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RESEARCH PAPER

Chronic activation of the low affinity site of β_1-adrenoceptors stimulates haemodynamics but exacerbates pressure-overload cardiac remodelling

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BACKGROUND AND PURPOSE

The β_1-adrenoceptor has at least two binding sites, high and low affinity sites (β_{1H} and β_{1L}, respectively), which mediate cardiostimulation. While β_{1H}-adrenoceptor can be blocked by all clinically used β-blockers, β_{1L}-adrenoceptor is relatively resistant to blockade. Thus, chronic β_{1L}-adrenoceptor activation may mediate persistent cardiostimulation, despite the concurrent blockade of β_{1H}-adrenoceptors. Hence, it is important to determine the potential significance of β_{1L}-adrenoceptors *in vivo*, particularly in pathological situations.

EXPERIMENTAL APPROACH

C57Bl/6 male mice were used. Chronic (4 or 8 weeks) β_{1L}-adrenoceptor activation was achieved by treatment, via osmotic mini pumps, with (-)-CGP12177 (10 $mg \cdot kg^{-1} \cdot day^{-1}$). Cardiac function was assessed by echocardiography and micromanometry.

KEY RESULTS

(-)-CGP12177 treatment of healthy mice increased heart rate and left ventricular (LV) contractility. (-)-CGP12177 treatment of mice subjected to transverse aorta constriction (TAC), during weeks 4–8 or 4–12 after TAC, led to a positive inotropic effect and exacerbated fibrogenic signalling while cardiac hypertrophy tended to be more severe. (-)-CGP12177 treatment of mice with TAC also exacerbated the myocardial expression of hypertrophic, fibrogenic and inflammatory genes compared to untreated TAC mice. Washout of (-)-CGP12177 revealed a more pronounced cardiac dysfunction after 12 weeks of TAC.

CONCLUSIONS AND IMPLICATIONS

β_{1L}-adrenoceptor activation provides functional support to the heart, in both normal and pathological (pressure overload) situations. Sustained β_{1L}-adrenoceptor activation in the diseased heart exacerbates LV remodelling and therefore may promote disease progression from compensatory hypertrophy to heart failure.

Abbreviations

β_{1L} or β_{1H}, low or high affinity binding site of β_1-adrenoceptor; FS, fractional shortening; HF, heart failure; HR, heart rate; LV, left ventricle or left ventricular; TAC, transverse aorta constriction

Introduction

The β_1-adrenoceptor is activated by (-)-noradrenaline (Alexander *et al.*, 2011) and blocked by all clinically used β-blockers. Some β-blockers, typified by (-)-CGP12177 and (-)-pindolol, not only block the β_1-adrenoceptor, but also activate it at higher concentrations (~100-fold) than those required to block it (Kaumann and Molenaar, 2008). To account for these findings, it was hypothesized that β-blockers such as (-)-CGP12177 and (-)-pindolol bind to the β_1-adrenoceptor at two different sites, one that *blocks* (-)-noradrenaline from activating the receptor, the β_{1H} site, and another that *activates* the receptor, the β_{1L} site. The concept of two separate binding sites on the β_1-adrenoceptor has been strongly supported by data from experiments carried out on cell lines containing recombinant β_1-adrenoceptors (Pak and Fishman, 1996; Konkar *et al.*, 2000a,b; Baker *et al.*, 2003; Joseph *et al.*, 2003; 2004; Baker, 2005; Kaumann and Molenaar, 2008), cells and tissues including those from β_1-adrenoceptor knockout mice (Konkar *et al.*, 2000a; Kaumann *et al.*, 2001) and molecular modelling (Baker *et al.*, 2008).

The physiological effects of activation of β_{1L}-adrenoceptors are observed in the heart *ex vivo*, from humans (Kaumann, 1996; Joseph *et al.*, 2003; Sarsero *et al.*, 2003) and other species [reviewed (Kaumann and Molenaar, 2008)], including mice (Kaumann *et al.*, 1998; 2001) where (-)-pindolol and related indolamines, (-)-CGP12177 and other β-blockers such as oxprenolol and (-)-alprenolol, cause cardiostimulant effects (Kaumann and Molenaar, 2008).

Blockade of β_1-adrenoceptors is a fundamental requirement for all β-blockers currently used for the management of heart failure (HF) (Bristow, 2000; Bristow *et al.*, 2003; Molenaar and Parsonage, 2005). Clinically used β-blockers block or activate β_{1L}-adrenoceptors only at much higher concentrations than those that block β_{1H}-adrenoceptors (Kaumann and Molenaar, 2008). Chronic activation of the sympatho–β-adrenergic system causes progression of HF and hastens mortality (Cohn *et al.*, 1984; Esler *et al.*, 1997; Bristow, 2000). Long-term administration of β-blockers usually results in haemodynamic improvement and reduced morbidity and mortality (Waagstein *et al.*, 1975; 1993; Colucci *et al.*, 1996; Packer *et al.*, 1996; 2001; MERIT, 1999). However, despite the use of β-blockers, HF remains progressive and the long-term prognosis after a diagnosis of HF is poor.

The sympathomimetic effects of some β-blockers, mediated through β_{1L}-adrenoceptors, could conceivably reduce the benefit of blockade of β_{1H}-adrenoceptors, thereby reducing their efficacy as a treatment for HF. Bucindolol is such a β-blocker (Bundkirchen *et al.*, 2002); it was shown to lack efficacy in HF (BEST, 2001). Pindolol is also known to aggravate arrhythmias in patients with ischaemic heart disease (Podrid and Lown, 1982) and (-)-CGP12177 causes arrhythmic Ca^{2+} transients in murine ventricular myocytes (Freestone *et al.*, 1999). Cardiostimulation by compounds with similar properties could conceivably be harmful in HF. This idea is based entirely on the known effects of chronic activation of β_{1H}-adrenoceptors in the context of human HF, and the finding that stimulation of both β_{1H} and β_{1L}-adrenoceptors is associated with activation of the the Gsα-protein/cyclic AMP-PKA pathway (Kaumann and Lynham, 1997; Sarsero *et al.*, 2003; Kaumann and Molenaar, 2008). However, before this hypothesis can be properly formulated, the possibility that activation of β_{1L}-adrenoceptors causes the progression of HF needs to be investigated.

We sought to address this question *in vivo* by, firstly, determining the effect of acute and chronic treatment with different doses of (-)-CGP12177, and, secondly, examining whether chronic β_{1L}-adrenoceptor stimulation with (-)-CGP12177 in the presence of pressure overload, induced by transverse aorta constriction (TAC), alters the development of cardiac hypertrophy and dysfunction (Du *et al.*, 2000; Gao *et al.*, 2005; Kiriazis *et al.*, 2008). Our results demonstrate that activation of β_{1L}-adrenoceptors has a chronic stimulating effect on cardiac function and that sustained β_{1L}-adrenoceptor activation in the heart exacerbates left ventricular (LV) remodelling and dysfunction.

Methods

Animals

All animal procedures were approved by a local Animal Ethics Committee and were in accordance with guidelines set out in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Ed). We consulted the ARRIVE and *British Journal of Pharmacology* guidelines for *in vivo* animal studies (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). C57Bl/6 male mice aged 10–12 weeks were used (n=153). A strain of mice with β_3-adrenoceptor knockout (β_3ARKO) and of FVB/n genetic background was used at 10–12 weeks of age (n=23). Animals were housed at two to four per cage, had free access to food and water and were maintained on a 12:12 h light-dark cycle. Analgesia (carprofen, 5 $mg \cdot kg^{-1}$) was administered at surgery. In mice that were anaesthetized for invasive procedures, adequate depth of surgical anaesthesia was verified with the absence of pedal reflex.

Echocardiography

Serial echocardiographic tests were performed in anaesthetized mice, using ketamine/xylazine/atropine (80/10/1.2 mg·kg^{-1}, i.p.) or isoflurane (~2%), and a Philips iE33 echocardiography system with a L15-7io compact linear-array transducer. After two-dimensional LV short-axis images had bee obtained, M-mode traces were acquired for measurement of LV wall thickness, LV chamber dimensions at diastole and systole, echocardiography-derived LV mass, fractional shortening (FS) and heart rate (HR), as described previously (Kiriazis *et al.*, 2008).

