages 631–633) in this issue.

1. Introduction

The sympathetic nervous system is an essential regulator of cardiovascular function, including control of heart rate, contractility and vascular tone. Inhibition of sympathetic activity represents an important pharmacological strategy in the treatment of hypertension, arrhythmias, coronary heart disease and chronic heart failure. However, the complexity

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of adrenergic receptor subtypes which are involved in sympathetic signaling is not yet matched by an equal number of pharmacological agents which can be applied for human therapy. Part of the discrepancy between the number of adrenergic receptor subtypes and the lack of sufficiently subtype selective ligands may result from the fact that the physiological relevance of subtype diversity in the adrenergic system is only partly understood [1,2].

Molecular cloning has led to the identification of three α_2-adrenoceptor subtypes, α_{2A}, α_{2B}, and α_{2C} [3,4]. A fourth subtype, α_{2D}, has been suggested from pharmacological studies but later turned out to be the rodent orthologue of the human α_{2A}-adrenoceptor [3,4]. Transgenic mouse models with targeted deletions in individual α_2-adrenoceptor genes have been developed to assign physiological and pharmacological functions to specific α_2-receptor subtypes (for reviews, see Refs. [2,5–8]). From these mouse studies, it was concluded that all three α_2-receptor subtypes may serve as presynaptic feedback regulators of catecholamine release from sympathetic neurons with α_{2A} being the functionally predominant subtype [9,10]. We have previously shown that α_{2C}-adrenoceptors in the murine adrenal medulla may operate as inhibitory feedback regulators to control the release of epinephrine from chromaffin cells [11].

Genomic sequencing of human α_2-adrenoceptor genes has led to the identification of a large number of genetic variants in all three receptors (for overview, see Refs. [12–16]). In particular, association studies have suggested that a deletion of four amino acids in the third intracellular loop of the α_{2C}-adrenoceptor (α_{2C}-DEL322–325) may be involved in the development of chronic heart failure and hypertension [17–19]. Direct evidence for a functional role of α_{2C}-adrenoceptors in heart failure is still lacking. Thus, we have further investigated the relationship between α_{2C}-receptor density, adrenal epinephrine secretion and cardiovascular function in mice with targeted deletion of α_{2C}-adrenoceptors. As an extension to previous investigations, this study primarily focuses on mice lacking a single α_{2C}-adrenoceptor gene copy. The results demonstrate that α_{2C} controls adrenal catecholamine secretion in a gene dosage-dependent manner with little or no receptor reserve.

2. Materials and methods

2.1. Generation and genotyping of α_{2C}-adrenoceptor-deficient mice

The generation of mouse lines lacking α_2-adrenoceptor subtypes has been described in detail [20–22]. The targeted α_{2C}-adrenoceptor allele was crossed back onto a C57BL/6J background for >12 generations. Mice used in this study were generated from intercrosses of heterozygous congenic $\alpha_{2C}^{+/-}$ mice. Genotypes were confirmed by polymerase chain reactions (Fig. 1a) performed with genomic DNA isolated from tail biopsies as described in detail [23]. Mice were maintained in a specified pathogen-free facility. All animal procedures were approved by the responsible animal care committees of the Universities of Freiburg and Würzburg. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

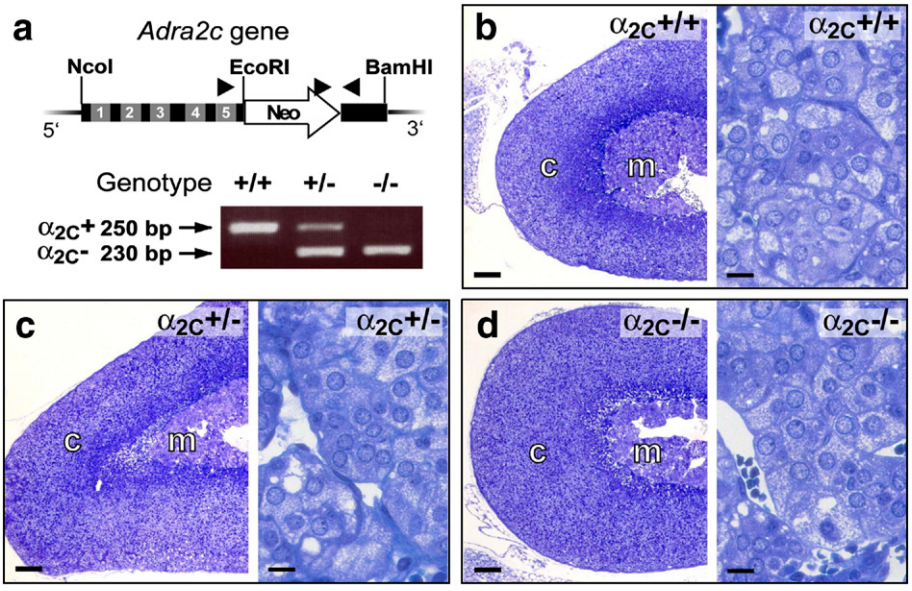

Fig. 1. Targeted deletion of the α_{2C}-adrenoceptor gene and adrenal histology in mice. (a) Schematic representation of the murine α_{2C}-receptor allele that was targeted for deletion by insertion of a neomycin cassette as described previously [22]. *Gray areas* indicate location of the sequences encoding the transmembrane regions of the α_{2C}-receptor (transmembrane domains six and seven were deleted by gene targeting [21]). *Arrowheads* show location of polymerase chain reaction primers used for genotyping. Lower panel: representative polymerase chain reactions to detect wild-type ($\alpha_{2C}^{+/+}$) and α_{2C}-deficient alleles of the α_{2C}-receptor gene. (b, c and d) Histological structure of the adrenal gland is not altered by deletion of the α_{2C}-adrenoceptor gene. Left panels: low magnification, bar 100 μm; c, cortex; m, medulla. Right panels: higher magnification of the adrenal medulla, bar 20 μm. 0.5 μm araldite sections stained with methylene blue.

