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RESEARCH PAPER

Myocardial oxidative stress contributes to transgenic β_2-adrenoceptor activation-induced cardiomyopathy and heart failure

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BACKGROUND AND PURPOSE
While maintaining cardiac performance, chronic β-adrenoceptor activation eventually exacerbates the progression of cardiac remodelling and failure. We examined the adverse signalling pathways mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species (ROS) after chronic β_2-adrenoceptor activation.

EXPERIMENTAL APPROACH
Mice with transgenic β_2-adrenoceptor overexpression (β_2-TG) and non-transgenic littermates were either untreated or treated with an antioxidant (N-acetylcysteine, NAC) or NADPH oxidase inhibitors (apocynin, diphenyliodonium). Levels of ROS, phosphorylated p38 mitogen-activated protein kinase (MAPK), pro-inflammatory cytokines and collagen content in the left ventricle (LV) and LV function were measured and compared.

KEY RESULTS
β_2-TG mice showed increased ROS production, phosphorylation of p38 MAPK and heat shock protein 27 (HSP27), expression of pro-inflammatory cytokines and collagen, and progressive ventricular dysfunction. β_2-adrenoceptor stimulation similarly increased ROS production and phosphorylation of p38 MAPK and HSP27 in cultured cardiomyocytes. Treatment with apocynin, diphenyliodonium or NAC reduced phosphorylation of p38 MAPK and HSP27 in both cultured cardiomyocytes and the LV of β_2-TG mice. NAC treatment (500 $mg \cdot kg^{-1} \cdot day^{-1}$) for 2 weeks eliminated the up-regulated expression of pro-inflammatory cytokines and collagen in the LV of β_2-TG mice. Chronic NAC treatment to β_2-TG mice from 7 to 10 months of age largely prevented progression of ventricular dilatation, preserved contractile function (fractional shortening 37 ± 5% vs. 25 ± 3%, ejection fraction 52 ± 5% vs. 32 ± 4%, both $P < 0.05$), reduced cardiac fibrosis and suppressed matrix metalloproteinase activity.

CONCLUSION AND IMPLICATIONS
β_2-adrenoceptor stimulation provoked NADPH oxidase-derived ROS production in the heart. Elevated ROS activated p38 MAPK and contributed significantly to cardiac inflammation, remodelling and failure.

LINKED ARTICLE
This article is commented on by Di Lisa *et al.*, pp. 1009–1011 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2010.01130.x

Abbreviations
α-MHC, α-myosin heavy chain; α-SMA, α-smooth muscle actin; β_2-TG, β_2-adrenoceptor transgenic; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5,tetramethylpyrrolidine; CTGF, connective tissue growth factor; DHE,

dihydroethidium; DPI, diphenyliodonium; EDPVR, end-diastolic pressure-volume relationship; EDV, end-diastolic volume; EF, ejection fraction; ESR, electron spin resonance; ESV, end-systolic volume; FS, fractional shortening; H_2DCF-DA, 2′,7′-dichlorodihydro-fluorescein diacetate fluorescence; HF, heart failure; HSP, heat shock protein; IL, interleukin; JNK, jun N-terminal kinase; LV, left ventricle; LVDd, LVDs, LV dimensions at end-diastole and end-systole; MAPK, mitogen-activated protein kinase; MCP-1 (CCL2), monocyte chemotactic protein-1; NAC, N-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NTG, non-transgenic; PCR, polymerase chain reaction; PEG-SOD, superoxide dismutase-polyethylene glycol; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; VCAM-1, vascular cell adhesion molecule 1

Introduction

Heart failure (HF) is a leading cause of mortality and morbidity. One common finding in HF is activation of the sympatho-adrenoceptor system, shown by increased sympathetic nerve firing rates, elevated plasma levels of catecholamines (Cohn, 1990) and increased cardiac noradrenaline spillover (Kaye *et al.*, 1995). Activation of the sympatho-adrenoceptor system mediates functional compensation through the β-adrenoceptor/stimulatory G-protein/protein kinase A signal pathway (Bristow, 2000). Such action is compatible with clinical findings that patients with unstable HF or NYHA class III/IV HF show impaired function or haemodynamic worsening following acute β-adrenoceptor blockade (Feldman *et al.*, 2008). On the other hand, there is convincing evidence for adverse biological consequences mediated by β-adrenoceptors. In support of this are the beneficial effects of β-adrenoceptor antagonists (β-blockers) in HF patients (Bristow, 2000) and animal models (Watanabe *et al.*, 2000) and the cardiomyopathy phenotype observed in transgenic mouse models with enhanced β-adrenoceptor signalling (Engelhardt *et al.*, 1999; Du *et al.*, 2000; Liggett *et al.*, 2000; Hardt *et al.*, 2002). β-Blockers are generally recommended for the treatment of patients with HF, and have been proven by clinical trials to be important in retarding HF progression, lessening the symptoms of HF and lowering the mortality compared with placebo (Hunt *et al.*, 2005). However, the signalling mechanism remains unclear based on these studies. Understanding the mechanisms responsible for the adverse consequences of chronic β-adrenoceptor activation is likely to be important in developing therapy capable of eliminating these while preserving compensatory effects.