Micromanometry

The relationship between acute doses of (-)-CGP12177 administered and effects on haemodynamic variables was determined. Mice were anaesthetized with ketamine/xylazine/atropine (80/10/1.2 mg·kg^{-1}, i.p.), and a 1.4 Fr Millar Mikro-tip catheter was positioned in the ascending aorta via the right carotid artery, and then advanced into the LV, as previously described (Du *et al.*, 2000; Gao *et al.*, 2005). (-)-CGP12177 was infused i.v. as a bolus (0.1 ml per mouse) from 0.001 to 10 mg·kg^{-1}. As the recovery from the effects of (-)-CGP12177 is very slow (Zakrzeska *et al.*, 2005), each dose of the drug was administered in separate animals.

Haemodynamic variables were also measured at the end of Experiment-1 and -2 (see below). Mice were anaesthetized with isoflurane (4% for induction and ~2% for maintenance). Steady state of aortic systolic pressure (SAP) and diastolic pressure, pulse pressure, HR, LV systolic pressure (LVSP) and end-diastolic pressure were measured. Indices of LV contractility and relaxation (dP/dt_{max} and dP/dt_{min}, respectively), contractility index (dP/dt_{max}/instantaneous pressure) and tau (relaxation index) were calculated using Chart5 software (ADInstruments, Bella Vista, NSW, Australia).

(-)-CGP12177 and osmotic mini pump implantation

(-)-CGP12177 was custom made by Sigma-Aldrich (Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India) at a purity of 98.2% (FW=315.8). To test the chronic effects of (-)-CGP12177 *in vivo*, mice anaesthetized with ketamine/xylazine/atropine (100/20/1.2 mg·kg^{-1}, i.p.) were implanted with an Alzet osmotic mini pump (model 2004, DURECT Corporation) containing (-)-CGP12177, dissolved in purified H_2O to the required concentration.

TAC and animal monitoring

The surgical procedures to induce TAC have been described previously (Du *et al.*, 2000; Gao *et al.*, 2005). In brief, animals were anaesthetized with a mixture of ketamine/xylazine/atropine (100/20/1.2 mg·kg^{-1}, i.p.). Animals were then intubated and ventilated and a midline incision was made at the upper sternum and the aorta was dissected between the right innominate and the left carotid arteries. The aorta was then constricted by approximately 70% to a lumen size of 0.4 mm. Sham-operated mice were subjected to the same surgery except for the banding of the aorta. The mortality during surgery and within the first 24 h after the surgery was about 5%. Each animal was closely monitored post surgery. If an animal was found to be unwell, routine treatment was applied following a Standard Operation Procedure, implemented by our local animal ethics committee, which included procedures such as keeping the animal warm and providing easy access to food and water. If the animal did not respond to treatment by 48 h, or showed signs of deterioration, it was killed humanely and this was counted as 'premature death'. The occurrence of HF was determined by subsequent autopsy findings.

Autopsy and histology

Following micromanometry, the chest cavity was inspected for evidence of pleural effusion, a blood sample was collected via cardiac puncture and atria, ventricles and lungs were dissected, blotted and weighed. Body mass (excluding mini pump) was recorded. The LV was frozen for chemical assay and a mid LV cross-sectional ring was fixed in neutral buffered formaldehyde (pH = 7), paraffin-embedded, cut into 5 μm sections and stained with Masson's trichrome or picrosirius red for analyses using Image-Pro Plus software (Media Cybernetics, Inc., Rockvill, MD, USA). Interstitial fibrosis was quantified in 8–10 representative views per LV section with results expressed as a % of the LV cross-sectional area. Cardiomyocyte diameter was determined from 10–12 randomly selected fields per LV section with the average from 70–100 cells used, as previously described (Gao *et al.*, 2005). Analyses were performed in a blinded fashion.

Gene expression

Total RNA was extracted from LV tissues and gene transcripts of atrial or brain natriuretic peptide, α- or β-myosin heavy chain, α-skeletal actin, α-smooth muscle actin, β_1-adrenoceptor, sarcoplasmic reticulum Ca^{2+}-ATPase, transforming growth factor-β, connective tissue growth factor, procollagen I, procollagen III, MMP2, IL-1β, IL-6, monocyte chemotactic protein-1, and NADPH oxidase 2 were determined, in duplicates, by quantitative real-time PCR as described previously (Gao *et al.*, 2005; Xu *et al.*, 2011). Gene expression levels were normalised to glyceraldehyde-3-phosphate dehydrogenase. The level of change was expressed as fold of sham-operated control.

Plasma renin

Plasma was assayed in duplicates for renin activity via the ProSearch International Australia Pty Ltd using radioimmunoassay.

Radioligand binding

LV myocardium was frozen in liquid nitrogen and stored at –70°C until used. Ventricular myocardium was homogenized in ice-cold Tris/Mg^{2+} assay buffer (in mM: Tris HCl 50, EGTA 5, EDTA 1, $MgCl_2$ 4, ascorbic acid 1, phenylmethylsulphonyl fluoride 0.5, and pH = 7.4), centrifuged at 50 000× *g* and the pellet resuspended in assay buffer. A single point binding experiment at high-affinity β-adrenoceptor binding sites (the site through which (-)-CGP12177 antagonizes the effects of catecholamines) was carried out with 2 nM (-)-[^{3}H]-CGP12177 (specific activity ~40 Ci·mmol^{-1}) with non-specific binding determined with 500 nM (-)-propranolol (Sarsero *et al.*, 1998; 1999). Low-affinity binding sites (the site through which (-)-CGP12177 elicits cardiostimulant effects) were

determined with 20 nM (-)-[^{3}H]-CGP12177 in the presence of 500 nM (-)-propranolol with non-specific binding determined with 20 μM (-)-CGP12177 (Sarsero *et al.*, 1998; 1999). Assays were carried out at 37°C for 120 min. The fraction of high and low affinity binding sites occupied was determined using the mass action equation and equilibrium dissociation constants of (-)-[^{3}H]-CGP12177 determined previously in mouse ventricle at high affinity (0.63 nM) and low affinity (105 nM) binding sites (Kaumann *et al.*, 2001). This was then used to determine the maximal density of binding sites, standardized against protein content. Protein content was determined using the Bradford reagent (Sigma-Aldrich).

Protocol of (-)-CGP12177 treatment of mice with TAC

C57Bl/6 mice underwent TAC or sham surgery. Four weeks post-surgery, echocardiography was performed under ketamine/xylazine/atropine anaesthesia. To reduce the risk of animal loss in the latter stages of TAC during cardiac functional assessment, isoflurane (4% induction, ~2% maintenance) was used for the rest of functional examinations by echocardiography and micromanometry. Just before the week-4 echocardiography test, half of the TAC mice were randomly allocated to the (-)-CGP12177 treatment group (TAC+CGP). Immediately after echocardiography, these TAC mice underwent surgery to implant mini pumps containing (-)-CGP12177 (10 mg·kg^{-1}·day^{-1}), as described above. The other half of the TAC mice were allocated to the untreated group (TAC). Drug treatment lasted either for 4 weeks (Experiment-1) or 8 weeks (Experiment-2), the latter group were implanted with a second, fresh mini pump, which replaced the initial mini pump. Final echocardiographic tests were conducted 4 weeks (i.e. week 8 after TAC, Experiment-1) or 8 weeks post mini pump implantation (i.e. week 12 after TAC, Experiment-2). In Experiment-1, micromanometry was conducted within 2 days after the last echocardiographic test but in the presence of (-)-CGP12177. In order to evaluate intrinsic cardiac function in the absence of (-)-CGP12177, in Experiment-2, mini pumps were removed following the last (12 weeks) echocardiography. Echocardiography was then repeated 3 days later, and micromanometry was performed on the following day. Figure 1 depicts the protocols used for these experiments.

Statistics

Results are presented as mean ± SEM, unless indicated otherwise. Statistical analyses were performed with SigmaStat v3.5 and GraphPad Prism 5 software, using ANOVA followed by Student-Neuman-Keuls *post hoc* test, or using Student's unpaired *t* test. Incidence was compared between groups by use of the χ^2 test. Differences were considered statistically significant at $P < 0.05$.