2.2. Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qPCR) was carried out as previously described [24,25]. In brief, adrenal glands were rapidly removed from decapitated mice. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 μg per sample) was DNase treated and reverse transcribed according to the manufacturer's instructions (QuantiTect Rev. Transcription Kit, Qiagen). For qPCR 35 μl of amplification mixture (Qiagen, Quantitect SYBR Green Kit) was used containing 20 ng of reverse transcribed RNA and 300 nM primers (MWG, Ebersberg, Germany) (Table 1). Reactions were run in triplicates (10 μl) on a MX3000P detector (Stratagene, Amsterdam, Netherlands). The cycling conditions were: 15 min polymerase activation at 95 °C and 40 cycles at 95 °C for 15 s, at 58 °C for 30 s and at 72 °C for 30 s. Absolute copy numbers were determined using standard curves of corresponding linear DNA-fragments (7 points from 10^9–10^2 copies diluted in 10 μg/ml carrier RNA (Qiagen)). Results were normalized to β-actin values.

2.3. Catecholamine release from isolated adrenal glands

Adrenal glands were prepared from mice after cervical dislocation and placed into modified Krebs–Henseleit solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03, oxygenated with a mixture of 95% O_2 and 5% CO_2 in the presence of a monoamine oxidase inhibitor (pargyline, 100 μM) and a catechol-*O*-methyltransferase inhibitor (tolcapone, 1 μM). The adrenal medullae were isolated from the glands and then placed in superfusion chambers, one per chamber, and were continuously superfused with Krebs–Henseleit solution [26]. After a 90-min period of stabilization, successive 5-min samples of the superfusate were collected into tubes containing 0.3 ml of 2 M perchloric acid. At the end of the experiments, the adrenal medullae were placed in 1 ml of 0.2 M perchloric acid and catecholamines were determined in superfusates and tissues. The effect of the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) on catecholamine release was determined by addition of 500 μM of DMPP in a single 5-min period. The inhibitory effect of α_2-adrenoceptor activation on catecholamine release was determined by addition of 100 nM medetomidine 15 min before nicotinic stimulation. The spontaneous outflow of norepinephrine and epinephrine was calculated as a fraction of the norepinephrine or epinephrine content of the tissue at the onset of the respective collection period (fractional rate; min^{-1}). The overflow elicited by nicotinic stimulation was calculated as the difference 'total norepinephrine or epinephrine outflow during and after stimulation' minus 'basal outflow', and was then expressed as a percentage of the norepinephrine or epinephrine content of the tissue at the onset of stimulation [27].

Table 1
Sequences of primers used for quantitative real-time PCR

Gene	Primer [5′→3′]		Product size (bp)
β-actin	TCCATCATGAAGTGTGACGT	s	112
	GAGCAATGATCTTGATCTTCAT	as	
α_{2A}AR	CGAGGTTATGGGTTACTGGTACT	s	91
	GTCAAGGCTGATGGCGCACAG	as	
α_{2B}AR	GCAGAGGTCTCGGAGCTAA	s	130
	GCCTCTCCGACAGAAGATA	as	
α_{2C}AR	GTGCGGCCTCAACGATGA	s	140
	CGTTTCTCGCTGAGCGTACGCGT	as	
DBH	GAGAACGCAGATCTCATCAT	s	145
	ACAGGCCATCTCGAGTCCTCT	as	
TH	AAACCTACCAGCCGGTGTA	s	219
	GCTAATGGCACTCAGTGCTT	as	
PNMT	CCTARCTCCGCAACAACTAC	s	131
	TATCAATGAGAACCCGTCCC	as	

Abbreviations: s, sense primer; as, antisense primer; α_2AR, α_2 adrenoceptor; DBH, dopamine β-hydroxylase; TH, tyrosine hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase.

2.4. [^{3}H]norepinephrine release from isolated atria

Release experiments were carried out as previously described [10,30]. Mice were killed by cervical dislocation and left and right atria were removed and preincubated in 2 ml medium containing 0.1 mM [^{3}H]norepinephrine (Amersham, Freiburg, Germany) for 45 min at 37 °C. They were transferred to 12 superfusion chambers equipped with platinum electrodes, one atrium per chamber, where they were superfused at 37 °C at a rate of 1.2 ml × min^{-1}. After 45 min of superfusion successive 2-min superfusate samples were collected. The preincubation medium consisted of (mM): NaCl 118, KCl 4.8, $CaCl_2$ 0.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, Na_2EDTA 0.03. The superfusion medium was the same but contained 2.5 mM $CaCl_2$ and 1 mM desipramine. The medium was saturated with 5% CO_2 in O_2. Six periods of electrical stimulation (20 pulses/50 Hz, 1 ms pulse width, 80 mA) were applied at 16 min intervals. At the end of the experiments, tissues were solubilized and tritium was determined in superfusate samples and tissues. The electrically evoked overflow of total tritium reflects exocytotic release of [^{3}H] norepinephrine and is termed thus in this paper.

2.5. Catecholamine determination

The assay of the catecholamines norepinephrine and epinephrine in tissues, urine and superfusate samples was performed by high performance liquid chromatography with electrochemical detection as previously described [28]. The lower limit of detection of catecholamines ranged from 350 to 1000 fmol.

2.6. Transverse aortic constriction

Male mice (8–10 weeks old) were anesthetized with 2.0% isoflurane [vol/vol] in 45 ml/min oxygen flow. After

thoracotomy, a 7.0 nylon suture was placed around a 27 G hypodermic needle to constrict the aortic arch. The degree of aortic stenosis was assessed by morphometric analysis of paraffin sections of the aortic arch as described [19].

