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RESEARCH PAPER

Knockout of β_1- and β_2-adrenoceptors attenuates pressure overload-induced cardiac hypertrophy and fibrosis

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Background and purpose: The role of β-adrenoceptors in heart disease remains controversial. Although β-blockers ameliorate the progression of heart disease, the mechanism remains undefined. We investigated the effect of β-adrenoceptors on cardiac hypertrophic growth using β_1- and β_2-adrenoreceptor knockout and wild-type (WT) mice.
Experimental approach: Mice were subjected to aortic banding or sham surgery, and their cardiac function was determined by echocardiography and micromanometry.
Key results: At 4 and 12 weeks after aortic banding, the left ventricle:body mass ratio was increased by 80–87% in wild-type mice, but only by 15% in knockouts, relative to sham-operated groups. Despite the blunted hypertrophic growth, ventricular function in knockouts was maintained. WT mice responded to pressure overload with up-regulation of gene expression of inflammatory cytokines and fibrogenic growth factors, and with severe cardiac fibrosis. All these effects were absent in the knockout animals.
Conclusion and implications: Our findings of a markedly attenuated cardiac hypertrophy and fibrosis following pressure overload in this knockout model emphasize that β-adrenoceptor signalling plays a central role in cardiac hypertrophy and maladaptation following pressure overload.
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Keywords: β-adrenoceptor; hypertrophy; fibrosis; catecholamines

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CTGF, connective tissue growth factor; IL, interleukin; MHC, myosin heavy chain; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; TAC, transverse aorta constriction; TGF-β, transforming growth factor-β

Introduction

Enhanced sympathetic nervous activity with elevated circulating levels of noradrenaline or noradrenaline spillover from diseased hearts correlates with the degree of haemodynamic abnormality and bears prognostic significance (Cohn *et al*., 1984; Kaye *et al*., 1995). While playing a pivotal role in regulating cardiac function, overt activation of β-adrenoceptors has also been implicated in the progression of heart disease. Chronic infusion of β-adrenoceptor agonists induces cardiac toxicity (Faulx *et al*., 2005). Likewise, increased neuronal noradrenaline release in mice deficient in presynaptic inhibitory α_2-adrenoceptors leads to cardiac hypertrophy and dysfunction (Hein *et al*., 1999; Brum *et al*., 2002). Furthermore, cardiac-restricted overexpression of β_1-, β_2-adrenoceptors, $G_s\alpha$ or protein kinase A, all lead to cardiomyopathy (Iwase *et al*., 1996; Engelhardt *et al*., 1999; Liggett *et al*., 2000; Antos *et al*., 2001).

There is strong evidence that enhanced sympathoadrenergic activity in the setting of pressure overload is detrimental. Following transverse aorta constriction (TAC), α_2-adrenoceptor knockout (KO) mice have an increased incidence of heart failure (Brede *et al*., 2002). Conversely, mice deprived of noradrenaline and adrenaline due to disruption of dopamine β-hydroxylase-KO respond to TAC with a blunted cardiac hypertrophy but a fully preserved function (Esposito *et al*., 2002). Although these findings suggest that endogenous

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catecholamines are important in the maladaptation to pressure overload, the subtypes of adrenoceptors responsible for this outcome remain unclear. In this regard, we previously showed that compared with wild-type (WT) controls, mice with cardiac-restricted overexpression of β_2-adrenoceptors developed a facilitated heart failure phenotype when subjected to TAC (Du *et al*., 2000).

In view of these findings, we hypothesized that β_1- and β_2-adrenoceptors mediate maladaptation in the setting of pressure overload. To address this question, we induced chronic TAC in mice lacking both β_1- and β_2-adrenoceptors ($\beta_{1/2}$-KO). For comparison, cardiac hypertrophy in response to chronic exposure to angiotensin-II was also examined. Our findings of a markedly attenuated cardiac hypertrophy in this model emphasize that β-adrenoceptor signalling plays a central role in cardiac maladaptation following pressure overload.

Materials and methods

Animals and surgery

All animal procedures were approved by the Institutional Ethics Committee and conformed to guidelines set out in the National Institute of Health Guide for the Care and Use of Laboratory Animals. $\beta_{1/2}$-KO mice were obtained from the Jackson Laboratory, USA (*Adrb1*^{tm1Bkk}*Adrb2*^{tm1Bkk}/J, stock no. 003810) and maintained by mating the double homozygote null mice. WT mice for the current studies were from the C57Bl/6J × DBA/2 background, as employed previously for comparison with $\beta_{1/2}$-KO mice (Chruscinski *et al*., 2001). As previously described (Du *et al*., 2000), we performed TAC or sham surgery on male mice at 4 months of age. At the time of surgery, the $\beta_{1/2}$-KOs had a lower body mass (23–33 g) than the WTs (30–39 g). Therefore, all WT animals had the aorta constricted to 0.5 mm o.d. using a probe, whereas a 0.4 mm o.d. probe was used in the majority of $\beta_{1/2}$-KOs.

For further comparison, TAC or sham operation was carried out in additional WT strains of mice (C57Bl/6J, 129sv and FVB/N) with genetic background relevant to that of $\beta_{1/2}$-KOs and then studied 12 weeks after surgery.

Echocardiography

Left ventricular (LV) function and dimensions were assessed by echocardiography in anaesthetized mice (300 mg kg^{-1} avertin, i.p.) using a Hewlett-Packard Sonos 5500 ultrasonograph with a 15 MHz linear-array probe, as described previously (Tan *et al*., 2003; Gao *et al*., 2005).

To confirm the absence of a functional β-adrenergic response, the effects of isoprenaline (4 μg kg^{-1}, i.p.) on fractional shortening and heart rate were assessed by echocardiography in non-operated WTs and $\beta_{1/2}$-KOs. Heart rate was also determined during echocardiography under the conscious state, a procedure known to subject mice to stress (Tan *et al*., 2003).

Haemodynamics by micromanometry and tail-cuff method

Haemodynamics was determined using a 1.4-Fr Millar catheter in close-chest anaesthetized mice, as previously described (Du *et al*., 2000; Gao *et al*., 2005). The catheter was positioned in the ascending aorta (proximal to the constrictive site) and the LV for pressure recordings. We initially studied mice at 12 weeks post-surgery anaesthetized with pentobarbitone (60 mg kg^{-1}) and atropine (1.2 mg kg^{-1}). In another batch of animals studied 4 weeks after surgery, micromanometry was conducted under avertin anaesthesia (400 mg kg^{-1}, i.p.) and the trans-stenotic pressure gradient was also determined by simultaneous pressure recording from both carotid arteries (Gao *et al*., 2005). For other strains of WT mice, catheterization was performed 12 weeks after surgery under pentobarbitone/atropine anesthesia.

To compare conscious systolic blood pressure (BP) between WT and $\beta_{1/2}$-KO mice, we adopted a tail-cuff method using a computerized BP monitor (model 31, IITC Inc., USA) and pulse amplifier. Mice were acclimatized to the recording conditions over several days preceding the assessment of systolic BP.