Previous studies have demonstrated profound changes in the β-adrenoceptor signalling system in the failing heart, notably β_1-adrenoceptor down-regulation and desensitization due apparently to overt and sustained stimulation, with largely unaltered β_2-adrenoceptors (Molenaar *et al.*, 2007; Feldman *et al.*, 2008), leading to an increased $\beta_2 : \beta_1$ ratio. Of β_1- and β_2-adrenoceptors, the former has been thoroughly investigated with consistent reports indicating detrimental consequences following β_1-adrenoceptor activation: apoptosis, inflammation, hypertrophy and fibrosis (Feldman *et al.*, 2008). The role of β_2-adrenoceptor activation is much less studied and remains controversial. Earlier *in vitro* studies suggested an anti-apoptotic action opposing that of β_1-adrenoceptors (Zhu *et al.*, 2001; 2003). Although combined therapy of β_2-adrenoceptor agonists and β_1-blocker was proposed by an experimental HF study (Ahmet *et al.*, 2008), adverse consequences following long-term use of β_2-adrenoceptor agonists, such as hypertrophy and dysfunction, have been reported (Ryall *et al.*, 2008). In addition, work carried out in human heart cautions against the use of β_2-adrenoceptor agonists in HF management (Hirono *et al.*, 2001; Cazzola *et al.*, 2005; Molenaar *et al.*, 2006). A recent study demonstrated that while β_2-adrenoceptors are exclusively localized with T-tubules in normal cardiomyocytes, in the failing myocardium they are distributed widely across the cell surface and adopt signalling characteristics of β_1-adrenoceptors, adding another layer of complexity to the signalling of β_2-adrenoceptor in HF (Nikolaev *et al.*, 2010).

Although β-blockers are commonly prescribed to HF patients, one unanswered question is whether there is clinical advantage to blocking β_2-adrenoceptor in addition to β_1-adrenoceptor. The COMET study is the only large clinical trial to compare the effects of carvedilol (β_1 + β_2-blocker) and metoprolol (β_1-blocker) in HF patients (Poole-Wilson *et al.*, 2003). In that trial, carvedilol showed a more significant reduction in mortality than metoprolol. This outcome had been taken as an example to argue that β_1 + β_2-blockers are more effective than β_1-adrenoceptor blockers in HF management. However, other known actions of carvedilol such as antioxidant and α_1-adrenoceptor blockade might also contribute to the overall efficacy.

Recent *in vitro* studies, including ours, have revealed several non-classical signalling molecules utilized by β_2-adrenoceptors, including β-arrestin 1 (Drake *et al.*, 2008; Gong *et al.*, 2008; Tilley *et al.*,

2009), p38 mitogen-activated protein kinase (MAPK) (Gong *et al.*, 2008; McAlees and Sanders, 2009) and reactive oxygen species (ROS) (Yin *et al.*, 2006; Gong *et al.*, 2008). However, such findings have been fragmentary in being either limited to *in vitro* systems, not revealing the signalling cascade, or in non-cardiac preparations.

To define the adverse and non-classic β_2-adrenoceptor signalling mechanisms in the heart, we used a transgenic model with cardiac-restricted β_2-adrenoceptor overexpression (β_2-TG) (Milano *et al.*, 1994), which leads to age-dependent onset and progression of cardiomyopathy and HF (Du *et al.*, 2000; Peter *et al.*, 2007). We tested *in vivo* and *in vitro* our hypothesis that there exists a β_2-adrenoceptor/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase/ROS/p38 MAPK signalling pathway leading to cardiac pathology and HF.