Results

Cardiac effects of chronic infusion of (-)-CGP12177 in healthy mice

We tested the cardiac effects of an infusion of (-)-CGP12177, via osmotic mini pumps, at six different doses (0.01, 0.1, 1, 10, 30 and 100 mg·kg^{-1}·day^{-1}) for a period of 4 weeks. Echocardiographic tests were performed 2 and 4 weeks after the start of the treatment. The (-)-CGP12177 treated groups showed significant increases in HR and FS compared to the vehicle control group, at both 2 and 4 weeks (Figure 2A). Such effects were dose-dependent within the range of 0.01 to 10 mg·kg^{-1}·day^{-1}, with no further increases in the cardiac parameters measured with doses of 30 and 100 mg·kg^{-1}·day^{-1} compared to that of the 10 mg·kg^{-1}·day^{-1} group. To determine whether chronic infusions of (-)-CGP12177 at doses of 1–100 mg·kg^{-1}·day^{-1} caused persistent alterations in cardiac function, osmotic mini pumps were removed and 3 days later, echocardiography was repeated. HR and FS values had returned towards control levels (Figure 2A). Chronic infusion of (-)-CGP12177 had no effect on LV or whole heart mass (data not shown). Based on these results, a dose of 10 mg·kg^{-1}·day^{-1} was selected for further studies on mice with TAC.

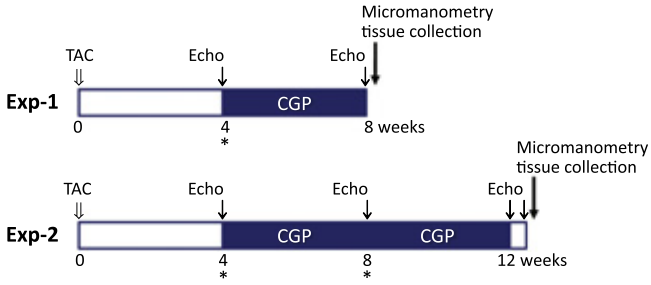

Figure 1

Experimental protocols used in this study on mice with transverse aorta constriction (TAC) for a period of either 8 weeks (Exp-1, Experiment-1) or 12 weeks (Exp-2, Experiment-2). Echo, echocardiography; *CGP*, (-)-CGP12177 delivered by osmotic mini pump (* indicates mini pump implantation surgery).

Anaesthetics, particularly a mixture of ketamine/zylazine, are known to reduce sympathetic nervous activity (Tan *et al.*, 2003), and this might mask the β_{1H}-adrenoceptor blocking action of (-)-CGP12177. Hence, to demonstrate the effects of (-)-CGP12177 on β_{1H}-adrenoceptors, we determined its effects on the HR of conscious mice that were stressed by being restrained (hand-held in a supine position for approximately 20 s); HR was assessed using echocardiography. We have previously shown that this procedure is associated with significant sympathetic activation due to the stress associated with being restrained (Tan *et al.*, 2003). HR was assessed before (baseline) and at 5, 30 and 70 min after i.p. injection of (-)-CGP12177 0.001 mg·kg^{-1}. After administration of (-)-CGP12177, the HR was 17% below the baseline at 5 min, and although it slightly recovered it was still lower at 70 min (Figure 3A); this appeared to be due to blockade of β_{1H}-adrenoceptors by (-)-CGP12177.

We further evaluated the acute dose-effect relationship of (-)-CGP12177 on haemodynamic measures in anaesthetized animals. (-)-CGP12177 induced a dose-dependent increase in HR and contractility, measured as dP/dt_{max}, dP/dt_{min} and contractility index (Figure 3B). The contractile response reached a plateau after approximately 0.1 mg·kg^{-1} of (-)-CGP12177 (i.v.).

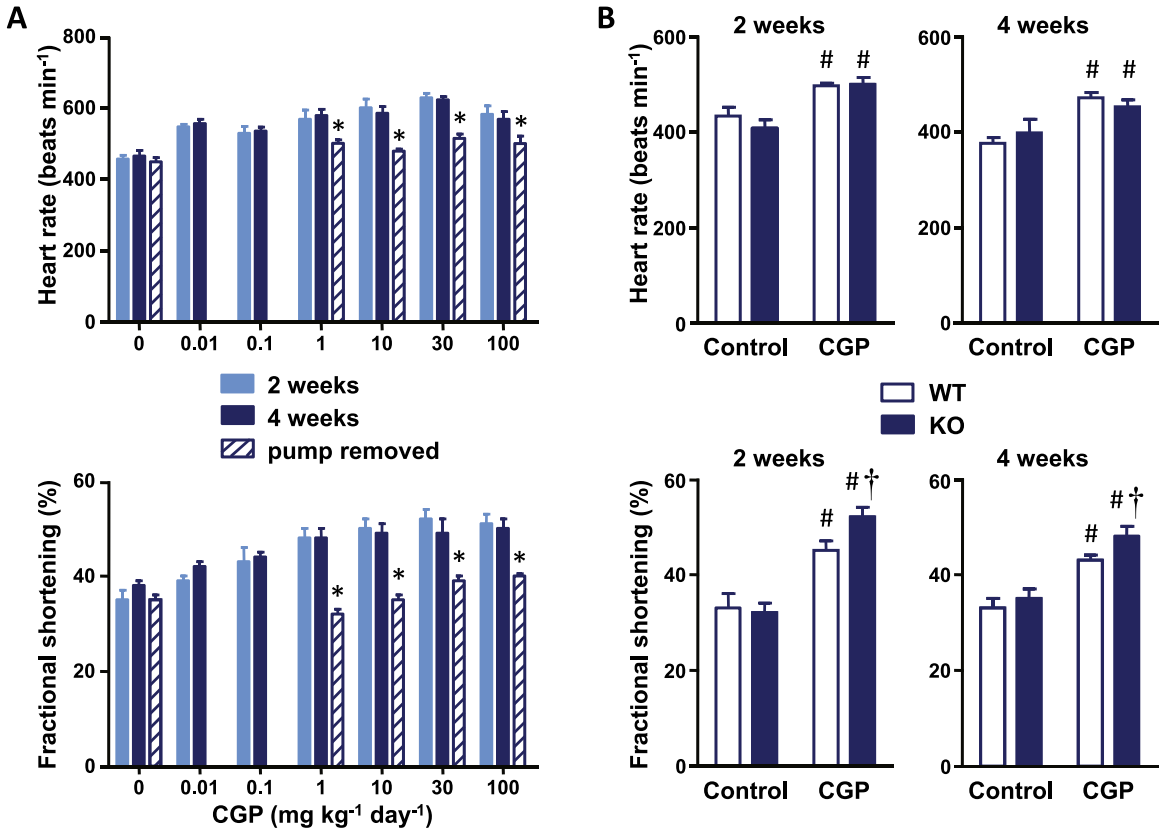

Figure 2

Cardiac effect of chronic infusion (2 and 4 weeks) of (-)-CGP12177 (CGP) in C57Bl/6 mice (A, n = 4–8 per group), and in β_3-adrenoceptor knockout (KO) or wild-type control FVB/n mice (B, n = 5–6 per group). (A) There was a statistically significant effect of drug dose on both heart rate and fractional shortening ($P < 0.05$, two-way ANOVA). Removal of the osmotic mini pump containing CGP abolished the increase in heart rate and fractional shortening (*$P < 0.05$ vs. 4-week, two-way ANOVA); (B) CGP had a stimulating effect on heart rate and fractional shortening in both control and KO mice. #$P < 0.05$ versus control with same genotype; †$P < 0.05$ versus WT with same treatment (one-way ANOVA).

Effect of (-)-CGP12177 on cardiac gene expression and β-adrenoceptor density

The levels of expression of 17 genes were examined in the LV of mice, untreated or treated with (-)-CGP12177 at 10 mg·kg^{-1}·day^{-1} for 4 weeks. There were no significant differences between the treated and untreated mice in any of the genes examined (data not shown). However, chronic infusion of (-)-CGP12177 at 10 and 30 mg·kg^{-1}·day^{-1}, but not 1 mg·kg^{-1}·day^{-1}, for 4 weeks caused a reduction in (-)-[^{3}H]-CGP12177 β_{1H}-adrenoceptor and β_2-adrenoceptor binding sites (Table 1).