Autopsy and histology

Atria, ventricles and lungs were dissected, blotted and weighed. Body mass and tibia length were measured. A mid LV cross-sectional ring was fixed in 10% buffered formaldehyde, paraffin-embedded, cut into 3-μm sections and stained with Masson's trichrome. The area of collagen was determined, using Optimas 6.2 image analysis software, by averaging 20 representative views of each LV section under × 10 objective, as previously described (Gao *et al*., 2005).

Treatment with angiotensin-II or prazosin in vivo

In a separate batch of animals, a subpressor dose of angiotensin-II (0.3 mg kg^{-1}day^{-1}) (Pillai *et al*., 2006) was administered via s.c. implantation of ALZET osmotic pumps for a period of 2 weeks. On day 14, animals were studied under avertin anaesthesia (400 mg kg^{-1}) by micromanometry to determine BP. Heart and LV weights were determined.

To test the potential influence of activated α_1-adrenoceptor as a consequence of higher levels of circulating catecholamines in the $\beta_{1/2}$-KO model on observed changes in baseline gene expression, non-operated $\beta_{1/2}$-KO mice received daily injections for 5 days with the α_1-adrenoceptor antagonist prazosin (8 mg kg^{-1}day^{-1}, s.c.) or saline. The dose was chosen based on previous studies showing effective blockade of α_1-adrenoceptor-mediated changes in cardiac gene expression *in vivo* (Farivar *et al*., 1995; Jalil *et al*., 1999). At the end, LVs were harvested for assay of ANP expression by real-time PCR.

Gene expression

Total RNA was extracted from LVs using TRIzol (Sigma, St Louis, MI, USA), treated with DNAase and reverse-transcribed with the use of random primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Gene transcripts of atrial or brain natriuretic peptide (ANP, BNP), α- or β-myosin heavy chain (α-, β-MHC), α-skeletal actin, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), transforming growth factor-β (TGF-β), connective tissue growth factor

(CTGF), procollagen III, tumour necrosis factor α, interleukin-1β (IL-1β), IL-6 and monocyte chemotactic protein-1 were determined by real-time PCR using SYBR green master mix (Invitrogen) on an ABI PRISM 7500 sequence detection system, as described previously (Gao *et al*., 2005). Primers were designed using Vector NTI (Invitrogen) and purchased from Sigma. PCR efficiency of various genes was predetermined to be comparable and gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to 18S or glyceraldehyde-3-phosphate dehydrogenase. The level of change is expressed as fold of WT sham.

Catecholamine determination

Blood samples were collected from anaesthetized animals by cardiac puncture. Catecholamines were extracted from plasma using alumina adsorption and quantified by HPLC with electrochemical detection, as previously described (Kaye *et al*., 1995).

Statistics

Results are presented as means ± s.e.mean. Statistical analyses were performed with SigmaStat 2.03 using two-way ANOVA followed by Bonferroni *t*-test, or using unpaired Student's *t*-test. Analysis of covariance was employed to compare regression lines. Differences were considered statistically significant at $P<0.05$.

Results

Phenotypic differences between $\beta_{1/2}$-KO and WT mice

Baseline parameters of cardiac function were largely unaltered in the $\beta_{1/2}$-KOs; however, striking differences occurred between genotypes after isoprenaline administration ($4\,\mu g\,kg^{-1}$, i.p.) or with restraint stress, as required for conscious echocardiography (Rohrer *et al*., 1999; Tan *et al*., 2003). In contrast to the substantial increases in heart rate and fractional shortening of WT animals under these conditions, the responses in $\beta_{1/2}$-KO mice were largely abolished (Table 1). We have shown that the cardiac response to isoprenaline was effectively blocked by the β_1-adrenoceptor antagonist atenolol (Tan *et al*., 2003). Furthermore, $\beta_{1/2}$-KO mice were characterized by elevated plasma levels of noradrenaline and adrenaline as well as dihydroxyphenylglycol (DHPG), the intraneuronal metabolite of noradrenaline (Table 1).

Table 1 Phenotype of $\beta_{1/2}$-adrenoceptor knockout mice ($\beta_{1/2}$-KO)

	Wild type	*$\beta_{1/2}$-KO*
Response to restraint stress		
Number	26	28
Conscious heart rate (beats min^{-1})	648 ± 14	486 ± 10*
Response to isoprenaline		
Number	8	8
ΔFractional shortening (%)	+ 25.8 ± 6.0	+ 3.6 ± 2.2*
ΔHeart rate (beats min^{-1})	+ 65.1 ± 8.8	+ 18.5 ± 6.3*
Plasma catecholamines		
Number	6	6
Adrenaline (ng ml^{-1})	1.57 ± 0.46	4.25 ± 0.61*
Noradrenaline (ng ml^{-1})	0.80 ± 0.18	3.35 ± 0.34*
Dihydroxyphenylglycol (ng ml^{-1})	1.66 ± 0.05	2.68 ± 0.30*

*$P<0.05$ vs wild type.

Attenuated LV hypertrophy in $\beta_{1/2}$-KO mice

Prior to 12 weeks post-TAC, 3 out of 17 (18%) WTs died of heart failure with lung congestion, chest fluid accumulation and atrial thrombus. All $\beta_{1/2}$-KO mice with TAC ($n=19$) survived to week 12 without signs of heart failure. LV mass post-TAC was increased in WT mice, largely due to an increase in wall thickness (Table 2), whereas there was a markedly attenuated hypertrophic response in $\beta_{1/2}$-KO mice (Figures 1a and b). Right ventricular and atrial weights were also more markedly increased in WT compared with $\beta_{1/2}$-KO mice following TAC (Figures 1c and d). Similar results were obtained when weights were normalized using tibia length (data not shown). Although non-littermate WT mice were studied for comparison with $\beta_{1/2}$-KOs, which have a mixed background (Rohrer *et al*., 1999), the hypertrophic response of WT animals to TAC was as expected and it was the $\beta_{1/2}$-KOs that gave a unique response. In fact, subjecting mice of various strains and body mass to 12 weeks TAC confirmed a robust and uniform hypertrophic response (online data Supplementary Table).

In WT mice with TAC, LV dP/dt_{max}, dP/dt_{min} and fractional shortening were preserved but LVEDP increased significantly (Table 2). Notably, despite a blunted hypertrophic response, LV function in $\beta_{1/2}$-KOs with TAC was maintained (Table 2). Although $\beta_{1/2}$-KO sham-operated mice tended to have a lower contractility compared with WTs, dP/dt in $\beta_{1/2}$-KO mice with TAC was significantly higher than that in the sham-operated $\beta_{1/2}$-KOs (Table 2).