Experimental procedures

Animals

All animal care and experimental procedures were approved by a local Animal Ethics Committee and the investigation conformed to the *Australian code of practice for the care and use of animals for scientific purposes* published by the Australian National Health and Medical Research Council (7th edition, 2004). Male β_2-adrenoceptor transgenic (β_2-TG) and non-transgenic (NTG) littermate mice, originally described by Milano *et al.* (Milano *et al.*, 1994), were used and housed in standard conditions. This colony was on a C57Bl6 × SJL mixed background and has maintained very stable transgene expression and cardiac phenotypes (Milano *et al.*, 1994; Du *et al.*, 2000; Gao *et al.*, 2003; Peter *et al.*, 2007). We studied animals at different ages (5–15 months of age, depending on the protocols), for comparison of findings from mice with pre-disease (5-month-old), during rapid progression of cardiomyopathy (7- to 10-month-old) and with advanced disease (15-month-old) with group sizes of 6–17 mice. To explore the adverse signalling of β_2-adrenoceptor, β_2-TG mice (7-month-old) were acutely treated with test agents including antioxidant (N-acetylcysteine, NAC), NADPH oxidase inhibitors (apocynin, diphenyliodonium chloride, DPI), and p38 MAPK inhibitor (SB 202190). Long-term efficacy of NAC therapy was examined in β_2-TG mice during 7–10 months of age when cardiomyopathy progresses rapidly (Du *et al.*, 2000).

Primary culture of ventricular myocytes

Cardiomyocytes were isolated from neonatal (0–2 days) Sprague-Dawley rats and plated at a density of 200 cells·mm^{-2}, as described previously (Yin *et al.*, 2003). Cultures were maintained for 72 h in Dulbecco's modified Eagle's medium (contains 1 g·L^{-1} D-glucose, Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, and then starved in serum-free medium for another 24 h.

Echocardiography

Echocardiography was performed and images were analysed, without knowledge of treatments, as described previously (Xu *et al.*, 2008). Animals were anaesthetized with a mixture of ketamine/xylazine/atropine (at 100/10/1.2 mg·kg^{-1}, respectively, i.p.). Left ventricle (LV) dimensions at end-diastole and end-systole (LVDd, LVDs) were measured. Fractional shortening was calculated as [(LVDd – LVDs)/LVDd] × 100%.

Micromanometry and organ weight

Left ventricle function was assessed by using 1.4F microtipped pressure-volume (P/V) catheters (SPR839) and the ARIA system (Millar Instrument Inc., Houston, TX, USA). Mice were anaesthetized with the mixture of ketamine/xylazine/atropine. P/V data were collected at steady state and during a transient occlusion of the inferior vena cava (Pacher *et al.*, 2008).

Measurement of ROS levels

Reactive oxygen species production in the LV myocardium was determined by three independent methods. First, superoxide production was determined by electron spin resonance (ESR) assay using the cell-permeable probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5,tetramethylpyrrolidine (CMH, Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany) and a Bruker EMX ESR spectrometer (Berthold Technologies GmbH and Co. Bad Wildbad, Germany), as described previously with minor modifications (Kuzkaya *et al.*, 2003). Briefly, freshly harvested LV tissue was incubated for 30 min in HEPES-Krebs buffer containing (in mmol·L^{-1}): NaCl 99, KCl 4.69, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.03, glucose 5.6, Na-HEPES 20, CMH 0.5, deferroxamine methanesulfonate 0.025, and N,N-diethyldithiocarbamate 0.005, pH 7.4, at 37°C. Tissues were then snap frozen in liquid N_2 and assayed using the ESR spectrometer. Tissues were then dried at 60°C for 48 h. LV superoxide production was quantified as the amplitude of the ESR spectra and normalized to dry tissue weight. Second, ROS production was assessed by lucigenin-enhanced chemiluminescence (Byrne *et al.*, 2003; Ritchie *et al.*, 2007). Briefly, LV tissues were dissected into small pieces (~1 mm in diameter) and incubated in the dark at 37°C for 1 h with NADPH

(100 μmol·L^{-1})-containing Krebs buffer in 96-well optiplates (PerkinElmer Inc., Waltham, MA, USA). Lucigenin (Sigma Aldrich, St. Louis, MO, USA) was then added into each well (final concentration 5 μmol·L^{-1}) and photon emissions were measured using the MicroLumat Plus luminometer (Berthold Technologies GmbH and Co. Bad Wildbad, Germany), with twenty repeats of 1 s count per well, at 37°C. LV superoxide production was quantified as the photon counts and normalized to dry tissue weight. Third, O_2^- formation was further analysed using OCT fresh-frozen LV sections (30 μm) incubated with dihydroethidium (DHE, Invitrogen Co.) for 1 h at 37°C. Images were obtained using a laser-scanning confocal microscope (LSM 510, Carl Zeiss Inc., Thornwood, NY, USA) with excitation/emission at 488/610 nm. DHE fluorescence was quantified using Image Pro 6.0 (Media Cybernetics Inc., Bethesda, MD, USA).