2.7. Hemodynamic measurements

For measurements in conscious, unrestrained mice, blood pressure and electrocardiograms were recorded by telemetry (DSI, Transoma Medical, USA, TA11-PAC10 for aortic pressure) 10–20 days after implantation of telemetry devices during day (7 a.m.–7 p.m.) and night (7 p.m.–7 a.m.). Mice which had been subjected to transverse aortic constriction were catheterized at the end of the respective observation period (Fig. 6). For left-ventricular catheterization with a 1.4 F pressure–volume catheter, mice were anesthetized with isoflurane (2 vol.% in O_2) and placed on a 37 °C table [19]. The microtip catheter was inserted into the right carotid artery and the pressure tip was advanced into the left ventricle [19].

2.8. Histology

After left-ventricular catheterization, hearts were fixed with 4% paraformaldehyde in phosphate-buffered saline, embedded in paraffin and stained with hematoxylin-eosin. Left-ventricular myocyte cross-sectional areas were analyzed by computer-assisted morphometry [19]. Adrenal glands were embedded in araldite and processed as described [29].

2.9. Statistical analysis

Hemodynamic and histological data as well as urinary catecholamine values were analyzed using ANOVA followed by Bonferroni post-hoc tests or Student *t* test, respectively. Survival curves were created by Kaplan–Meier plots and tested via log-rank test. A p value of less than 0.05 was considered as statistically significant. Results are displayed as means±SEM.

3. Results

3.1. Generation of α_{2C}-adrenoceptor-deficient mice

Mice with intact (α_{2C}+/+) or deleted α_{2C}-adrenoceptor genes (α_{2C}+/− and α_{2C}−/−) were born at the expected Mendelian ratios and did not show any signs of developmental or structural defects (Fig. 1a) (Ref. [22] and data not shown). The size and microscopical structure of the adrenal gland did not differ between genotypes (Fig. 1). In order to determine the expression of the three α_2-adrenoceptor subtypes and catecholamine synthesizing enzymes in the adrenal medulla, quantitative RT-PCR was performed (Fig. 2). In heterozygous α_{2C}+/− mice, α_{2C} mRNA copy numbers were reduced from 30±1 copies per 10^5 β-actin

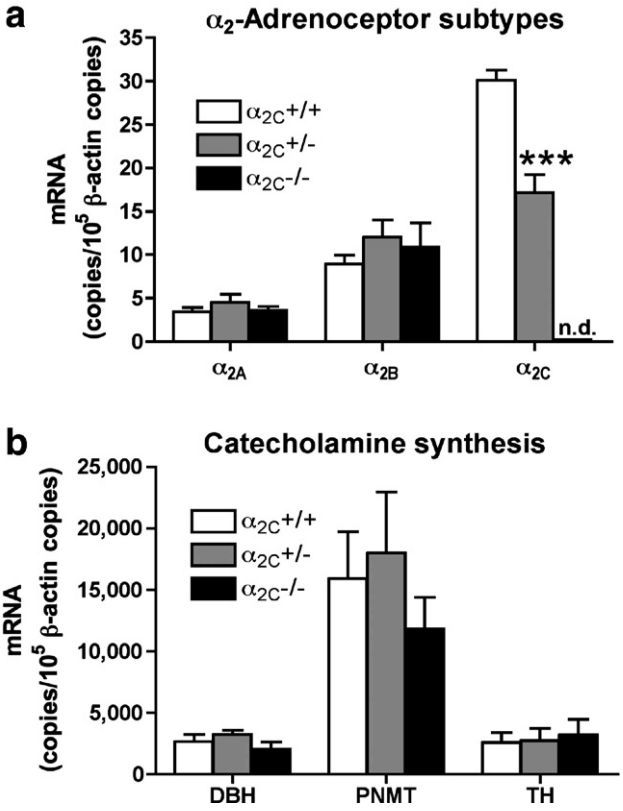

Fig. 2. Expression of α_2-adrenoceptor subtypes and catecholamine synthesis enzymes in the adrenal gland after targeted deletion of the α_{2C}-adrenoceptor gene in mice. (a) α_{2C} mRNA showed the highest expression of all three α_2-subtypes in adrenal glands from wild-type mice. Levels of α_{2C} mRNA were significantly reduced in α_{2C}+/− adrenal glands. (b) mRNA expression of catecholamine synthesizing enzymes in the adrenal gland. Abbreviations: DBH, dopamine β-hydroxylase; TH, tyrosine hydroxylase; PNMT phenylethanolamine *N*-methyltransferase. Means±SEM, $n=5$ per genotype. ***$p<0.001$ vs. α_{2C}+/+.

mRNA copies in wild-type specimens to 17±2 copies per 10^5 β-actin copies in α_{2C}+/− adrenal glands ($p<0.001$, $n=5$ per genotype). In specimens from α_{2C}−/− mice, no α_{2C} mRNA was detected (Fig. 2a). Expression levels of α_{2A}- or α_{2B}-adrenoceptor mRNA or catecholamine synthesizing enzymes were not significantly affected by deletion of the α_{2C}-adrenoceptor gene (Fig. 2a,b).