As expected, catheter-derived systolic aortic pressure (SAP) was higher in TAC mice vs their respective sham groups (Table 2). However, we found, initially in the 12-week batch using pentobarbitone/atropine, and later on in the 4-week batch using avertin, that pressure values were lower in $\beta_{1/2}$-KO mice of sham-operated and TAC groups compared with WT counterparts in which similar pressures were measured irrespective of the anaesthetics used (Table 2). This was in contrast to previous reports showing a similar mean BP in awake, unrestrained WT and $\beta_{1/2}$-KO mice (Rohrer *et al*., 1999). We confirmed this in non-operated mice by measuring conscious tail-cuff systolic BP (WT 118 ± 3 mm Hg, $n=8$, vs $\beta_{1/2}$-KO 109 ± 3 mm Hg, $n=6$ per group, $P>0.05$).

The pressure gradient across the band was also determined in mice at 4 weeks after surgery by dual catheterization, and found to be comparable between $\beta_{1/2}$-KO and WT animals with TAC (Figure 2b). As employed by other studies (Knowles *et al*., 2001; Esposito *et al*., 2002), we plotted LV/body mass ratio against SAP or pressure gradient. $\beta_{1/2}$-KOs clearly had a blunted hypertrophic response compared with WT counterparts (Figures 2a and c). Data from $\beta_{1/2}$-KO mice with 12-week TAC were not included in the LV/body mass vs SAP regression analysis considering the much underestimated SAP levels under pentobarbitone anaesthesia.

Table 2 Functional data of wild-type (WT) and $\beta_{1/2}$-adrenoceptor knockout mice ($\beta_{1/2}$-KO) with sham operation or transverse aorta constriction (TAC)

	WT		*$\beta_{1/2}$-KO*	
	Sham	*TAC*	*Sham*	*TAC*
4 weeks—haemodynamics (avertin)				
Number	5	5	5–6	14
Heart rate (beats min^{-1})	481 ± 4	399 ± 21*	506 ± 11	489 ± 10**
SAP (mm Hg)	103 ± 6	180 ± 5*	95 ± 7	148 ± 4*,**
LVSP (mm Hg)	103 ± 5	182 ± 8*	92 ± 4	150 ± 5*,**
LVEDP (mm Hg)	3.8 ± 0.4	8.3 ± 1.8*	3.1 ± 0.1	4.4 ± 0.6**
dP/dt_{max} (mm Hg s^{-1})	8287 ± 425	8076 ± 703	6244 ± 272**	7611 ± 312*
dP/dt_{min} (mm Hg s^{-1})	9593 ± 545	10 650 ± 899	6807 ± 163**	8214 ± 319*,**
12 weeks—haemodynamics (pentobarbitone)				
Number	6	8	6	13
Heart rate (beats min^{-1})	327 ± 7	335 ± 12	415 ± 6**	410 ± 15**
SAP (mm Hg)	107 ± 6	176 ± 8*	85 ± 6	120 ± 6*,**
LVSP (mm Hg)	107 ± 5	177 ± 9*	86 ± 6	125 ± 7*,**
LVEDP (mm Hg)	4.4 ± 0.5	7.2 ± 0.9*	2.7 ± 0.5	3.3 ± 0.3**
dP/dt_{max} (mm Hg s^{-1})	7182 ± 247	7439 ± 470	6073 ± 656	7083 ± 395
dP/dt_{min} (mm Hg s^{-1})	7039 ± 339	8713 ± 29*	5146 ± 500**	7088 ± 414*,**
12 weeks—echocardiography (avertin)				
Number	9	14	9	19
Heart rate (beats min^{-1})	458 ± 11	428 ± 11	427 ± 12	438 ± 10
LVDd (mm)	4.40 ± 0.11	4.62 ± 0.11	3.42 ± 0.05**	3.32 ± 0.06**
LVSd (mm)	2.82 ± 0.13	3.15 ± 0.11*	2.24 ± 0.07**	2.11 ± 0.06**
Wth (mm)	0.64 ± 0.03	1.09 ± 0.05*	0.63 ± 0.03	0.70 ± 0.02**
FS (%)	36 ± 2	32 ± 1	34 ± 2	37 ± 1**

Abbreviations: FS, fractional shortening; LVEDP, LV end diastolic pressure; LVSP, LV systolic pressure; LVDd or LVSd, LV diastolic or systolic dimension; SAP, systolic aortic pressure; Wth, mean wall thickness at diastole.
*$P<0.05$ vs respective sham group; **$P<0.05$ vs respective WT group.

Angiotensin-II-induced cardiac hypertrophy
At the dose (0.3 mg kg^{-1}day^{-1}) tested (Pillai *et al*., 2006), angiotensin-II stimulates cardiac hypertrophy independent of changes in systolic BP (saline vs angiotensin-II infusion: WT: 97 ± 5 vs 105 ± 5 mm Hg, $\beta_{1/2}$-KO: 103 ± 7 vs 104 ± 4 mm Hg, $n = 6$–7 per group). Our results show that the degree of LV hypertrophy in the $\beta_{1/2}$-KO mice tended to be blunted compared with WT animals (+18 ± 4 vs +29 ± 4% over respective vehicle-treated controls, $P = 0.096$; Figure 1e).

Altered hypertrophy-associated gene expression in $\beta_{1/2}$-KO mice
Expression levels of ANP, BNP, β-MHC and α-skeletal actin were increased by 6- to 22-fold in the LV of WT mice with TAC, whereas SERCA expression was reduced (Figures 3a–f). Notably, compared with WT shams, $\beta_{1/2}$-KOs at baseline had elevated mRNA levels of all genes tested except for a lower SERCA2a level (Figures 3a–f). Prazosin treatment for 5 days did not alter ANP expression level (6.7 ± 1.0-fold for the saline group vs 6.0 ± 0.6-fold for the prazosin group relative to saline-treated WTs, $n = 5$ each). Furthermore, in line with their blunted hypertrophic response, there were only modest or insignificant changes in the expression of these genes in $\beta_{1/2}$-KO mice with TAC relative to their sham controls (Figures 3a–f).

Mitogen-activated protein kinases are key players in mediating expression of hypertrophy-associated fetal genes. To test whether the activated fetal gene profile seen in the $\beta_{1/2}$-KO mice at baseline was related to activation of mitogen-activated protein kinases, we determined, by western blots, the level of phosphorylated and total extracellular signal-activated kinases, p38 mitogen-activated protein kinase and Jun N-terminal kinase of the LV from non-operated $\beta_{1/2}$-KO and WT mice. Our results showed that these three kinases were not activated in the $\beta_{1/2}$-KO LVs relative to WT values (online Supplementary Figure).

Attenuated LV fibrosis and inflammation in $\beta_{1/2}$-KO mouse hearts
Perivascular and interstitial fibrosis were evident in hypertrophied LV of WT mice, but was absent in $\beta_{1/2}$-KO mice with TAC (Figures 4a and b). This finding was corroborated by increased mRNA levels, relative to shams, of TGF-β, CTGF and procollagen III only in the LVs of WT mice with TAC (Figures 4c–e). Basal expression level of CTGF in the LV of $\beta_{1/2}$-KO mice was higher.