Reactive oxygen species production in neonatal rat cardiomyocytes was determined using cell-permeable probe, 2′,7′-dichlorodihydro-fluorescein diacetate fluorescence (H_2DCF-DA), as we described previously (Laskowski *et al.*, 2006; Gong *et al.*, 2008). Briefly, Serum-starved cells were loaded with 10 μmol·L^{-1} H_2DCFDA in Dulbecco's modified Eagle's medium without phenol red in the dark for 30 min at 37°C. Cellular fluorescence intensity was measured using Victor 3 Plate Reader (Perkin-Elmer) following β_2-adrenoceptor stimulation.

Gene expression

RNA transcripts of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, monocyte chemotactic protein-1, vascular cell adhesion molecule-1, transforming growth factor-β, connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), NOX2 and NOX4 subunits of NADPH oxidase, human β_2-adrenoceptor transgene, α-myosin heavy chain (α-MHC) were determined by SYBR Green polymerase chain reaction with an ABI PRISM 7700 Sequence Detection System (Xu *et al.*, 2008) and normalized by that of glyceraldehyde 3-phosphate dehydrogenase.

β_2-adrenoceptor binding assay

β_2-adrenoceptor binding assay was performed as described previously with minor modifications (Sheridan *et al.*, 2000). LVs were homogenized and crude membranes prepared. Membrane proteins (~5 μg) were incubated with 200 pmol·L^{-1} [^{125}I](–)iodocyanopindalol (Perkin Elmer, 81.4 TBq·mmol^{-1}) for 1 h with or without the β_2-adrenoceptor antagonist ICI 118551 (1 μmol·L^{-1}, Sigma) at 37°C, filtered through a 96-Well Filter Plate (PerkinElmer) and counted in a γ-counter.

Western blot

Left ventricle tissues or cells were lysed in lysis buffer (Yin *et al.*, 2003). Equal amounts of protein (30 μg) were separated on 4–12% SDS-PAGE. Membranes were incubated with antibodies against phospho-p38 MAPK, p38 MAPK, phospho-heat shock protein (HSP) 27, HSP27 (Cell Signaling, 1:2000) or α-tubulin (Sigma, 1:20 000), and exposed using enhanced chemiluminescence reagent. Band intensity was quantified using the software Quantity One (version 4.5.2; Bio-Rad).

Histology

Hearts were fixed in 10% formalin in phosphate-buffered saline, paraffin-embedded, serially sectioned (5 μm) and stained with Picrosirius red or Masson's trichrome. Images were captured and analysed, without knowledge of treatments, by using the Image-pro plus 6.0 System, as described previously (Xu *et al.*, 2008).

Cardiomyocyte apoptosis

TUNEL staining was performed in OCT fresh-frozen LV (5 μm slices). TUNEL-positive nuclei of myocytes were counted and expressed as a percentage of total cardiomyocytes in an entire section (4000–6000 cells) (Gao *et al.*, 2003).

Statistical analysis

Results were expressed as mean ± SEM. Between-group comparisons were made by one- or two-way ANOVA or two-way repeated measures ANOVA with a Bonferroni *post hoc* analysis using GraphPad 5 (GraphPad Inc., La Jolla, CA, USA), as appropriate. The least-square method was used for linear correlation and regression. $P < 0.05$ was considered as statistically significant.

Materials

The suppliers of the materials used here were as follows: Apocynin, DPI, SB202190, NAC, CGP 20712A, Isoprenaline, PEG-SOD all from Sigma-Aldrich, USA. H_2DCF-DA: was from Invitrogen, USA and for the anaesthetic mixture, ketamine was from Parnell Laboratories Pty Ltd, Australia; xylazine was from Troy Laboratories Pty Ltd, Australia and atropine was from Pfizer Australia. Receptor and drug nomenclatures follow Alexander *et al.* (2009).