3.2. Regulation of adrenal catecholamine secretion by α_{2C}-adrenoceptors

Feedback regulation of adrenal catecholamine secretion was investigated in adrenal tissue slices incubated in vitro in superfusion chambers (Fig. 3). Basal release of catecholamines was significantly higher in adrenal glands from α_{2C}+/− and α_{2C}−/− mice than from wild-type control mice (Fig. 3a,b). Upon stimulation with the nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), release increased 2–3 folds without differences between genotypes (Fig. 3a,b). Activation of α_2-adrenoceptors by medetomidine reduced DMPP-stimulated release of epinephrine by 88±4%

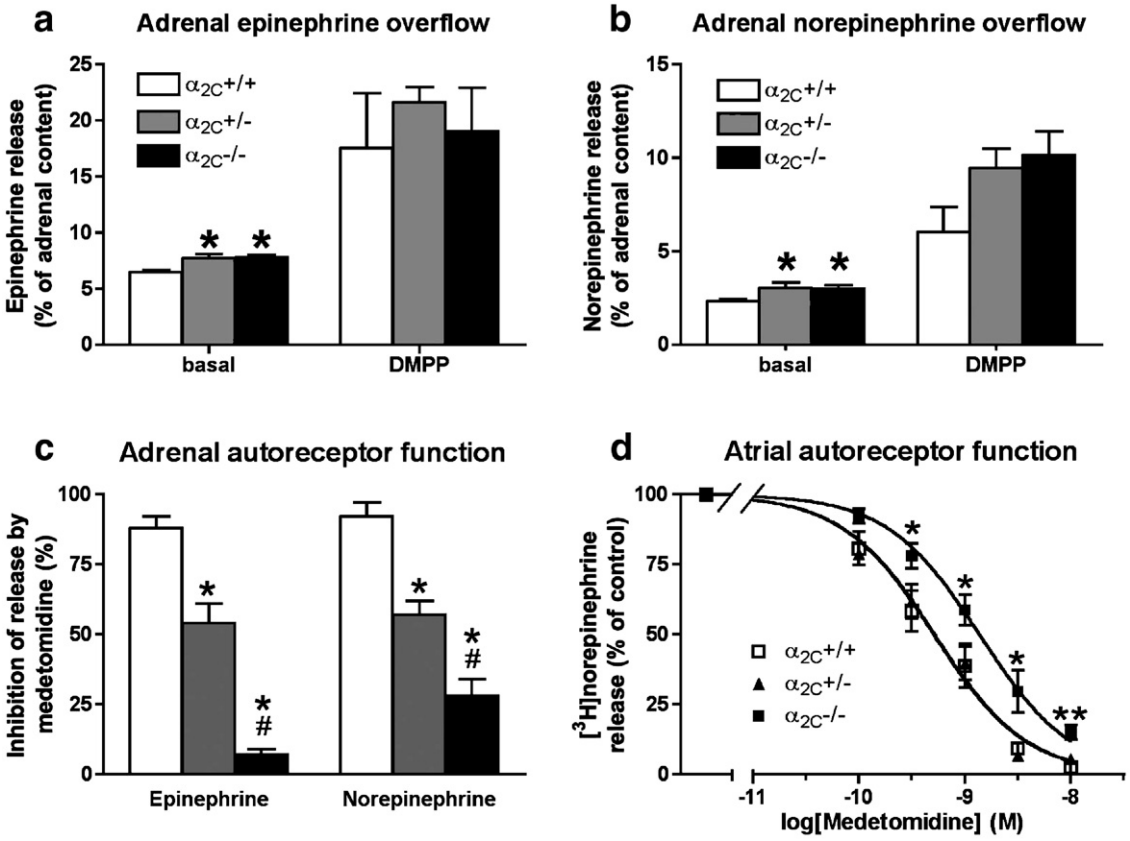

Fig. 3. Feedback control of catecholamine secretion from isolated adrenal glands and atria in vitro. Secretion of epinephrine (a) and norepinephrine (b) from slices of adrenal gland tissue was measured in superfusion chambers in vitro. Basal release was significantly higher in specimens from α_{2C}+/− and α_{2C}−/− mice. Catecholamine secretion stimulated by the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) did not differ between genotypes. (c) Inhibition of DMPP-stimulated catecholamine release by the α_2-adrenoceptor agonist, medetomidine (100 nM) from slices of adrenal glands. α_2-mediated inhibition was significantly blunted in α_{2C}+/− and α_{2C}−/− adrenal glands. Means±SEM, n=4 per genotype, *p<0.05 vs. α_{2C}+/+, #p<0.05 α_{2C}−/− vs. α_{2C}+/−. (d) Effect of medetomidine on electrically evoked overflow of [^{3}H]norepinephrine from atria. *p<0.05, **p<0.01 vs. α_{2C}+/+ means±SEM, n=12.

(n=4) and release of norepinephrine by 92±5% (n=4), respectively (Fig. 3c). In α_{2C}+/− adrenal glands, the inhibitory effect of medetomidine was significantly blunted to 54±7% (p<0.01, n=4) for epinephrine release and to 57±5% (p<0.05, n=4) for norepinephrine overflow, respectively. In α_{2C}−/− adrenal glands, α_2-mediated inhibition of epinephrine