Effect of (-)-CGP12177 on β_3-adrenoceptors

Controversy exists about whether the cardiostimulant effects of (-)-CGP12177 in human atrium are caused by activation of β_3-adrenoceptors (Skeberdis *et al.*, 2008) or β_{1L}-adrenoceptors (Christ *et al.*, 2011). To determine whether the increased HR and contractility induced by chronic infusion of (-)-CGP12177 into the mouse was due to stimulation of β_3-adrenoceptors, we studied the effects of a 2- or 4-week treatment with (-)-CGP12177 at 10 mg·kg^{-1}·day^{-1} in both β_3-adrenoceptor knockout and wild-type FVB/n mice. There were no differences in basal HR and FS values between the genotypes (Figure 2B). Also (-)-CGP12177 caused increases in HR to a similar extent in both the β_3-adrenoceptor knockout and wild-type mice, when tested at 2 and 4 weeks post drug administration ($P > 0.05$, Figure 2B). However, (-)-CGP12177 caused a slightly greater increase in FS in β_3-adrenoceptor knockout mice, indicating a small cardiodepressant effect of (-)-CGP12177 mediated through activation of β_3-adrenoceptors.

Effect of (-)-CGP12177 in mice with chronic TAC

The cardiac effects of (-)-CGP12177 after chronic TAC were studied in two experiments (Figure 1) with (-)-CGP12177 treatment (at 10 mg·kg^{-1}·day^{-1}) for 4 weeks (i.e. weeks 4–8 after TAC, Experiment-1) or 8 weeks (i.e. weeks 4–12 after TAC, Experiment-2). At 4 weeks post-TAC, mice were randomly assigned to receive either (-)-CGP12177 (10 mg·kg^{-1}·day^{-1}) or no treatment for the subsequent 4 or 8 weeks.

For Experiment-1, TAC for 4 weeks increased LV wall thickness at systole and diastole as well as LV mass compared to sham-operated mice, and there were no differences between the two TAC groups before the administration of (-)-CGP12177 (Table 2). At 8 weeks post-TAC (no treatment),

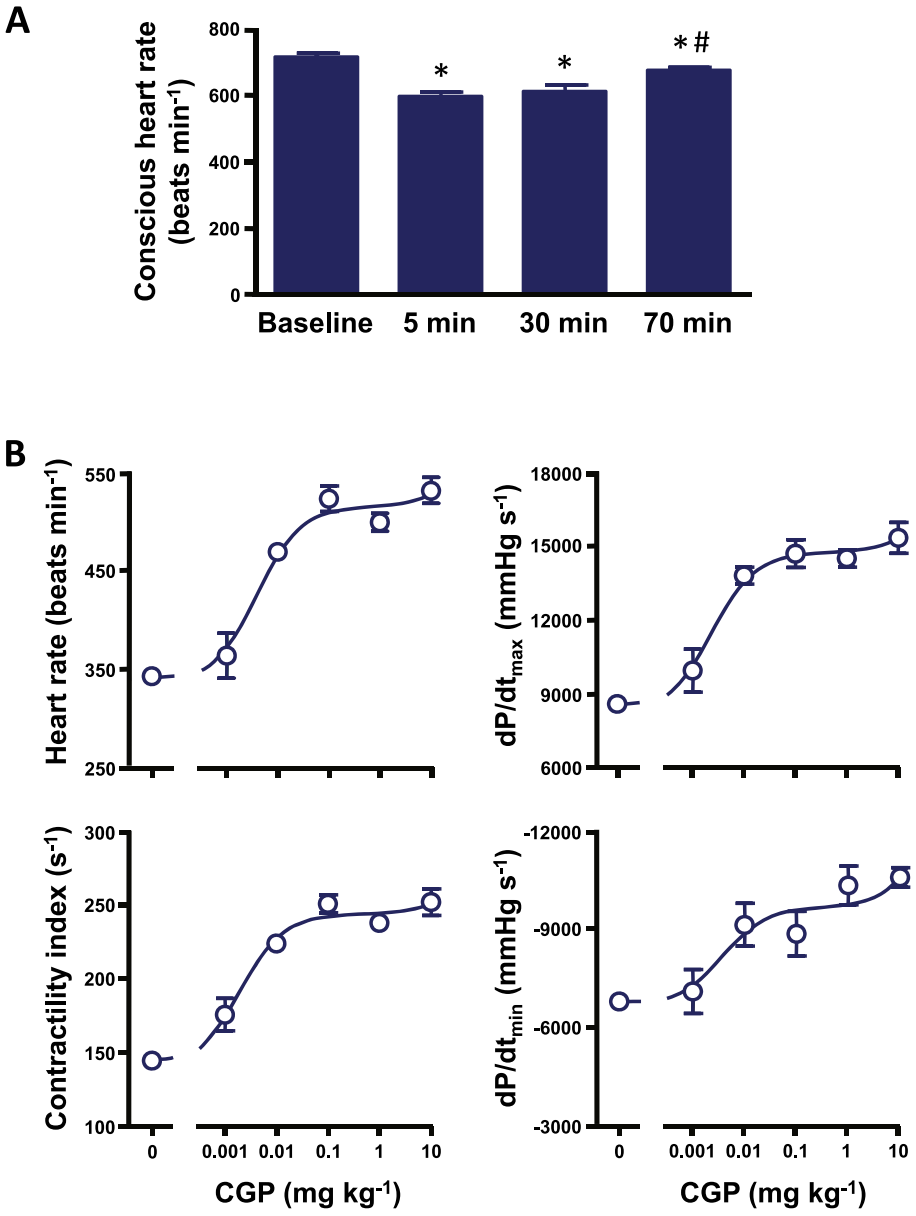

Figure 3

Changes in cardiac functional parameters induced by acute i.v. infusion with (-)-CGP12177 (CGP) in C57Bl/6 mice. (A) Heart rate at baseline and 5, 30 and 70 min post-bolus injection of CGP (at 0.001 mg·kg^{-1}) in conscious and restrained mice (n = 5). *P < 0.05 versus baseline; $^{\#}P$ < 0.05 versus 5 and 30 min timepoints (one-way repeated measures ANOVA). (B) CGP dose-haemodynamic response relationship. Each animal received a single dose; n = 5–6 mice per group with the baseline data combined from all animals. The drug effect was statistically significant for all parameters (P < 0.05, one-way ANOVA). If error bars are not visible they are contained within the symbol.

there was a significant increase in SAP and LVSP, measured proximal to the constriction site, compared to sham-operated controls (P < 0.05, Table 3). Pulse pressure was increased by 3.6-fold (Table 3). Other haemodynamic parameters remained unchanged compared to those of the sham-operated group. Cardiac hypertrophy was manifested as a 60–70% increase in LV:body mass or heart:body mass ratios (Figure 4). Furthermore, TAC resulted in a 1.3-fold increase in LV cardiomyocyte diameter and a threefold increase in LV interstitial fibrosis (Figure 5A, B). (-)-CGP12177 exacerbated the cardiac hypertrophy at 8 weeks post TAC, as indicated by the significant further increase in atria:body mass and a tendency for the LV:body mass and heart:body mass to increase compared to the untreated TAC group (Figure 4); the cardiomyocyte diameter was also increased by a further 6% compared to the untreated TAC group (Figure 5A). In addition, in Experiment-1 the treatment with (-)-CGP12177 was associated with significant increases in pulse pressure, LVSP, dP/dt_{max}, dP/dt_{min} and contractility index compared with the untreated TAC group (Table 3).