Results

Transgenic β_2-adrenoceptor activation causes enhanced ROS production

First, we assayed ROS formation in the LV of 5-month-old β_2-TG mice (n = 6–9/group). As shown

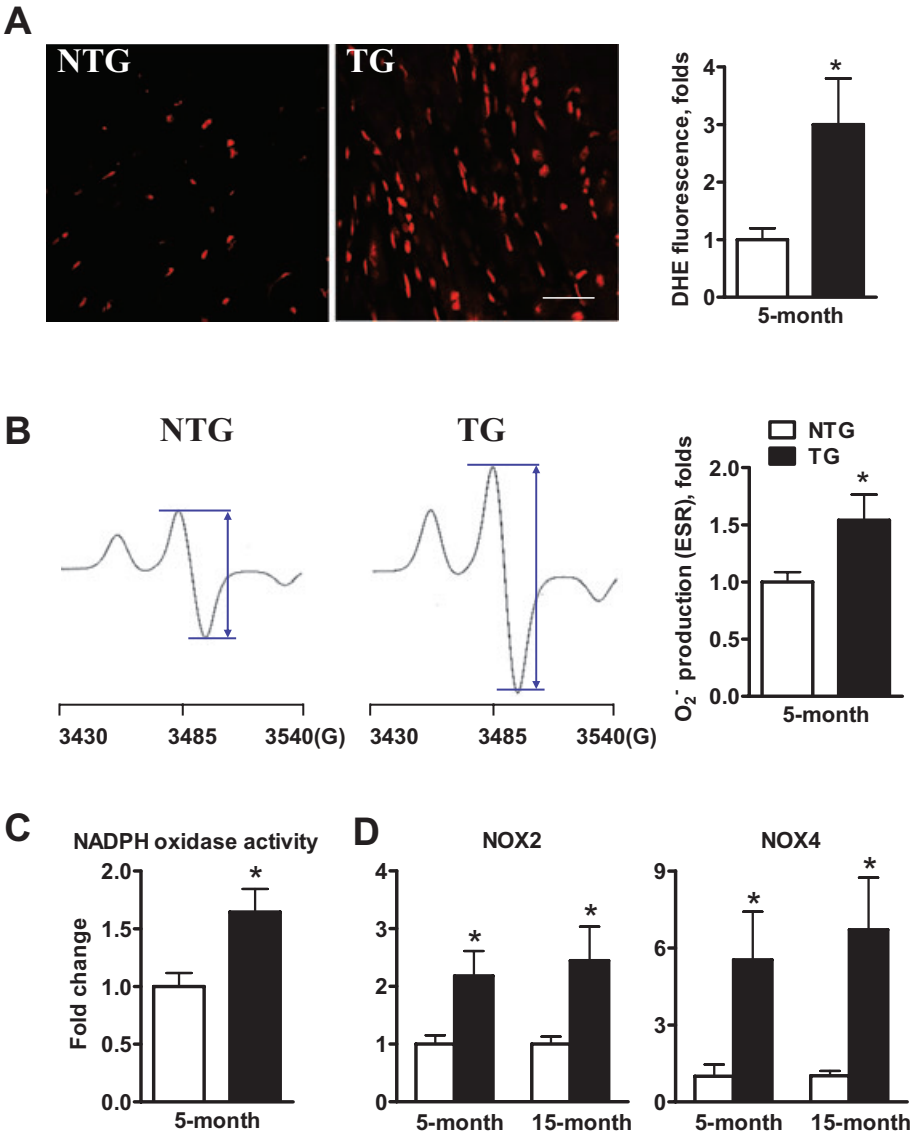

Figure 1

Increased ROS production in the LV of β_2-TG mice. A, representative fluorescent probe DHE staining for oxidative fluorescent signal of LV sections from NTG and β_2-TG mice (5-month-old) and densitometric analysis of DHE fluorescence. (Bar = 50 μm). B, ROS production was determined in freshly harvested LV (5-month-old) by electron spin resonance assay using superoxide probe CMH, or C, lucigenin-enhanced chemiluminescence assay for NADPH oxidase activity. D, quantitative real-time PCR for mRNA expression of NOX2 and NOX4 isoforms in the LV of NTG and β_2-TG mice at both 5 and 15 months of age. Data are presented as relative changes to age-matched NTG mice (n = 5–9/group). *P < 0.05 versus NTG mice.

in Figure 1A, LV sections stained with the fluorescent probe DHE revealed a marked increase in oxidative fluorescent signal in the LV of 5-month-old β_2-TG mice. Likewise, superoxide production by ESR assay increased by about 50% in the LV of 5-month-old β_2-TG compared with NTG mice (P < 0.05, Figure 1B). Further, NADPH oxidase was shown to be an important source of ROS by results from NADPH-enhanced lucigenin assay showing a 64% increase in NADPH oxidase activity in LVs of 5-month-old β_2-TG versus NTG mice (P < 0.05, Figure 1C). In keeping with these findings, in β_2-TG at 5- and 15-months (n = 5–7/group) of age, gene expression in the LV was up-regulated by over twofold for NOX2 (both P < 0.05), and sixfold for NOX4 (both P < 0.05) relative to NTG mice (Figure 1D).