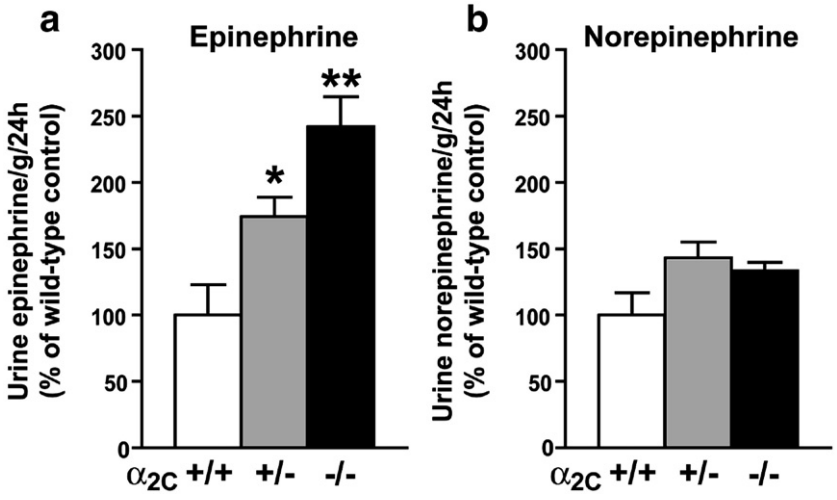

Fig. 4. Urine catecholamine levels in wild-type and α_{2C}-adrenoceptor-deficient mice. (a) 24 h epinephrine excretion was significantly increased in α_{2C}+/− and α_{2C}−/− mice as compared with wild-type littermates. (b) Norepinephrine excretion was not altered in α_{2C}+/− and α_{2C}−/− mice as compared with wild-type (α_{2C}+/+) mice. *p<0.05, **p<0.01, α_{2C}+/− vs. α_{2C}+/+, means±SEM, n=6–8 mice per genotype.

secretion was almost completely absent (7±2%, $p<0.001$, $n=4$). In contrast, 28±6% ($p<0.001$, $n=4$) of the stimulated norepinephrine release could not be inhibited in $\alpha_{2C}-/-$ adrenal glands. Taken together, these results demonstrate that step-wise reduction of α_{2C}-adrenoceptors is paralleled by a reduction of the feedback inhibition.

3.3. Atrial feedback inhibition by α_{2C}-adrenoceptors

To examine whether inactivation of one allele or two alleles of the α_{2C}-adrenoceptor gene affects feedback inhibition at peripheral sympathetic nerve terminals, the effect of medetomidine on electrically stimulated norepinephrine outflow from atria was determined. The α_2-agonist medetomidine inhibited norepinephrine release in a concentration-dependent manner in atria of all three genotypes (Fig. 3d). The resulting pK_D values from atria of wild-type and heterozygous α_{2C}-deficient mice did not differ significantly ($\alpha_{2C}+/+$ 9.3±0.1 vs. $\alpha_{2C}-/-$ 9.3±0.1, $n=12$). The concentration response curve for medetomidine was significantly ($p<0.001$ vs. $\alpha_{2C}+/+$ and $\alpha_{2C}+/-$) shifted to the right in atria from $\alpha_{2C}-/-$ mice ($\alpha_{2C}+/-$ 8.9±0.1, $p<0.001$ vs. $\alpha_{2C}+/+$, $n=12$). In contrast to the results obtained from adrenal glands these results indicate that inactivation of a single allele of the α_{2C}-receptor gene did not affect the potency of feedback inhibition from atria.

3.4. Renal catecholamine secretion

In order to assess the in vivo relevance of α_{2C}-adrenoceptors for adrenal catecholamine regulation, renal excretion of catecholamines was determined in 24 h urine samples (Fig. 4a,b). Consistent with the in vitro data, urine epinephrine levels were increased by 74±15% ($p<0.01$, $n=4$) in heterozygous and by 142±23% ($p<0.001$, $n=4$) in homozygous α_{2C}-deficient mice (Fig. 4a). Urine norepinephrine excretion was not significantly affected by deletion of the α_{2C}-adrenoceptor gene (Fig. 4b).

3.5. Cardiovascular consequences of α_{2C}-adrenoceptor deletion

As the long-term consequences of α_2-adrenoceptor deletion for resting blood pressure in mice are unknown, telemetric pressure transducers were inserted into the left carotid artery and implanted subcutaneously. Two weeks after surgery, blood pressure and heart rate were determined for five consecutive days in 2-min intervals (Fig. 5). In all genotypes, nighttime