Expression of several pro-inflammatory cytokines, tumour necrosis factor α, IL-1β, IL-6 and monocyte chemotactic protein-1, was elevated post-TAC in WTs, but not in $\beta_{1/2}$-KO LVs (Figures 5a–d). There were no genotype-dependent differences in the basal expression of these genes.

Discussion

This study demonstrated that deletion of β_1- and β_2-adrenoceptors largely abolished chronic pressure overload-induced cardiac hypertrophy and fibrosis. The upregulated expression of inflammatory cytokines and fibrogenic growth

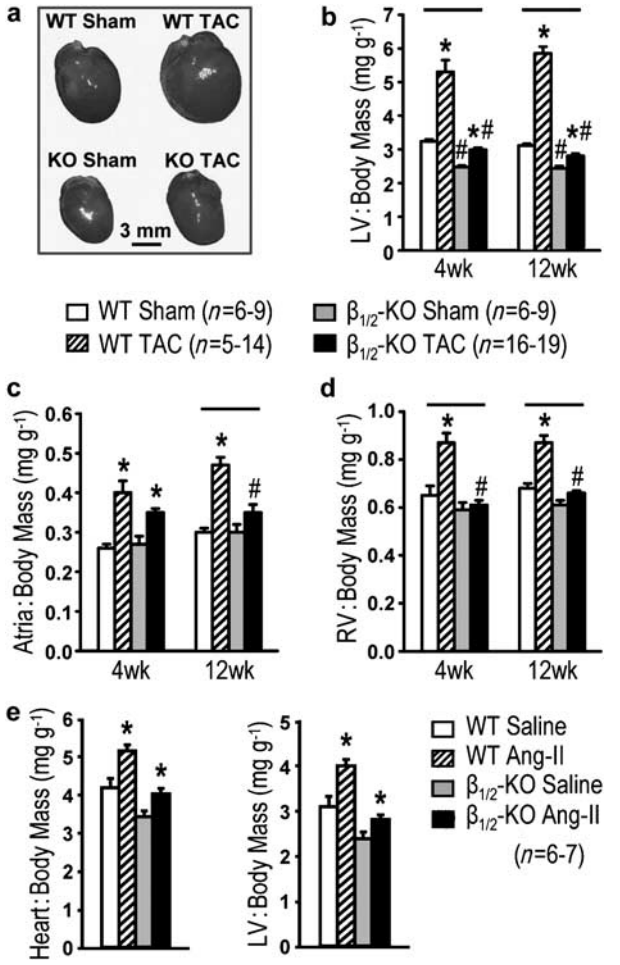

Figure 1 Photographs of hearts at 12 weeks post-surgery (**a**) and gravimetric data normalized for body mass showing marked increases in normalized left ventricle (LV), right ventricle (RV) and atrial weights in wild-type (WT) mice post-aortic constriction, whereas these increases were attenuated in $\beta_{1/2}$-knockout (KO) mice (**b**–**d**). Extent of cardiac hypertrophy in response to 2-week treatment with angiotensin-II (Ang-II) with results expressed as heart or LV mass over body mass ratio (**e**). $^{*}P<0.05$ vs respective sham/vehicle treated group; $^{\#}P<0.05$ vs respective WT group; horizontal line denotes significant interaction between genotype and response to TAC.

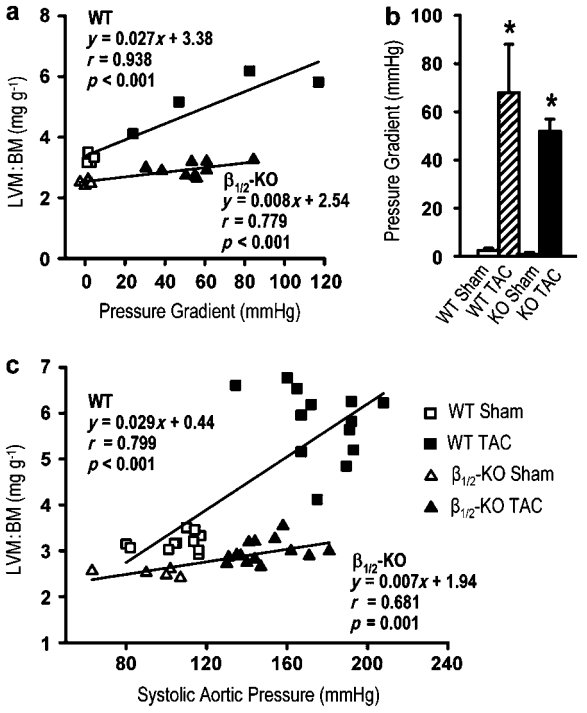

Figure 2 Correlations between extent of pressure overload and left ventricular (LV) hypertrophy expressed as gravimetric LV mass normalized for body mass (LVM:BM). Plots of LVM:BM against the trans-stenotic systolic pressure gradient in mice at 4 weeks post-surgery (**a**). Linear regression relations reveal a significantly steeper slope for wild-type (WT) compared with $\beta_{1/2}$-knockout (KO) mice ($P<0.005$). Bar graph summarizes the mean pressure gradients per group ($n=4$–10) (**b**). Plots of LVM:BM against systolic aortic pressures measured in $\beta_{1/2}$-KO mice at 4 weeks (with avertin anaesthesia) and in WT mice at 4 and 12 weeks (with avertin or pentobarbitone/atropine anaesthesia) post-surgery (**c**). A blunted hypertrophic response is evident by the reduced slope of the regression line for $\beta_{1/2}$-KO mice ($P<0.001$). $^{*}P<0.05$ vs respective sham group.

factors, seen in hypertrophic hearts of WT mice, was abolished in the $\beta_{1/2}$-KO hearts. All these changes occurred despite the fact that at the baseline, $\beta_{1/2}$-KO animals had over threefold increases in circulating levels of catecholamines and an activated fetal gene profile. These findings emphasize a primary role of β-adrenoceptors in the cardiac maladaptation to chronic pressure overload.