We examined the extent of the changes in cardiac expression of genes related to hypertrophy, fibrosis and

Table 1

Maximal density of β_{1H} + β_2-adrenoceptor and β_{1L}-adrenoceptor binding sites determined with (-)-[^{3}H]-CGP12177 and effect of (-)-CGP12177 treatment in healthy hearts and those subjected to transverse aorta constriction (TAC)

(-)-CGP12177 dose	*n*	$\beta_{1H} + \beta_2$	β_{1L}
4 weeks			
No treatment	8	7.58 ± 0.44	48.4 ± 5.4
1 mg·kg^{-1}·day^{-1}	3	5.76 ± 0.18	34.0 ± 7.3
10 mg·kg^{-1}·day^{-1}	3	5.53 ± 0.48*	43.5 ± 0.6
30 mg·kg^{-1}·day^{-1}	4	5.55 ± 0.39*	47.5 ± 2.9
Experiment-2			
TAC	6	7.30 ± 0.32	50.7 ± 7.5
TAC+10 mg·kg^{-1}·day^{-1}	6	6.08 ± 0.19$^{\#}$	47.4 ± 2.9

Values expressed as fmol mg^{-1} protein. *P < 0.05 compared to no treatment [one-way ANOVA for multiple groups (No treatment, 1, 10, 30 mg·kg^{-1}·day^{-1})]; $^{\#}P$ < 0.01 versus TAC (Student's unpaired t test).

Table 2

Experiment-1: Cardiac functional parameters obtained by echocardiography at 4 weeks post-sham or transverse aorta constriction (TAC) surgery and immediately before commencement of (-)-CGP12177 (CGP) treatment

	Sham (*n* = 8)	TAC (*n* = 20)	TAC+CGP (*n* = 21)
Heart rate (beats·min^{-1})	470 ± 13	487 ± 11	475 ± 4
LV diastolic dimension (mm)	4.21 ± 0.07	4.16 ± 0.07	4.34 ± 0.08
LV systolic dimension (mm)	2.92 ± 0.10	2.96 ± 0.06	2.99 ± 0.07
Diastolic wall thickness (mm)	0.69 ± 0.04	0.98 ± 0.03*	0.98 ± 0.04*
Systolic wall thickness (mm)	1.08 ± 0.05	1.41 ± 0.03*	1.45 ± 0.04*
Fractional shortening (%)	31 ± 2	29 ± 1	31 ± 1
LV mass (mg)	107 ± 9	165 ± 6*	178 ± 8*

*P < 0.05 versus Sham (one-way ANOVA).
LV, left ventricle.

inflammation. The untreated TAC group showed multi-fold up-regulation of fetal genes including β-myosin heavy chain, α-skeletal actin, atrial and brain natriuretic peptides, together with an approximately 50% down-regulation of α-myosin heavy chain, sarcoplasmic reticulum Ca^{2+}-ATPase and β_1-adrenoceptor (Figure 6). This group also showed an up-regulation of procollagen-I and connective tissue growth factor (Figure 6). Compared with the untreated TAC group, the (-)-CGP12177 treatment further increased the expression of β-myosin heavy chain, atrial and brain natriuretic peptides, and up-regulated the majority of fibrotic-related (procollagen-I, procollagen-III, MMP2, connective tissue growth factor, TGF-β) and inflammatory genes (IL-1β, monocyte chemotactic protein-1 and NADPH oxidase 2) studied (Figure 6). Plasma renin activity was elevated in both groups with TAC but was not affected by treatment with (-)-CGP12177 (Figure 6), although the kidney:body mass ratio was higher in the TAC+CGP relative to that of TAC group (Figure 4).

In the mouse TAC model, the development of cardiac decompensation effects and HF is dependent on the duration of TAC (Du *et al.*, 2000; Gao *et al.*, 2005). Therefore, in Experiment-2, we investigated whether a prolonged treatment with (-)-CGP12177 of mice with TAC would exert significant influence on cardiac function and hypertrophy. As for Experiment-1, at 4 weeks post-TAC, which was before the commencement of (-)-CGP12177, the two TAC groups were comparable (Figure 7). Compared with mice in Experiment-1 that had TAC for 8 weeks, the prolonged (12 weeks) TAC in Experiment-2 was associated with more severe cardiac hypertrophy and increased lung wet weight indicating pulmonary congestion (Figure 4). Echocardiographic tests (done under isoflurane anaesthesia) revealed a progressive increase in LV dimensions and decline in FS during the 4 to 12 weeks after TAC (Figure 7). (-)-CGP12177 did not influence the extent of LV dilatation and decline in FS (Figure 7). Interestingly, a functional examination conducted 3 days after removal of the osmotic mini pump, for washout of (-)-CGP12177, revealed more severe LV dysfunction. Compared with the untreated TAC group, FS, dP/dt_{max} and dP/dt_{min} were reduced and tau was increased, 3 days after (-)-CGP12177 was withdrawn from TAC mice that had

Table 3

Experiment-1: Cardiac haemodynamic parameters obtained by micromanometry in untreated mice with transverse aorta constriction (TAC) for 8 weeks and TAC mice receiving (-)-CGP12177 at 10 mg·kg^{-1}·day^{-1} for weeks 4 to 8 (TAC+CGP)

	Sham (n = 8)	TAC (n = 16)	TAC+CGP (n = 18)
Heart rate (beats·min^{-1})	435 ± 21	462 ± 14	486 ± 11
Systolic aortic pressure (mmHg)	94 ± 4	172 ± 7*	200 ± 4*#
Diastolic aortic pressure (mmHg)	65 ± 3	66 ± 2	71 ± 2
Pulse pressure (mmHg)	29 ± 1	106 ± 6*	129 ± 4*#
LV systolic pressure (mmHg)	98 ± 3	175 ± 7*	204 ± 3*#
LV end-diastolic pressure (mmHg)	6 ± 1	8 ± 1	9 ± 1
dP/dt_{max} (mmHg·s^{-1})	8465 ± 1499	9039 ± 536	11 005 ± 359*#
dP/dt_{min} (mmHg·s^{-1})	–7718 ± 928	–8990 ± 469	–11 260 ± 500*#
Contractility index (s^{-1})	160 ± 18	184 ± 7	201 ± 7#
Tau (ms)	13 ± 1	12 ± 1	10 ± 1

Aortic pressures were measured proximal to the constriction site. dP/dt_{max} and dP/dt_{min}: maximal rates of rise and fall of left ventricular (LV) pressure, respectively.
*P < 0.05 versus Sham, #P < 0.05 versus TAC (one-way ANOVA).