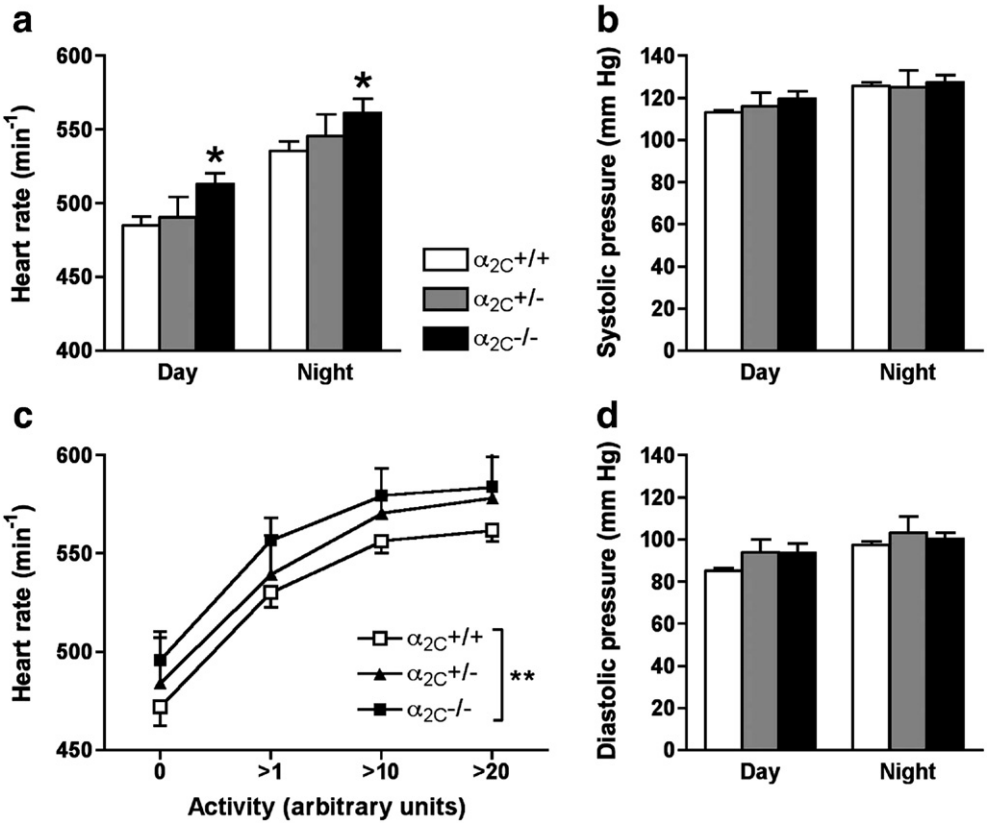

Fig. 5. Effect of α_{2C}-adrenoceptor deletion on hemodynamic parameters. Heart rate (a), systolic (b) and diastolic pressure (c) were assessed in mice 2 weeks after implantation of a telemetric pressure transducer. $\alpha_{2C}-/-$ mice showed significant tachycardia during the day and also at night (a). *$p<0.05$ vs. $\alpha_{2C}+/+$. (c) Heart rate increased significantly with locomotor activity. Genotype was significantly associated with increased heart rate at all activity levels. 2-way ANOVA, ***$p<0.01$. Means±SEM, $n=5$ mice per genotype.

systolic and diastolic blood pressure and heart rate were significantly higher than daytime values (Fig. 5a,b,d). At night, heart rate was 4.9% higher in α_{2C}−/− mice than in α_{2C}+/+ mice ($p<0.05$, $n=5$ mice per genotype) (Fig. 5a). When the effect of locomotor activity on heart rate was assessed, increased heart rate correlated significantly with the degree of activity. In addition, α_{2C} genotype significantly affected heart rate at rest and at all levels of locomotor activity (Fig. 5c).

As increased sympathetic activity is a well-known risk factor of adverse cardiovascular outcome, we tested whether mice with targeted deletion of the α_{2C}-adrenoceptor gene were more prone to develop heart failure after left-ventricular pressure overload as compared to wild-type mice. Cardiac pressure overload was induced by transverse aortic constriction (TAC) under anesthesia. Perioperative mortality did not differ between genotypes (α_{2C}+/+ 20%, α_{2C}+/− 17%, α_{2C}−/− 26%). The aortic banding operation resulted in similar degrees of aortic stenosis in WT, α_{2C}+/− and α_{2C}−/− animals (aortic stenosis lumen area: α_{2C}+/+ 17.0±2.5%, α_{2C}+/− 12.1±1.5%, α_{2C}−/− 14.3±2.7% of sham control, $n=5$–9 mice per genotype group). Postoperative survival, hemodynamic parameters and left-ventricular hypertrophy were followed for up to 7 months after aortic constriction (Fig. 6a). None of the α_{2C}+/+ mice died until the end of the observation period