β_2-adrenoceptor activation induces phosphorylation of p38 MAPK and HSP27 via NADPH oxidase and ROS

p38 MAPK is a critical signalling molecule downstream of β_2-adrenoceptor activation (Peter *et al.*, 2007; Gong *et al.*, 2008). We therefore examined the

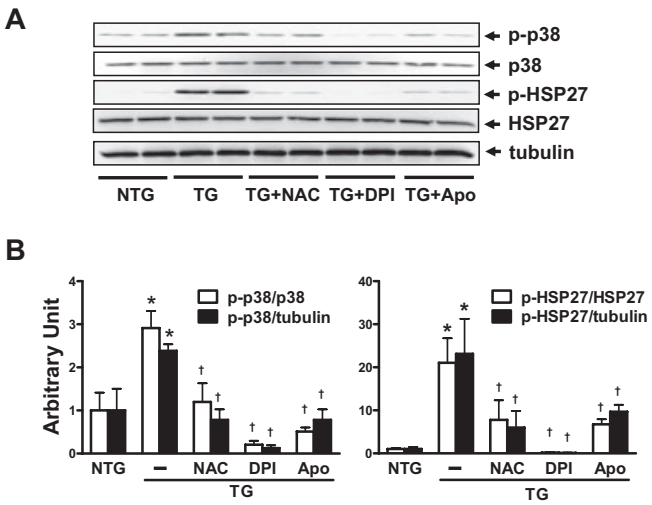

Figure 2

p38 MAPK activation by NADPH oxidase-derived ROS in the LV of β_2-TG. A, β_2-TG mice (5- to 7-month-old) were treated with N-acetylcysteine (NAC, 250 mg·kg^{-1}, i.p.), or NADPH inhibitors, apocynin (Apo, 2 mg·kg^{-1}, i.p.) or diphenyliodonium chloride (DPI, 1 mg·kg^{-1}, i.p.). The LV was collected 1 h after the treatment. Western blot analyses were performed using antibodies against phospho-p38 MAPK, p38 MAPK, phospho-HSP27, HSP27 and tubulin respectively. B, Levels of phosphorylated and total p38 MAPK or HSP27 and tubulin, quantified by densitometry and presented as changes relative to age-matched NTG mice. *P < 0.05 versus NTG mice; †P < 0.05 versus vehicle-treated β_2-TG group, n = 6/group.

role of NADPH oxidase and ROS in mediating p38 MAPK activation in the β_2-TG model. LVs of 7-month-old β_2-TG mice exhibited a threefold increase in phospho-p38 MAPK and a 21-fold increase over NTG in phosphorylation of its downstream molecule, HSP27 (Figure 2, both P < 0.05). These changes seen in the β_2-TG were significantly reduced by treatment with the antioxidant, NAC (250 mg·kg^{-1}, i.p.), or NADPH oxidase inhibitors, apocynin (2 mg·kg^{-1}, i.p.) or DPI (1 mg·kg^{-1}, i.p., n = 6/group, all P < 0.05).

The increase of ROS production and phosphorylation of p38 MAPK and HSP27 was also observed in primary cultured neonatal cardiomyocytes. β_2-adrenoceptor stimulation increased ROS production by about 90%, which was abolished by pretreatment with the β_2-adrenoceptor antagonist, ICI 118551 (1 μmol·L^{-1}). Pretreatment with either the antioxidant, NAC (1 mmol·L^{-1}), or NADPH oxidase inhibitors, apocynin (100 μmol·L^{-1}) or DPI (10 μmol·L^{-1}) also abolished β_2-adrenoceptor stimulation-induced ROS production (Figure 3A, all P < 0.05). The levels of phospho-p38 MAPK and phospho-HSP27, which were increased following β_2-adrenoceptor stimulation, were abolished by pretreatment with β_2-adrenoceptor antagonist, ICI 118551. Pretreatment of cultured cardiomyocytes with either the antioxidants, NAC or superoxide dismutase-polyethylene glycol (PEG-SOD, 25 U·mL^{-1}), or NADPH oxidase inhibitors, apocynin or DPI, also significantly reduced β_2-adrenoceptor stimulation-induced phosphorylation of p38 MAPK and HSP27 (Figure 3B, n = 3/group, all P < 0.05).