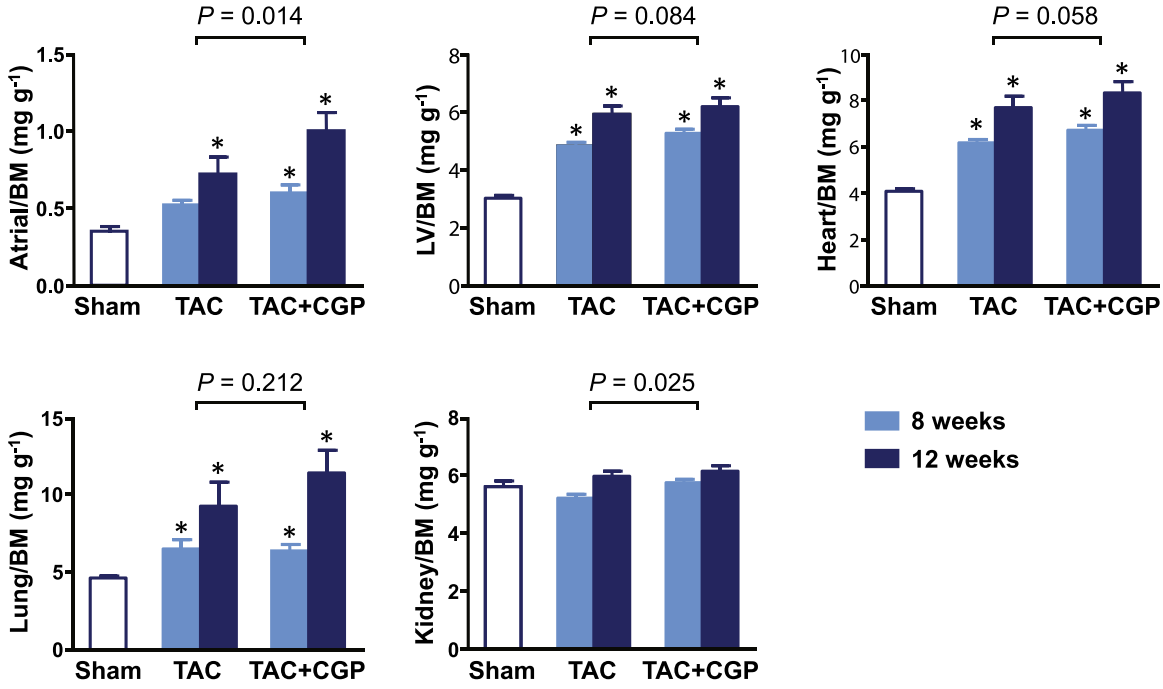

Figure 4

Organ weights normalized to body mass (BM) in mice subjected to sham surgery or transverse aorta constriction (TAC) for either 8 (Experiment-1) or 12 weeks (Experiment-2) with and without treatment with (-)-CGP12177 (CGP) at 10 mg·kg^{-1}·day^{-1}. Sham data from 8 and 12 weeks were combined. There was a significant increase in tissue:BM ratio between 8 and 12 weeks post TAC for all tissues (P < 0.05, two-way ANOVA). P values shown indicate comparison between TAC and TAC+CGP groups (two-way ANOVA). TAC+CGP mice had increased kidney:BM and atrial:BM ratios, and the LV:BM and heart:BM ratios tended to be increased but this was not significant. n = 14–21. *P < 0.05 versus sham-operated control.

been treated with the drug for 8 weeks (Figure 7, Table 4). These data strongly indicate a more severe systolic and diastolic dysfunction at this time-point, which only became evident once the inotropic effect of (-)-CGP12177 was terminated.

Chronic infusion of (-)-CGP12177, 10 mg·kg^{-1}·day^{-1}, for 8 weeks to mice with TAC caused a reduction in β_{1H}-adrenoceptor and β_2-adrenoceptor density, but not β_{1L}-adrenoceptor binding sites compared to the TAC only group (Table 1, P < 0.01).

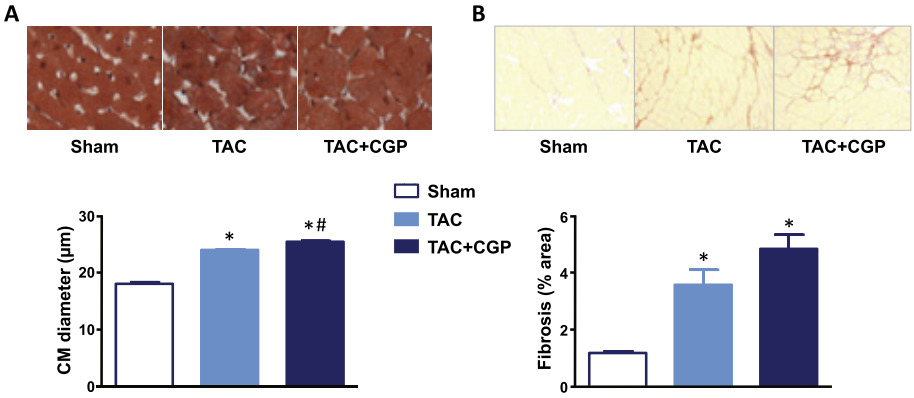

Figure 5

Histological changes in the left ventricle taken from mice subjected to sham surgery or transverse aorta constriction (TAC) and TAC with (-)-CGP12177 treatment (*TAC+CGP*) from Experiment-1. (A) Chronic TAC caused an increase in cardiomyocyte (CM) diameter, which was further increased by (-)-CGP12177 (40× magnification, n = 6–10). (B) Interstitial fibrosis measured by picrosirus red staining for collagen with results expressed as percentage of view field (10 × magnification, n = 6–10). The fibrosis tended to be more severe in the TAC+CGP group (P = 0.09) compared to the untreated TAC group. *P < 0.05 versus sham-operated control; $^{\#}P$ < 0.05 versus TAC (one-way ANOVA).

Table 4

Experiment-2: Cardiac haemodynamic parameters obtained by micromanometry in untreated mice with transverse aorta constriction (TAC) for 12 weeks, and 3 days after (-)-CGP12177 was withdrawn from TAC mice treated with the drug from weeks 4 to 12 (TAC ± CGP)

	TAC (n = 10)	TAC ± CGP (n = 11)
Heart rate (beats·min^{-1})	517 ± 19	475 ± 16
Systolic aortic pressure (mmHg)	193 ± 6	180 ± 8
Diastolic aortic pressure (mmHg)	71 ± 4	64 ± 3
Pulse pressure (mmHg)	122 ± 5	117 ± 5
LV systolic pressure (mmHg)	202 ± 6	179 ± 10
LV end-diastolic pressure (mmHg)	6 ± 1	7 ± 1
dP/dt_{max} (mmHg·s^{-1})	11 106 ± 635	9056 ± 627*
dP/dt_{min} (mmHg·s^{-1})	–11 663 ± 735	–8251 ± 540*
Contractility index (s^{-1})	203 ± 8	192 ± 8
Tau (ms)	10 ± 1	14 ± 1*

dP/dt_{max} and dP/dt_{min}: maximal rates of rise and fall of left ventricular (LV) pressure, respectively.
*P < 0.05 versus TAC control group (Student's unpaired t test).

The incidence of chronic HF, determined from autopsy findings of mice that died prematurely or at the end of the study (following micromanometry), as indicated by the presence of chest fluid, pulmonary congestion and/or left atrial thrombus (Du *et al.*, 2000), was not significantly different between the TAC (9 of 32) and TAC+CGP (14 of 34) mice from Experiments-1 and -2 combined (P = 0.393, χ^2 test). These data include three premature deaths (as specified in the Methods).

Discussion and conclusions

This is the first study that has addressed the cardiac action of β_{1L}-adrenoceptors *in vivo* under physiological and pathological conditions. We have made a few important findings. First, activation of β_{1L}-adrenoceptors with (-)-CGP12177 in healthy mice induced a dose-related increase in HR and cardiac contractile function. This cardiac action of (-)-CGP12177 was largely independent of β_3-adrenoceptors. Second, under conditions of chronic pressure overload, activation of β_{1L}-adrenoceptors with (-)-CGP12177 tended to exacerbate the degree of cardiac hypertrophy and related hypertrophic, fibrotic and inflammatory gene profile. In addition, there was a trend for more severe cardiac remodelling in mice with TAC treated with (-)-CGP12177 for an extended period. Collectively, our findings suggest potentially harmful effects of β-blockers that activate β_{1L}-adrenoceptors in cardiac physiology and, importantly, under chronic pressure overload.

Our *in vivo* studies revealed β_{1H}-blocking as well as β_{1L}-stimulating actions of (-)-CGP12177 and indicated the impor-