We observed that disruption of β_1- and β_2-adrenoceptors markedly attenuated hypertrophic growth following 4 and 12 weeks of TAC. Relations between the extent of LV hypertrophy and SAP or pressure gradient revealed a 3- to 4-fold smaller regression coefficient in $\beta_{1/2}$-KO than WT animals. A blunted hypertrophy response to TAC in the $\beta_{1/2}$-KO mice occurred in the setting of elevated circulating catecholamines, strongly suggesting a pivotal role of β-adrenoceptor in promoting the development of hypertrophy. This is in keeping with previous reports of an attenuated pressure-overload hypertrophy in mice depleted of noradrenaline and adrenaline (dopamine β-hydroxylase-KO) (Esposito *et al*., 2002), and more pronounced hypertrophy, fibrosis and heart failure deaths in the α_2-adrenoceptor-deficient mice with elevated circulating catecholamine levels due to absence of presynaptic inhibition of noradrenaline exocytosis (Brede *et al*., 2002). Despite a threefold increase in circulating levels of catecholamines, KO of the major β-adrenoceptors was able to markedly attenuate pressure overload-induced hypertrophy and fibrosis, clearly suggesting that it is β-, not α_1-adrenoceptors, which mediate cardiac maladaptation. Although our study was limited by the lack of data from single β-adrenoceptor KO models, there is strong evidence that the deleterious effects of cardiac β-adrenergic activation are mainly mediated by β_1-adrenoceptors (Lohse *et al*., 2003; Bernstein *et al*., 2005).

In the $\beta_{1/2}$-KO mice, a fetal gene profile coexists with absence of hypertrophy at baseline and a markedly attenuated pressure-overload hypertrophy. Actually, uncoupling of hypertrophy-related fetal gene profile and hypertrophic growth has been noticed in recent years by studies using genetically engineered mouse models (Hill *et al*., 2002; O'Connell *et al*., 2006), cultured cardiomyocytes with gene

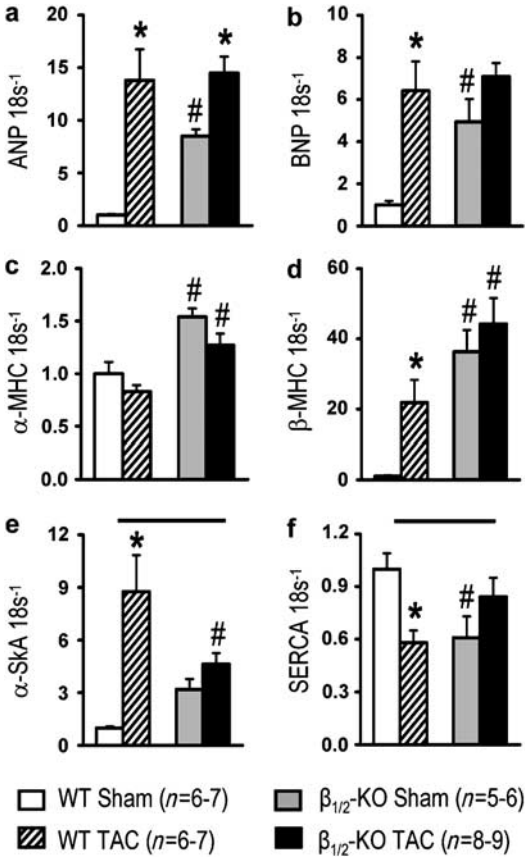

Figure 3 Gene expression of atrial natriuretic peptide (ANP) (**a**), brain natriuretic peptide (BNP) (**b**), α-myosin heavy chain (α-MHC) (**c**), β-myosin heavy chain (β-MHC) (**d**), α-skeletal actin (α-SkA) (**e**) and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (**f**) in the left ventricular (LV) myocardium of wild-type (WT) and $\beta_{1/2}$-knockout (KO) mice 12 weeks post-transverse aortic constriction (TAC). *$P<0.05$ vs respective sham group; $^{\#}P<0.05$ vs respective WT group; horizontal line denotes significant interaction between genotype and response to TAC.

targeting (Jeong *et al*., 2005) or drug interventions (van Eickels *et al*., 2001; Patrizio *et al*., 2007). Our study provides an additional set of conditions in which an activated fetal gene profile does not lead to hypertrophy and, upon chronic pressure overload, is associated with attenuated hypertrophy. Interestingly, despite that many of the hypertrophy-related markers are not modified from respective baseline levels, a mild but significant hypertrophic response is present in the $\beta_{1/2}$-KO mice after TAC. This is suggestive of alternative/redundant pathways mediating the development of cardiac hypertrophy under such conditions. Studies on genetically engineered mouse models targeting ANP and natriuretic peptide receptor-A convincingly show antihypertrophic properties of this pathway (Du, 2007). Several studies have reported an attenuated pressure-overload hypertrophy and fibrosis by disruption of natriuretic peptide receptor-A (Knowles *et al*., 2001), cardiac overexpression of pro-ANP gene (Franco *et al*., 2004) or stimulation of the cGMP-dependent protein kinase signalling pathway that is coupled with natriuretic peptide receptor-A (Takimoto *et al*., 2005). Thus, under as yet undefined conditions, the fetal gene profile is not necessary for hypertrophic growth and that elevated natriuretic peptides *per se* might activate endogenous inhibitory signalling counteracting hypertrophic growth.

The systolic pressures at the ascending aorta or LV were lower in $\beta_{1/2}$-KO than WT mice with sham surgery or TAC when anaesthetized with pentobarbitone or avertin. Thus, a lower degree of pressure overload in $\beta_{1/2}$-KO than WT mice might account for a less severe hypertrophy. However, this is unlikely for two reasons. First, our regression analyses showed that irrespective of the degree of pressure overload or pressure gradient across the banding site, hypertrophic growth in the $\beta_{1/2}$-KO mice was markedly attenuated. The pressure gradient measured from $\beta_{1/2}$-KO mice was comparable to that of other reports showing significant LV hypertrophy (Liao *et al*., 2004; Tsujimoto *et al*., 2005; Palazzesi *et al*., 2006). Second, the conscious BP was similar based on this and also previous reports on this model (Rohrer *et al*., 1999; Bernstein, 2002). Such a lower BP in the $\beta_{1/2}$-KO mice is most likely due to their aberrant haemodynamic response to the two injectable anaesthetics.

Pharmacological and genetic activation of β-adrenoceptor signalling induce myocardial fibrosis (Engelhardt *et al*., 1999; Liggett *et al*., 2000; Faulx *et al*., 2005). We reported that mice with cardiac β_2-adrenoceptor overexpression respond to TAC with a more severe interstitial fibrosis than that of WT littermates (Du *et al*., 2000). The absence of fibrosis in $\beta_{1/2}$-KOs in settings of pressure overload and elevated circulating catecholamines provides a strong evidence for β-adrenoceptor, rather than α_1-adrenoceptor, in mediating hypertrophic and fibrotic signalling under diseased conditions. Indeed, the upregulation of TGF-β, CTGF and procollagen III in the hypertrophic WT hearts was absent in $\beta_{1/2}$-KOs with TAC. An increased CTGF gene expression alone in $\beta_{1/2}$-KO mouse hearts is unable to stimulate collagen synthesis and there is evidence that cooperation of CTGF and other fibrogenic factors is necessary for induction of fibrosis (Lam *et al*., 2003; Chuva de Sousa Lopes *et al*., 2004).