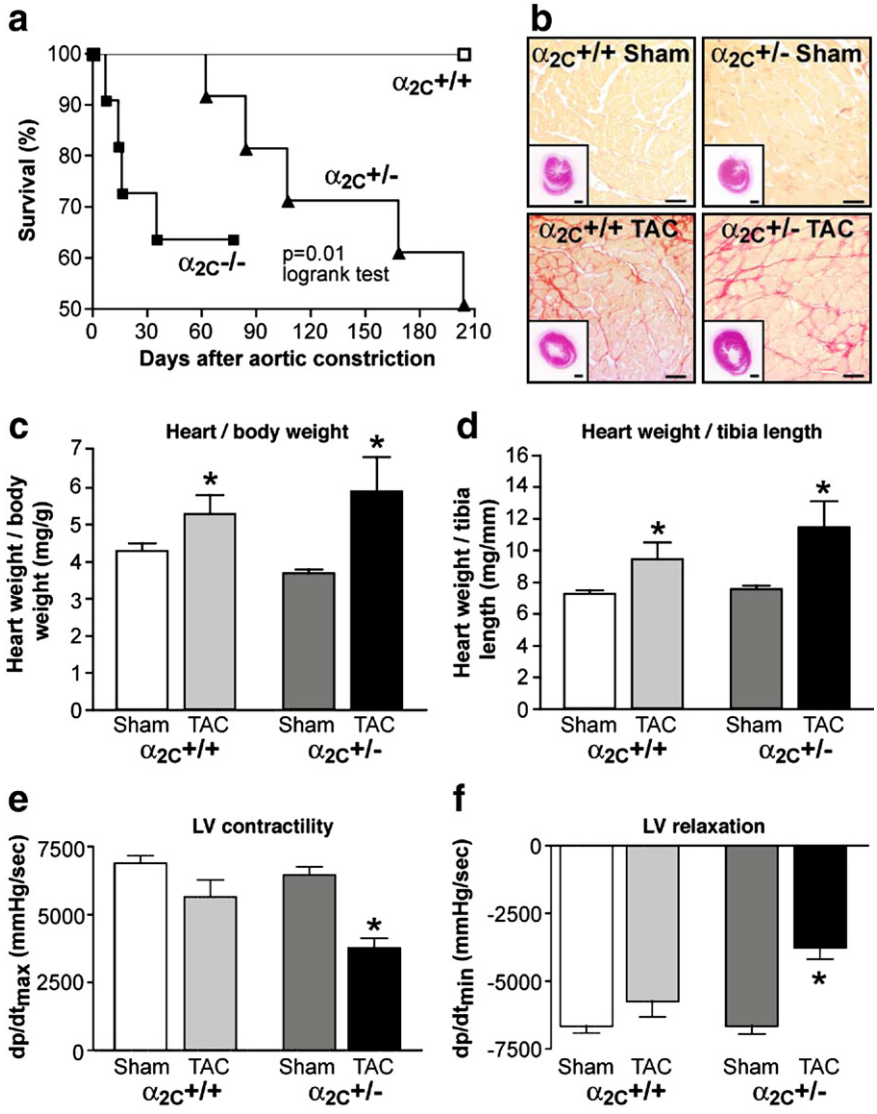

Fig. 6. Increased mortality of α_{2C}-adrenoceptor-deficient mice after transverse aortic constriction (TAC). (a) Survival of mice was followed for up to 7 months after TAC. Mortality was significantly increased in α_{2C}+/− mice and even more so in α_{2C}−/− mice (30% mortality: 32 days for α_{2C}−/− and 102 days for α_{2C}+/− mice). Kaplan–Meier plots, *$p<0.01$, log-rank test, $n=15$ mice per genotype. (b) Sirius red staining of midventricular sections from sham-operated or aortic-constricted (TAC) mice 7 months after the operation. Interstitial fibrosis after TAC was apparent by abundant red staining in α_{2C}+/+ and α_{2C}+/− mice (lower panels). Bar, 50 μm. Inserts: Hematoxylin-eosin stained midventricular cardiac sections. Bar, 1 mm. (c and d) Heart weight/body weight and heart weight/tibia length ratios were significantly increased in α_{2C}+/+ and α_{2C}+/− mice after TAC ($n=5$–9 mice per genotype, *$p<0.05$, TAC vs. sham). (e and f) Left-ventricular contractility (dp/dt_{max}) as well as left-ventricular relaxation (dp/dt_{min}) were reduced in α_{2C}+/− mice after TAC. All data are means±SEM, $n=5$–9 mice per genotype, *$p<0.05$, TAC vs. sham.

whereas survival was significantly reduced in α_{2C}+/− and even more in α_{2C}−/− mice after aortic banding (Fig. 6a). The survival curve of α_{2C}−/− mice after TAC did not differ significantly from our previous report [19]. None of the sham-operated control animals died postoperatively until the end of the observation period.

We have previously reported that increased mortality of α_{2C}−/− mice after TAC resulted from chronic heart failure [19]. Thus, in this study we further focused on the characterization of the α_{2C}+/− mice after aortic constriction (Fig. 6). In response to TAC, wild-type and α_{2C}+/− mice developed cardiac fibrosis and hypertrophy (Fig. 6b,c). Heart weight to body weight ratio increased by 59% in α_{2C}+/− mice after aortic constriction as opposed to 23% in α_{2C}+/+ animals (Fig. 6c). Similar increases were identified for heart weight to tibia length ratios after TAC (α_{2C}+/− +32% vs. α_{2C}+/+ +51%, Fig. 6d). Functional analysis with a microtip catheter revealed a decrease in left-ventricular contractility (dp/dt_{max}) by 40.6% in α_{2C}+/− mice after TAC but no significant change in α_{2C}+/+ mice (Fig. 6e). Similarly, left-ventricular relaxation (dp/dt_{min}) was reduced by 44% in α_{2C}+/− mice after TAC without a significant reduction in α_{2C}+/+ mice (Fig. 6f).

4. Discussion

The present study provides novel insight into the feedback regulation of catecholamine release from the adrenal medulla. Heterozygous deletion of the α_{2C}-adrenoceptor in mice reduced the efficacy of an α_2-agonist to inhibit adrenal catecholamine secretion by approximately 50%. As a consequence of partial disruption of adrenomedullary feedback inhibition, urine epinephrine excretion was elevated in heterozygous α_{2C}+/− mice and these mice were more susceptible to develop heart failure after transverse aortic constriction. Thus, in contrast to feedback control in sympathetic nerves, adrenal feedback regulation lacks a significant receptor reserve.

Previous studies in mice deficient in α_2-adrenoceptor subtypes have mostly focused on the role of the three α_2-receptor subtypes, α_{2A}, α_{2B}, and α_{2C}, in sympathetic and central adrenergic feedback inhibition. In vitro evidence suggested that all three α_2-adrenoceptor subtypes can inhibit release of norepinephrine from sympathetically innervated tissues [9,10]. In isolated mouse atria, α_{2A}-receptors were identified to serve as feedback regulators at high neuronal action potential frequencies whereas the α_{2C}-subtype inhibited norepinephrine release at lower levels of neuronal activity [10]. In contrast, in vivo α_{2A}- and α_{2C}-receptors were found to differentially control sympathetic and adrenomedullary catecholamine secretion, respectively [11].

In the present study, heterozygous deletion of α_{2C}-adrenoceptors resulted in diminished adrenomedullary feedback control of epinephrine release, indicating low or absent receptor reserve in the adrenal medulla. This finding indicates that adrenal feedback control differs fundamentally from sympathetic feedback control. In peripheral sympathetic or central adrenergic neurons, α_2-adrenergic receptors are expressed at very high levels, reaching 200–300 fmol/mg of membrane protein in the brain [30,31]. In this situation, activation of only a small percentage of receptors (<20%) elicits full inhibition of transmitter release [32,33]. The remaining 80% of receptors which are not necessary to achieve maximal inhibition of neurotransmitter secretion constitute a “receptor reserve” [32,33].

These observations are also consistent with results of experiments performed with gene-targeted mice with dysfunctional α_{2A}-receptors. Upon heterozygous deletion of the α_{2A}-adrenoceptor in mice, a significant phenotype became apparent only after activation of the receptor with a partial agonist [34]. Secondly, in a gene-targeted mouse expressing an α_{2A}-receptor variant (α_{2A}-D79N) at 20% of the density of the wild-type α_{2A}-receptor, presynaptic feedback inhibition in sympathetic nerves was not altered [20], suggesting that 20% of functional α_{2A}-receptors are required for presynaptic feedback control in adrenergic neurons.