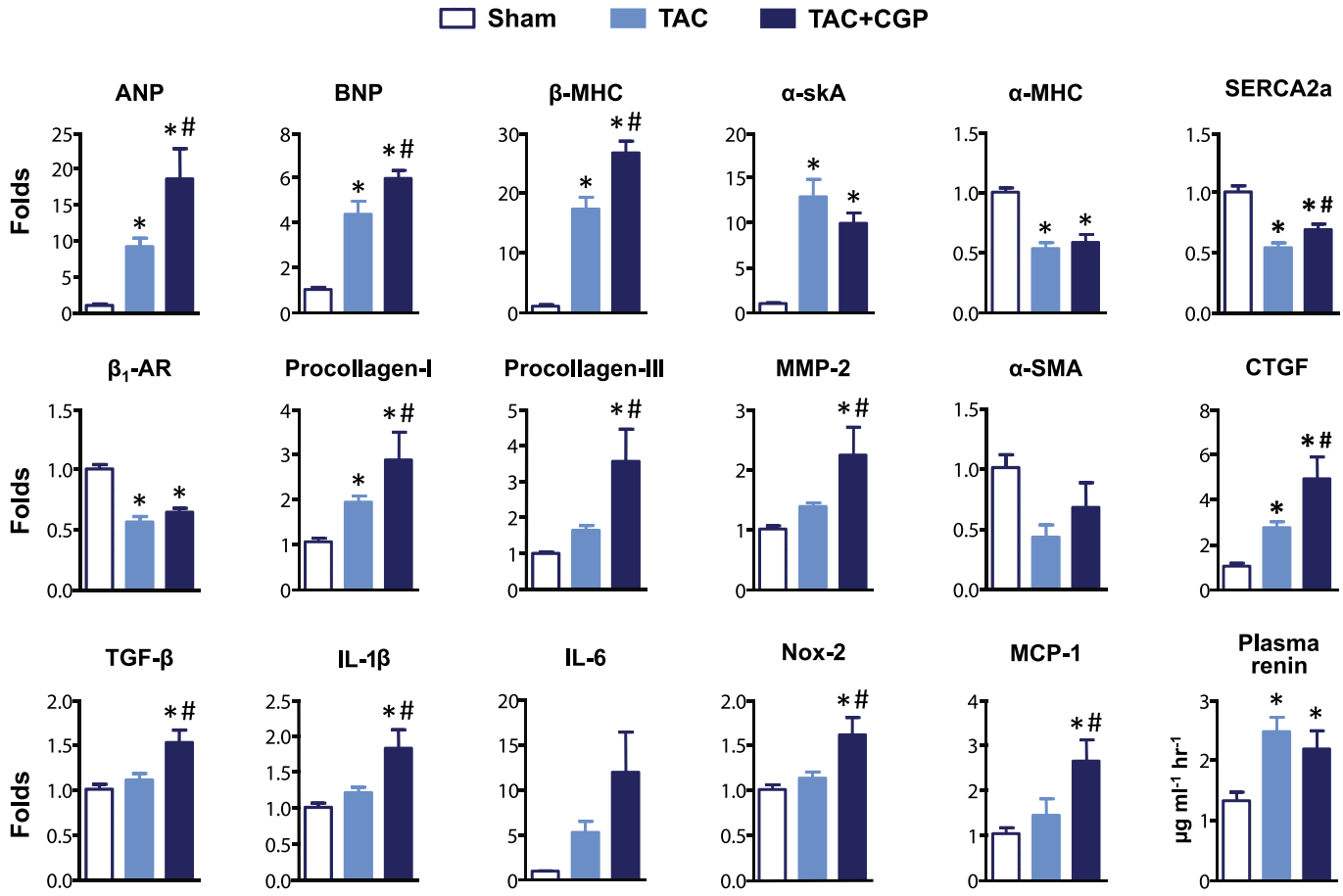

Figure 6

Changes in gene expression of the left ventricular myocardium and plasma renin from mice subjected to sham surgery, transverse aorta constriction (TAC) or TAC together with (-)-CGP12177 treatment (TAC+CGP) from Experiment-1. Expression of the following selected genes were determined by quantitative real-time PCR: atrial or brain natriuretic peptide (ANP, BNP), α- or β-myosin heavy chain (α-, β-MHC), α-skeletal actin (α-skA), α-smooth muscle actin (α-SMA), β_1-adrenoceptor (β_1-AR), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), procollagen-I, procollagen-III, *MMP*-2, IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1), and NADPH oxidase 2 (Nox-2). Gene expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase as house-keeping gene and expressed as fold change of sham group value. n = 4–8. *P < 0.05 versus sham-operated control; $^{\#}P$ < 0.05 versus TAC (one-way ANOVA).

tance of experimental conditions. At the lowest dose (0.001 mg·kg^{-1}) tested, (-)-CGP12177 produced small, insignificant increases in HR in animals anaesthetized with ketamine and xylazine, but significant reductions in HR in conscious and stressed mice. Both experimental conditions are known to be distinct in terms of the sympathetic nervous activity and their affect on β-adrenoceptor-mediated responses. This is reflected by a marked difference in mean HR under anaesthetized compared to conscious conditions (343 compared to 713 beats·min^{-1}). Our data demonstrated an 'opposing action' of (-)-CGP12177 at 0.001 mg·kg^{-1} on HR as a measure of cardiac β_1-adrenoceptor activity, which is probably explained by the difference in the degree of β_1-adrenoceptor activation by cardiac sympathetic drive.

β_{1L}-adrenoceptor-mediated cardiac stimulating effects have been well documented using isolated cardiac tissues or cardiomyocytes from many species including man (Kaumann and Molenaar, 2008). However, there has been an absence of *in vivo* studies to address their pathophysiological significance. Therefore, in the present study, we tested the cardiac effects of chronic treatment with (-)-CGP12177 administered via osmotic mini pump. While (-)-CGP12177 blocks β_{1H}-adrenoceptors in the nanomolar range (Kaumann and Molenaar, 2008), our *in vivo* observations from healthy animals clearly showed a dose-dependent cardiac stimulating action of (-)-CGP12177, which was fully developed at doses of 10–30 mg·kg^{-1}·day^{-1}, equivalent to 30–95 μmol·kg^{-1} range. In comparison with the scale of the increase induced by full activation of β_1-adrenoceptors with agonists such as isoprenaline that we observed previously (Du *et al.*, 2002), the magnitude of the cardiac stimulation induced by (-)-CGP12177 was relatively weak. This is in line with the results from *in vitro* studies in the mouse right and left atrium (Kaumann *et al.*, 1998), human atrial and/or ventricular tissues (Kaumann, 1996; Sarsero *et al.*, 2003) and other species (Kaumann and Molenaar, 2008). In an *in vitro* rat heart preparation treated with (-)-propranolol 200 nM, (-)-CGP12177 at 10 μM stimulated an increase in PKA to a level about 30% of

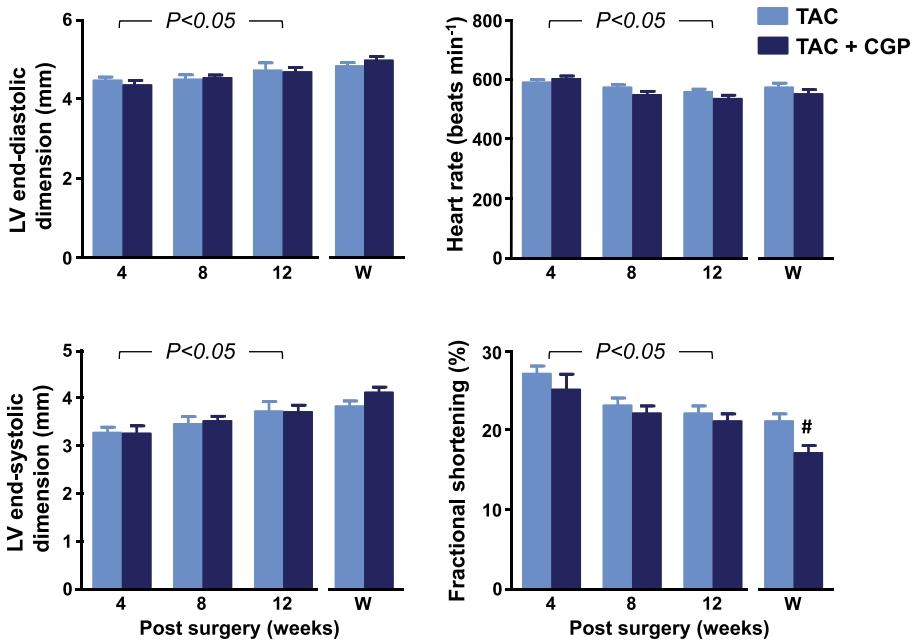

Figure 7

Changes in echocardiographic measurements of left ventricular (LV) function and dimensions in mice with transverse aorta constriction (TAC) for 12 weeks either untreated or treated with (-)-CGP12177 from week 4 to week 12 (TAC+CGP) (Experiment-2). The 4-week echocardiography values were obtained just prior to mini pump implantation in the (-)-CGP12177-treated group, and show that both TAC groups (TAC vs. TAC + CGP) had similar parameters at this pretreatment timepoint. Across the 12 weeks both TAC groups were similar, with only an effect of time evident (horizontal lines with *P* value on top of graphs, from two-way repeated measures ANOVA). Immediately after the 12 week echocardiography, mini pumps were removed from the (-)-CGP12177 treated animals, and an additional echocardiography was performed 3 days later to allow for drug washout (W). n = 10–12 per group. $^{\#}P < 0.05$ versus TAC at same timepoint (Student's unpaired t test).

that evoked by a saturating concentration of (-)-isoprenaline (Kaumann and Lynham, 1997). Adverse cardiac effects induced by chronic treatment with isoprenaline at higher doses are well known and manifested by enhanced oxidative stress, inflammation, cardiomyocyte apoptosis and fibrosis (Sun *et al.*, 2005; Zhang *et al.*, 2005; Chen *et al.*, 2010). We observed that chronic administration of (-)-CGP12177 at doses up to 100 mg·kg^{-1} did not result in any adverse effects, as measured by echocardiography, heart weight, histology and gene expression, which is probably in part due to the fact that full activation of β_{1L}-adrenoceptors with (-)-CGP12177 is equivalent to a modest degree of stimulation of β_{1H}-adrenoceptors. Another interesting feature of β_{1L}-adrenoceptor activation with (-)-CGP12177 is the persistence of the cardiac stimulating effect. This was indicated by haemodynamic responses to (-)-CGP12177 given as a bolus, or throughout the 4-week period studied, suggesting the lack of desensitization of β_{1L}-adrenoceptors under these conditions.