There has been no study to examine, utilizing the β-adrenoceptor KO strains, the development of pressure-overload hypertrophy, except a recent one on the $\beta_{1/2}$-KO model (Palazzesi *et al*., 2006). In that study, no phenotypic difference between $\beta_{1/2}$-KO and littermate controls was identified either at baseline or following a 4-week period of TAC (Palazzesi *et al*., 2006). Considering the mixed background of the $\beta_{1/2}$-KO strain used in the present study (C57B6/129sv/DBA/FVB) (Rohrer *et al*., 1999), we also tested another three relevant strains of WT mice by inducing TAC. All these WT mice developed severe hypertrophic growth, as one would have expected (online Supplementary Table). Thus, the attenuated hypertrophy following pressure overload in the $\beta_{1/2}$-KO mice is a unique phenotype. We validated the genotype of the $\beta_{1/2}$-KO mice at both genomic DNA (β_2-adrenoceptor) and message RNA levels (β_1-adrenoceptor, data not shown), and documented blunted chronotropic and inotropic responses to isoprenaline or restraint stress, phenotypes consistent with previous reports (Rohrer *et al*., 1999; Bernstein, 2002; Esposito *et al*., 2002). Inhibition of pressure-overload hypertrophy in mice by some β-blockers has been reported (Marano *et al*., 2002; Liao *et al*., 2004; Patrizio *et al*., 2007). Our findings are very similar to that

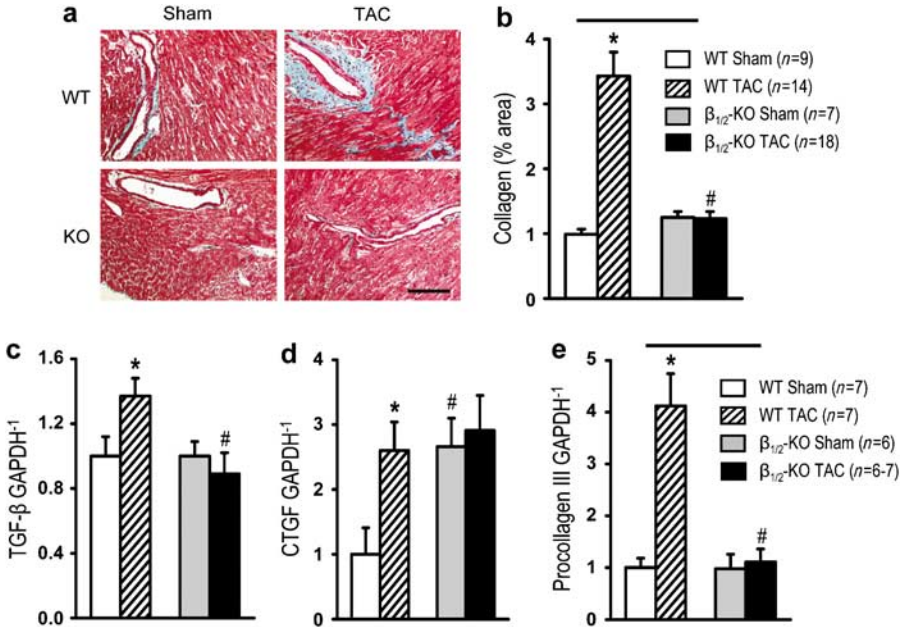

Figure 4 Representative left ventricular (LV) sections 12 weeks post-surgery stained with Masson's trichrome showing perivascular and interstitial fibrosis (blue staining) in wild-type (WT) hearts with transverse aortic constriction (TAC) for 12 weeks (**a**). Bar graph (**b**) summarizes group data. Gene expression of transforming growth factor-β (TGF-β) (**c**), connective tissue growth factor (CTGF) (**d**) and procollagen III (**e**) in the LV myocardium from WT and $\beta_{1/2}$-knockout (KO) mice 12 weeks post-TAC relative to sham-operated groups ($n=6$–7 per group). *$P<0.05$ vs respective sham group; $^{\#}P<0.05$ vs respective WT group; horizontal line denotes significant interaction between genotype and response to TAC. Bar = 300 μm.

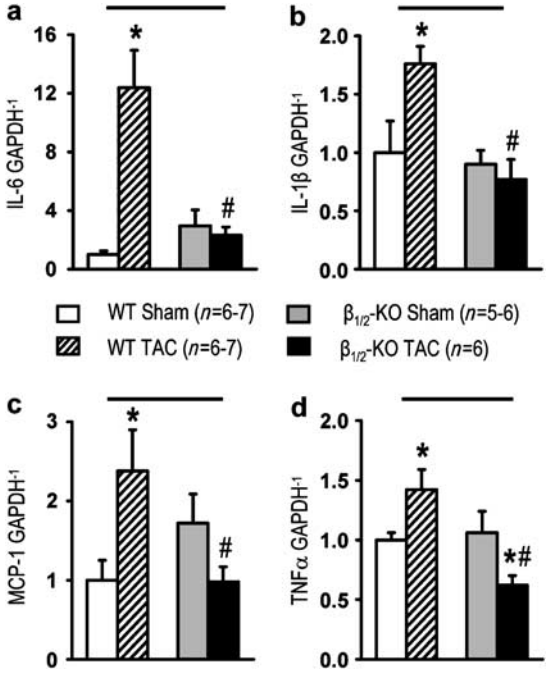

Figure 5 Interleukin-6 (IL-6) (**a**), IL-1β (**b**), monocyte chemotactic protein-1 (MCP-1) (**c**) and tumour necrosis factor α (TNFα) (**d**) gene transcripts were largely elevated in the wild-type (WT), but not in $\beta_{1/2}$-knockout (KO) mouse left ventricles (LVs) following 12 weeks transverse aortic constriction (TAC) with respect to the sham group. *$P<0.05$ vs respective sham group; $^{\#}P<0.05$ vs respective WT group; horizontal line denotes significant interaction between genotype and response to TAC.

previously reported in the dopamine β-hydroxylase-KO model (Esposito *et al*., 2002), and in line with the hypertrophic and fibrotic phenotypes seen in mice with cardiac overexpression of β_1-, β_2-adrenoceptor, $G_{s\alpha}$ or protein kinase A (Iwase *et al*., 1996; Engelhardt *et al*., 1999; Liggett *et al*., 2000). Recent research on hypertensive patients has revealed that the severity of LV hypertrophy is in proportion to the scale of sympathetic nervous activity but correlates weakly with the degree of hypertension (Burns *et al*., 2007).