Inflammation and extracellular matrix remodelling in β_2-TG hearts

mRNA expression of pro-inflammatory cytokines was increased in the LV of aged (15-month-old), and in that of adult (5-month-old) β_2-TG mice (Table 1, all P < 0.05 vs. NTG), with the exception of TNF-α. Higher mRNA levels of CTGF, α-SMA, procollagen-1 and procollagen-3 were also observed in the LV of β_2-TG mice at both ages compared with their age-matched NTG littermates (Table 1, all P < 0.05). Compared with NTG mice, both latent and active forms of MMP-2 were significantly higher in the LV of β_2-TG at both ages studied (Figure 4A, all P < 0.05). Further, increased collagen content in the LV was evident in 5-month-old β_2-TG mice (P < 0.05 vs. age-matched NTG), and became more pronounced in the 15-month-old β_2-TG group (P < 0.05 vs. either 15-month-old NTG or 5-month-old β_2-TG mice, Figure 4B).

β_2-TG mice exhibited progressive cardiac remodelling and dysfunction

TG mice (5-month-old) showed enhanced systolic function and active relaxation at diastole in comparison with their NTG counterparts (Table 2, Figure 5A). End-diastolic volume, end-systolic volume, ejection fraction (EF) and stroke volume were however comparable between 5-month-old NTG and TG mice. Heart rates of 5-month-old TG mice were also higher than those of NTG mice. β_2-TG mice at this age already exhibited LV stiffening, measured as the end-diastolic pressure-volume relationship (P < 0.05 vs. NTG, Table 2, Figure 5B).

β_2-TG mice developed cardiomyopathy as they aged (Table 2, Figure 5). Aged TG mice (15-month-old) showed significant reduction in parameters including dP/dt_{max}, dP/dt_{min}, dV/dt_{min}, EF, stroke volume, preload-adjusted maximal power and preload-recruitable stroke work relationship (M_w), together with increased LVEDP, τ, and LVDd or LVDs (all P < 0.05) in comparison either with 5-month-old TG mice or with age-matched NTG mice. LV stiffening was more exaggerated in 15-month-old TG mice (P < 0.05 vs. both 15-month-old NTG mice and 5-month-old TG mice, respectively, Table 2, Figure 5B).