In contrast, sequential deletion of α_{2C}-adrenoceptor genes resulted in a step-wise reduction in adrenal feedback control. Heterozygous deletion of α_{2C}-receptors caused a reduction of α_{2C}-mRNA copy numbers by 43% (Fig. 2) which was accompanied by a concomitant increase in whole body epinephrine spillover. Protein levels of α_2-adrenoceptors in the adrenal medulla were below the level of detection by standard radioligand binding (i.e. <10 fmol/mg membrane protein, data not shown). Thus, adrenal chromaffin cells contain α_2-adrenoceptors at a density which is one order of magnitude lower than receptor density in adrenergic neurons in the CNS (200–300 fmol/mg) [30]. These findings are consistent with a recent report in which the density of adrenal α_2-adrenoceptors was estimated to be 30±5 fmol/mg membrane protein [35]. Thus, in the adrenal medulla feedback regulation operates without “spare” or “reserve” α_2-receptors. The presence of a high number of spare receptors sensitizes receptor signal transduction such that lower concentrations of receptor agonists are sufficient to elicit full responses. Teleological, feedback control without spare receptors may be better suited to regulate the high concentrations of the adrenal hormone epinephrine directly at the site of its release.

An important question would be why only epinephrine and not norepinephrine levels were elevated in the urine of α_{2C}+/− and α_C−/− mice despite the fact that α_2-agonist can inhibit the release of both catecholamines from adrenal glands in vitro? The answer may lie in the different amounts of epinephrine and norepinephrine stored and released from the adrenal medulla. The quantity of norepinephrine stored and released from the adrenal medulla may not be sufficient to result in a detectable elevation of plasma or urine norepinephrine concentrations. In the mouse adrenal gland, norepinephrine represents 25% and epinephrine 75% of the total catecholamine content [36]. In isolated adrenal glands, basal or DMPP-stimulated release of epinephrine (in % of stored epinephrine) was 3-fold higher than norepinephrine release (see Fig. 3a,b). The total amounts of epinephrine (approximately 900 pmol/mg adrenal weight)

and norepinephrine (approximately 100 pmol/mg adrenal weight) released from the adrenal gland in vitro differ by a factor of 9–10. Thus, in the mouse in vivo adrenal norepinephrine may represent only a small percentage of plasma and urine norepinephrine with the majority being released from sympathetic nerves.

In chronic heart failure, catecholamine release from sympathetic nerve and adrenal medulla is activated [19,35]. Once cardiac dysfunction reaches a critical point, the degree of sympathetic activation correlates strongly with the severity of failure and overrides the effects of presynaptic inhibitory receptors. Thus, presynaptic/adrenal α_2-receptors may be more important to control catecholamines in a healthy situation rather than providing a brake during maximal activation of sympathetic tone during cardiac disease. Dysfunction of this feedback control may thus predispose patients to develop or accelerate cardiac hypertrophy and failure.

The findings of the present study may be relevant to evaluate the significance of polymorphisms in the human α_{2C}-adrenoceptor gene. Recently, a number of genetic polymorphisms have been identified in human adrenergic receptor genes [15,17,37–40]. While the pathophysiological mechanism of some of these genetic variants still remains unknown, several observations suggest that a deletion of four amino acids in the intracellular region which is involved in G-protein coupling of the α_{2C}-adrenoceptor (α_{2C}-Del322–325) may be of particular relevance [17]. When expressed at low densities in Chinese hamster ovary cells, the human α_{2C}-Del receptor was 90% deficient in coupling to several intracellular second messenger systems including inhibition of adenylyl cyclase [17]. However, as 8% of Caucasians and 60% of the African-American population carry this polymorphism, most people who have been identified to date are heterozygous carriers, i.e. they have one intact α_{2C}-receptor gene and one dysfunctional copy.

The α_{2C}-Del allele has been associated with elevated arterial blood pressure and exaggerated yohimbine-induced increases in blood pressure and heart rate in healthy adults [18]. In addition, two studies have demonstrated that the α_{2C}-Del allele was associated with the development of heart failure in African-Americans as well as in Caucasians [19,39]. In a retrospective analysis of patients with dilated cardiomyopathy, α_{2C}-Del genotype was associated with lower mortality [41]. However, a recent study in African-Americans carrying the α_{2C}-Del allele did not identify an association with increased blood pressure, heart rate or incidence of hypertension [42]. Until now, none of the clinical studies has included a haplotype analysis to search for a possible association between α_{2C}-adrenoceptor genotype and cardiovascular disease. Interestingly, Small et al. have identified 24 haplotypes of the human α_{2C}-adrenoceptor gene which resulted in significant expression differences between haplotypes in recombinant cell lines in vitro [38].

In the present study, diminished feedback control of adrenal epinephrine secretion resulted in tachycardia and increased susceptibility to develop heart failure after left-ventricular pressure overload. Patients with decompensated heart failure show increases in both plasma epinephrine and plasma norepinephrine, whereas patients with chronic stable heart failure usually have increases in norepinephrine only [43–46]. It has been suggested that increased epinephrine levels are related to catabolic metabolic balance and cachexia in patients with chronic heart failure [47].

The pathophysiological importance of adrenal feedback control has been emphasized by a recent report demonstrating an essential role of upregulation of the receptor kinase GRK2 in chromaffin cells in animal models of chronic heart failure [35]. However, the treatment of patients with chronic heart failure with the α_2-agonist moxonidine has unexpectedly resulted in increased mortality rates [48].

The present study demonstrates that feedback inhibition of adrenal catecholamines by α_{2C}-adrenoceptors operates without a detectable receptor reserve. Thus, alterations in α_{2C}-receptor density and/or coupling efficiency to intracellular G-protein signaling pathways may directly affect the release of epinephrine from the adrenal gland. These findings may help to identify the pathophysiological relevance of genetic variants of the human α_{2C}-adrenoceptor and devise new strategies for pharmacological control of diseases with increased adrenomedullary activity.

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