Whereas the mechanism responsible for our findings is not entirely clear, we have observed that elevated expression of pro-inflammatory cytokines and fibrogenic growth factors in hypertrophied WT hearts was absent in $\beta_{1/2}$-KOs. Increasing evidence suggests a pro-inflammatory action of β-adrenoceptor activation. Whereas treatment of animals with isoprenaline stimulates expression of pro-inflammatory cytokines (Murray *et al*., 2000), β-blockade therapy in patients with dilated cardiomyopathy reversed the elevated circulating levels of cytokines (Ohtsuka *et al*., 2001). The upregulated expression of inflammatory cytokines in pressure-overloaded hearts is likely due to enhanced oxidative stress following β-adrenoceptor activation (Lohse *et al*., 2003; Remondino *et al*., 2003; Tsujimoto *et al*., 2005). Inflammatory cytokines are known to contribute to the development of cardiac hypertrophy, fibrosis and dysfunction (Mann, 2003; Prabhu, 2004; Tsujimoto *et al*., 2005). In addition, the elevated basal levels of ANP and BNP in the $\beta_{1/2}$-KO mouse hearts might act as endogenous antihypertrophic and antifibrotic factors, as discussed previously. Furthermore, absence of major β-adrenoceptors likely attenuates intracellular Ca^{2+} signalling and cascade mediated by calcineurin and calmodulin/calmodulin kinase pathways (Lohse *et al*., 2003).

We observed in the $\beta_{1/2}$-KO mouse heart, a persistent fetal gene profile and a smaller heart/body mass ratio relative to

several relevant WT strains. We cannot exclude the possibility that the hearts of $\beta_{1/2}$-KO mice remain in a 'premature' status due to loss of β-adrenoceptors. During embryonic stage, β-adrenoceptors are highly expressed in the heart and they function in response to circulating catecholamines (Slotkin *et al*., 1994). Earlier studies showed that blockade of β-adrenoceptors during gestation and/or early postpartum reduced the heart size (Kudlacz *et al*., 1990; Maltin *et al*., 1990); a change that was reversible after withdrawal of β-blockers. Such changes might persist into adulthood in the $\beta_{1/2}$-KO mice. By as yet undefined mechanisms, hypertrophic growth of the $\beta_{1/2}$-KO mice in response to chronic exposure to pressure overload or angiotensin-II is blunted. Interestingly, a smaller heart size was reported in mice with genetic disruption of α_{1A}- and α_{1B}-adrenoceptor genes (ABKO), indicating that α_1-adrenergic signalling is also important for cardiac development (O'Connell *et al*., 2006). However, adult ABKO mice differ from the $\beta_{1/2}$-KO mice in that ABKO mice respond to pressure overload with a similar degree of hypertrophy, relative to WT controls, but fetal gene expression was absent.

In conclusion, we have shown that disruption of both β_1- and β_2-adrenoceptors effectively blocked chronic pressure overload-induced hypertrophy and fibrosis with a preserved cardiac function. Thus, β-adrenoceptors play a central role in mediating hypertrophic and fibrotic signalling. Extrapolation of these findings to the clinical setting would imply a pro-hypertrophic action of sympathetic nervous activation in hypertensive patients, as indicated by recent clinical findings (Burns *et al*., 2007), and a direct antihypertrophic efficacy of β-blockade. To extend these findings, further studies are warranted to delineate the differential role of β_1- and β_2-adrenoceptors in the setting of pressure overload. Also, conditional cardiac-specific β-adrenoceptor KO models would be ideal for this purpose.

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Conflict of interest

The authors state no conflict of interest.

References

Antos CL, Frey N, Marx SO, Reiken S, Gaburjakova M, Richardson JA *et al.* (2001). Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase A. *Circ Res* **89**: 997–1004.

Bernstein D (2002). Cardiovascular and metabolic alterations in mice lacking β_1- and β_2-adrenergic receptors. *Trends Cardiovasc Med* **12**: 287–294.

Bernstein D, Fajardo G, Zhao M, Urashima T, Powers J, Berry G *et al.* (2005). Differential cardioprotective/cardiotoxic effects mediated by β-adrenergic receptor subtypes. *Am J Physiol Heart Circ Physiol* **289**: H2441–H2449.

Brede M, Wiesmann F, Jahns R, Hadamek K, Arnolt C, Neubauer S *et al.* (2002). Feedback inhibition of catecholamine release by two different α_2-adrenoceptor subtypes prevents progression of heart failure. *Circulation* **106**: 2491–2496.

Brum PC, Kosek J, Patterson A, Bernstein D, Kobilka B (2002). Abnormal cardiac function associated with sympathetic nervous system hyperactivity in mice. *Am J Physiol Heart Circ Physiol* **283**: H1838–H1845.

Burns J, Sivananthan MU, Ball SG, Mackintosh AF, Mary DA, Greenwood JP (2007). Relationship between central sympathetic drive and magnetic resonance imaging-determined left ventricular mass in essential hypertension. *Circulation* **115**: 1999–2005.

Chruscinski A, Brede ME, Meinel L, Lohse MJ, Kobilka BK, Hein L (2001). Differential distribution of β-adrenergic receptor subtypes in blood vessels of knockout mice lacking β_1- or β_2-adrenergic receptors. *Mol Pharmacol* **60**: 955–962.

Chuva de Sousa Lopes SM, Feijen A, Korving J, Korchynskyi O, Larsson J, Karlsson S *et al.* (2004). Connective tissue growth factor expression and Smad signaling during mouse heart development and myocardial infarction. *Dev Dyn* **231**: 542–550.

Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS *et al.* (1984). Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N Engl J Med* **311**: 819–823.

Du XJ (2007). Divergence of hypertrophic growth and fetal gene profile: the influence of β-blockers. *Br J Pharmacol* **152**: 169–171.

Du XJ, Autelitano DJ, Dilley RJ, Wang B, Dart AM, Woodcock EA (2000). β_2-Adrenergic receptor overexpression exacerbates development of heart failure after aortic stenosis. *Circulation* **101**: 71–77.

Engelhardt S, Hein L, Wiesmann F, Lohse MJ (1999). Progressive hypertrophy and heart failure in β_1-adrenergic receptor transgenic mice. *Proc Natl Acad Sci USA* **96**: 7059–7064.

Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ *et al.* (2002). Genetic alterations that inhibit *in vivo* pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circulation* **105**: 85–92.

Farivar RS, Crawford DC, Chobanian AV, Brecher P (1995). Effect of angiotensin II blockade on the fibroproliferative response to phenylephrine in the rat heart. *Hypertension* **25**: 809–813.

Faulx MD, Ernsberger P, Vatner D, Hoffman RD, Lewis W, Strachan R *et al.* (2005). Strain-dependent β-adrenergic receptor function influences myocardial responses to isoproterenol stimulation in mice. *Am J Physiol Heart Circ Physiol* **289**: H30–H36.

Franco V, Chen YF, Oparil S, Feng JA, Wang D, Hage F *et al.* (2004). Atrial natriuretic peptide dose-dependently inhibits pressure overload-induced cardiac remodeling. *Hypertension* **44**: 746–750.

Gao XM, Kiriazis H, Moore XL, Feng XH, Sheppard K, Dart A *et al.* (2005). Regression of pressure overload-induced left ventricular hypertrophy in mice. *Am J Physiol Heart Circ Physiol* **288**: H2702–H2707.

Hein L, Altman JD, Kobilka BK (1999). Two functionally distinct α_2-adrenergic receptors regulate sympathetic neurotransmission. *Nature* **402**: 181–184.