We detected a decrease in β_{1H} ($+\beta_2$)-, but not β_{1L}-adrenoceptor binding site numbers following the 4-week infusion of (-)-CGP12177 at doses over 10 mg·kg^{-1}·day^{-1}. Similar changes were observed in hypertrophic hearts without and with (-)-CGP12177 treatment. In these experiments, (-)-[^{3}H]-CGP12177 binds to both β_{1H} and β_2-adrenoceptors. Due to insufficient quantities of tissues, we did not delineate between β_{1H} and β_2-adrenoceptors. Our assay for $\beta_{1H}+\beta_2$–adrenoceptor binding would largely reflect changes in β_{1H}-adrenoceptors as this subtype accounts for ~80% of β-adrenoceptors in the heart. A reduction in β_1-adrenoceptor mRNA was observed in LVs from mice with TAC or the TAC+CGP (10 mg·kg^{-1}·day^{-1}) group when compared to the control mice. It has been well documented that β_1- but not β_2-adrenoceptor signalling is desensitized in heart disease and failure, partially through loss of receptors from cellular membranes (i.e. down-regulation), and through reduced gene transcription (Bristow *et al.*, 1986; Bristow, 2000; Lohse *et al.*, 2003). Such changes are due, for the most part, to sustained agonist stimulation. While β_{1H}- and β_{1L}-adrenoceptor binding sites are separate, they are on the same receptor. Therefore it is feasible, although not proven, that chronic agonist activation of β_{1L}-adrenoceptors also reduces β_{1H}-adrenoceptor binding sites, due to a loss of β_1-adrenoceptors.

Previous studies on the mouse TAC model, including ours (Sheridan *et al.*, 2000; Gao *et al.*, 2005), have documented the dynamic evolution from compensatory hypertrophy to functional decompensation and ultimately HF. We carefully examined the consequences of chronic activation of β_{1L}-adrenoceptors with (-)-CGP12177 in mice with TAC. Cardiostimulant effects of (-)-CGP12177 were evident from both the non-invasive echocardiography and the invasive micromanometry conducted during the treatment period up to 12 weeks after TAC. Our results indicate that the ventricular remodelling (increase in atrial weight, cardiomyocyte size and hypertrophic gene pattern as well as expression of fibro-

genic or inflammatory genes) in mice treated with (-)-CGP12177 for 4–8 weeks after TAC is more severe compared with untreated TAC mice. These effects occurred despite such treatment resulting in a moderate increase in ventricular contractile function. This is the first time that β_{1L}-adrenoceptor activation has been shown to adversely affect cardiac remodelling *in vivo*, at the organ, cellular and molecular levels, under conditions of pressure overload hypertrophy.

Higher levels of SAP, pulse pressure and LVSP were observed at week-8 after TAC in the presence of (-)-CGP12177, compared to the corresponding untreated TAC group. While these parameters are commonly used to assess the degree of pressure overload, such a difference is unlikely to be explained by a difference in the degree of pressure overload because TAC surgery and animal allocation to untreated or (-)-CGP12177 treatment at week-4 was carried out without information of ultimate grouping. Furthermore, functional assessments at 4 weeks did not reveal any differences between the TAC and TAC+CGP groups before the commencement of (-)-CGP12177 treatment. Such differences in the presence of (-)-CGP12177 are most likely due to an enhanced contractility as a consequence of β_{1L}-adrenoceptor stimulation, which further augmented SAP when the LV pumps blood against a fixed resistance (aortic constriction). In Experiment-2 when haemodynamic measures were performed after washout of (-)-CGP12177, there was a trend for the levels of SAP and LVSP to be lower than those of the untreated TAC group. This is attributable to the development of ventricular contractile dysfunction reflected by a significant reduction in dP/dt_{max} and FS in the treated group.

Chronic use of β_1-adrenoceptor agonists such as isoprenaline in rodents is well known to induce cardiac pathology and dysfunction (Sun *et al.*, 2005; Zhang *et al.*, 2005; Chen *et al.*, 2010). Whereas chronic activation of β_{1L}-adrenoceptors with (-)-CGP12177 resulted in apparent functional enhancement, this action was not associated with changes in gene expression in the myocardium of normal mice without TAC. However, in the presence of TAC, chronic β_{1L}-adrenoceptor stimulation by (-)-CGP12177 exacerbated the extent of gene expression known to lead to adverse remodelling and dysfunction in hearts with pressure-overload hypertrophy.

End-stage human HF is associated with a loss of both β_{1H}-adrenoceptor (+β_2-adrenoceptor) and β_{1L}-adrenoceptor binding sites (Sarsero *et al.*, 2003) and desensitization of inotropic responses mediated through activation of β_{1H}-adrenoceptors (Bristow *et al.*, 1986; Molenaar *et al.*, 2007) and β_{1L}-adrenoceptors (Sarsero *et al.*, 2003) in human heart tissues. Human HF is characterized by increased sympathetic nervous activity, noradrenaline spillover and activation of β_{1H}-adrenoceptors (Cohn *et al.*, 1984; Esler *et al.*, 1997; Bristow, 2000; Bristow *et al.*, 2003). Chronic activation of β_1-adrenoceptors causes progression of HF resulting in adverse remodelling, haemodynamic dysfunction, morbidity and mortality. Further clarification of the pathological role of β_1-adrenoceptors was obtained from transgenic mice with 15-fold overexpression of cardiac β_1-adrenoceptors, which exhibited initial enhancement of heart function at a young age, followed by progressive deterioration that included hypertrophy and HF, sharing similar features with human HF (Engelhardt *et al.*, 1999). Despite progress in the management of human HF by the use of β-blockers that reverse adverse cardiac remodelling and improve cardiac function, the long-term prognosis of patients with HF is, unfortunately, still extremely poor. The present study provides interesting evidence for a role for β_{1L}-adrenoceptor activation in promoting adverse cardiac remodelling and functional dysfunction.

While the pharmacological significance of β_{1L}-adrenoceptors has been shown *ex vivo*, its role *in vivo* under pathological conditions remains speculative. In this study on mice with chronic pressure overload, activation of β_{1L}-adrenoceptors by the exogenous agonist (-)-CGP12177 led to adverse consequences. Thus, in the setting of heart disease, activation of β_{1L}-adrenoceptors by some β-blockers (Kaumann and Molenaar, 2008) is likely and their use should be avoided. However, future studies are required to test this possibility.

Conclusion

Using (-)-CGP12177 as a β_{1L}-adrenoceptor agonist, we demonstrated a functional stimulating effect mediated by β_{1L}-adrenoceptors in healthy and diseased mouse heart *in vivo*, which was maintained throughout the study period of 4 or 8 weeks. While activation of β_{1L}-adrenoceptors using (-)-CGP12177 in healthy animals did not result in adverse consequences, such an intervention exacerbates cardiac remodelling in the setting of chronic pressure overload and functional decompensation. Our results suggest that cardiostimulation through β_{1L}-adrenoceptors by a β-blocker could potentially worsen cardiac disease, thereby decreasing the beneficial effects of blockade of β_{1H}-adrenoceptors.

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Conflicts of interest

The authors declare no conflicts of interest.

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