Hill JA, Rothermel B, Yoo KD, Cabuay B, Demetroulis E, Weiss RM *et al.* (2002). Targeted inhibition of calcineurin in pressure-overload cardiac hypertrophy. Preservation of systolic function. *J Biol Chem* **277**: 10251–10255.

Iwase M, Bishop SP, Uechi M, Vatner DE, Shannon RP, Kudej RK *et al.* (1996). Adverse effects of chronic endogenous sympathetic drive induced by cardiac $G_S\alpha$ overexpression. *Circ Res* **78**: 517–524.

Jalil A, Horiuchi M, Nomoto M, Kobayashi K, Saheki T (1999). Catecholamine metabolism inhibitors and receptor blockades only partially suppress cardiac hypertrophy of juvenile visceral steatosis mice with systemic carnitine deficiency. *Life Sci* **64**: 1137–1144.

Jeong MY, Kinugawa K, Vinson C, Long CS (2005). AFos dissociates cardiac myocyte hypertrophy and expression of the pathological gene program. *Circulation* **111**: 1645–1651.

Kaye DM, Lefkovits J, Jennings GL, Bergin P, Broughton A, Esler MD (1995). Adverse consequences of high sympathetic nervous activity in the failing human heart. *J Am Coll Cardiol* **26**: 1257–1263.

Knowles JW, Esposito G, Mao L, Hagaman JR, Fox JE, Smithies O *et al*. (2001). Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A-deficient mice. *J Clin Invest* **107**: 975–984.

Kudlacz EM, Navarro HA, Eylers JP, Slotkin TA (1990). Adrenergic modulation of cardiac development in the rat: effects of prenatal exposure to propranolol via continuous maternal infusion. *J Dev Physiol* **13**: 243–249.

Lam S, van der Geest RN, Verhagen NA, van Nieuwenhoven FA, Blom IE, Aten J (2003). Connective tissue growth factor and IGF-I are produced by human renal fibroblasts and cooperate in the induction of collagen production by high glucose. *Diabetes* **52**: 2975–2983.

Liao Y, Asakura M, Takashima S, Ogai A, Asano Y, Shintani Y *et al*. (2004). Celiprolol, a vasodilatory β-blocker, inhibits pressure overload-induced cardiac hypertrophy and prevents the transition to heart failure via nitric oxide-dependent mechanisms in mice. *Circulation* **110**: 692–699.

Liggett SB, Tepe NM, Lorenz JN, Canning AM, Jantz TD, Mitarai S *et al*. (2000). Early and delayed consequences of β_2-adrenergic receptor overexpression in mouse hearts: critical role for expression level. *Circulation* **101**: 1707–1714.

Lohse MJ, Engelhardt S, Eschenhagen T (2003). What is the role of β-adrenergic signaling in heart failure? *Circ Res* **93**: 896–906.

Maltin CA, Delday MI, Hay SM (1990). The effect of clenbuterol administration *in utero* and throughout lactation on pre- and post-natal muscle development in the rat. *Growth Dev Aging* **54**: 143–150.

Mann DL (2003). Stress-activated cytokines and the heart: from adaptation to maladaptation. *Annu Rev Physiol* **65**: 81–101.

Marano G, Palazzesi S, Fadda A, Vergari A, Ferrari AU (2002). Attenuation of aortic banding-induced cardiac hypertrophy by propranolol is independent of β-adrenoceptor blockade. *J Hypertens* **20**: 763–769.

Murray DR, Prabhu SD, Chandrasekar B (2000). Chronic β-adrenergic stimulation induces myocardial proinflammatory cytokine expression. *Circulation* **101**: 2338–2341.

O'Connell TD, Swigart PM, Rodrigo MC, Ishizaka S, Joho S, Turnbull L (2006). α_1-Adrenergic receptors prevent a maladaptive cardiac response to pressure overload. *J Clin Invest* **116**: 1005–1015.

Ohtsuka T, Hamada M, Hiasa G, Sasaki O, Suzuki M, Hara Y (2001). Effect of β-blockers on circulating levels of inflammatory and anti-inflammatory cytokines in patients with dilated cardiomyopathy. *J Am Coll Cardiol* **37**: 412–417.

Palazzesi S, Musumeci M, Catalano L, Patrizio M, Stati T, Michienzi S *et al*. (2006). Pressure overload causes cardiac hypertrophy in β_1-adrenergic and β_2-adrenergic receptor double knockout mice. *J Hypertens* **24**: 563–571.

Patrizio M, Musumeci M, Stati T, Fasanaro P, Palazzesi S, Catalano L *et al*. (2007). Propranolol causes a paradoxical enhancement of cardiomyocyte foetal gene response to hypertrophic stimuli. *Br J Pharmacol* **152**: 216–222.

Pillai JB, Gupta M, Rajamohan SB, Lang R, Raman J, Gupta MP (2006). Poly(ADP-ribose) polymerase-1-deficient mice are protected from angiotensin II-induced cardiac hypertrophy. *Am J Physiol* **291**: H1545–H1553.

Prabhu SD (2004). Cytokine-induced modulation of cardiac function. *Circ Res* **95**: 1140–1153.

Remondino A, Kwon SH, Communal C, Pimentel DR, Sawyer DB, Singh K *et al*. (2003). Adrenergic receptor-stimulated apoptosis in cardiac myocytes is mediated by reactive oxygen species/c-Jun NH_2-terminal kinase-dependent activation of the mitochondrial pathway. *Circ Res* **92**: 136–138.

Rohrer DK, Chruscinski A, Schauble EH, Bernstein D, Kobilka BK (1999). Cardiovascular and metabolic alterations in mice lacking both β_1- and β_2-adrenergic receptors. *J Biol Chem* **274**: 16701–16708.

Slotkin TA, Lau C, Seidler FJ (1994). Adrenergic receptor overexpression in the fetal rat: distribution, receptor subtypes, and coupling to adenylate cyclase activity via G-proteins. *Toxicol Appl Pharmacol* **129**: 223–234.

Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER *et al*. (2005). Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* **11**: 214–222.

Tan TP, Gao XM, Krawczyszyn M, Feng X, Kiriazis H, Dart AM *et al*. (2003). Assessment of cardiac function by echocardiography in conscious and anesthetized mice: importance of the autonomic nervous system and disease state. *J Cardiovasc Pharmacol* **42**: 182–190.

Tsujimoto I, Hikoso S, Yamaguchi O, Kashiwase K, Nakai A, Takeda T *et al*. (2005). The antioxidant edaravone attenuates pressure overload-induced left ventricular hypertrophy. *Hypertension* **45**: 921–926.

van Eickels M, Grohe C, Cleutjens JP, Janssen BJ, Wellens HJ, Doevendans PA (2001). 17β-Estradiol attenuates the development of pressure-overload hypertrophy. *Circulation* **104**: 1419–1423.

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