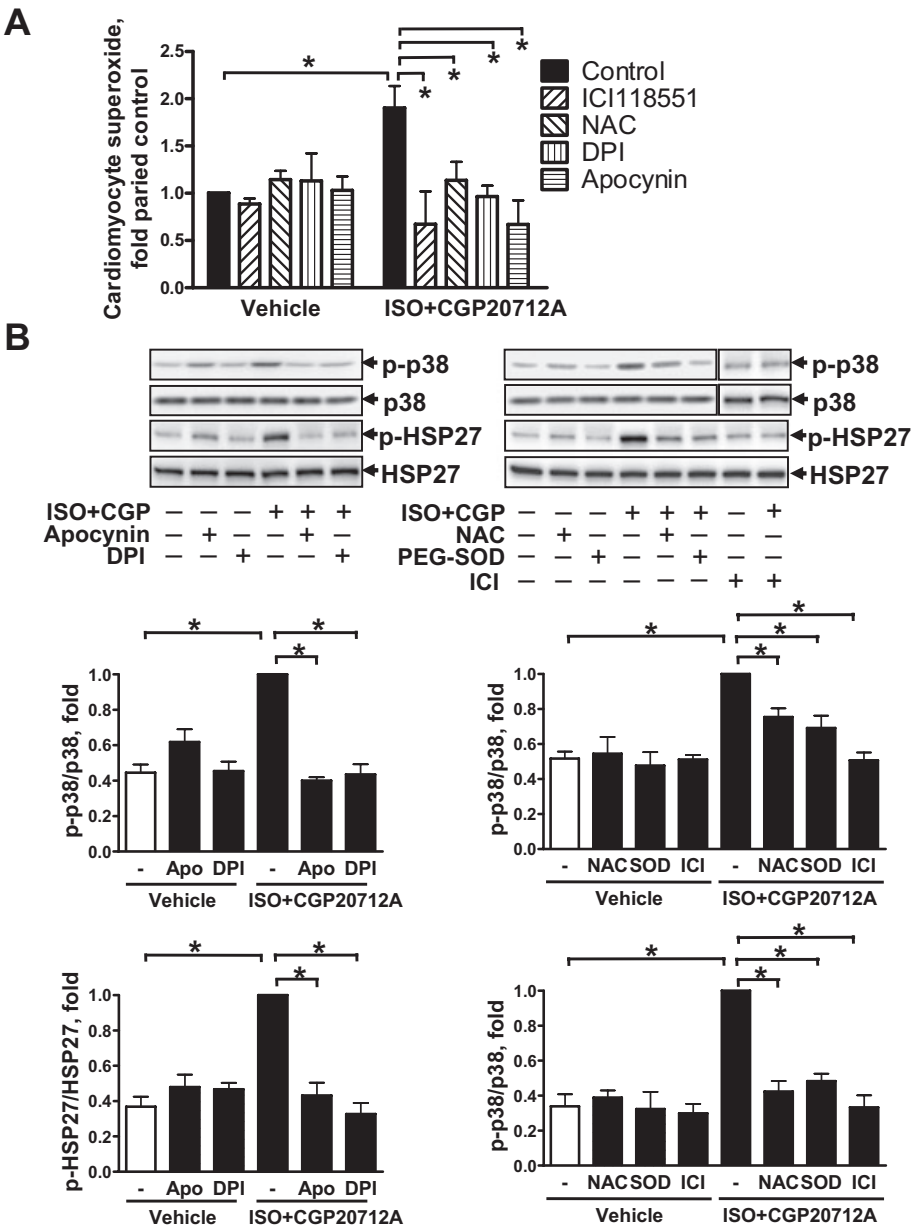

Figure 3

p38 MAPK activation by NADPH oxidase-derived ROS in cultured cardiomyocytes following β_2-adrenoceptor stimulation. A, Serum-starved cells were loaded with 10 μmol·L^{-1} H_2DCFDA for 30 min at 37°C. Cells were then preincubated with CGP20712A (CGP, 1 μmol·L^{-1}, selective β_1-adrenoceptor antagonist), together with ICI118551 (ICI, 1 μmol·L^{-1}, selective β_2-adrenoceptor antagonist), NAC (1 mmol·L^{-1}), DPI (10 μmol·L^{-1}) or apocynin (100 μmol·L^{-1}), respectively, for 30 min, and then stimulated with isoprenaline (ISO, 1 μmol·L^{-1}) for 10 min. Cellular fluorescence intensity was measured using a fluorescence reader and expressed as the -fold increase of unstimulated cells. B, Cells were preincubated with CGP, together with apocynin, DPI, NAC, PEG-SOD (25 U·mL^{-1}) or ICI, respectively, for 30 min, and then stimulated with ISO for 10 min. Western blot analyses were performed using antibodies against phospho-p38 MAPK, p38 MAPK, phospho-HSP27 and HSP27 respectively. The blot shown is a representative of three similar experiments. Pooled data are presented as relative changes to ISO + CGP stimulated cells. *P < 0.05 versus ISO + CGP.

NAC inhibited oxidative stress and restored gene expression in β_2-TG hearts

N-acetylcysteine (500 mg·kg^{-1}·day^{-1}) was given to 7-month-old β_2-TG mice for 2 weeks. This dose was chosen based on previous studies in mice, showing abolition of the oxidative stress and disease progression (Byrne *et al.*, 2003). LV sections stained with fluorescent probe DHE revealed a diminished oxidative fluorescent signal in the LV of NAC-treated β_2-TG mice (Figure 6A). NAC treatment significantly

Table 1

LV gene expression of inflammatory cytokines and profibrotic factors in β_2-TG mice

	5 months old		15 months old		7–8 months old		
	NTG	β_2-TG	NTG	β_2-TG	NTG	β_2-TG	β_2-TG + NAC
TNF-α	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	1.7 ± 0.3*	1.0 ± 0.3	2.1 ± 0.4*	0.3 ± 0.1‡
IL-1β	1.0 ± 0.1	3.2 ± 0.9*	1.0 ± 0.2	3.4 ± 0.7*	1.0 ± 0.5	4.3 ± 1.2*	0.2 ± 0.2‡
IL-6	1.0 ± 0.2	6.9 ± 2.0*	1.0 ± 0.3	9.1 ± 2.5*	1.0 ± 0.5	16.2 ± 4.3*	6.9 ± 2.0‡
MCP-1 (CCL2)	1.0 ± 0.2	3.1 ± 0.6*	1.2 ± 0.3	2.7 ± 0.5*	–	–	–
VCAM-1	1.0 ± 0.1	1.4 ± 0.3	1.3 ± 0.2	2.0 ± 0.2*	–	–	–
TGF-β	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	–	–	–
CTGF	1.0 ± 0.1	1.7 ± 0.2*	1.0 ± 0.1	6.9 ± 0.7*†	1.0 ± 0.2	3.5 ± 0.3*	1.2 ± 0.1‡
α-SMA	1.0 ± 0.1	1.9 ± 0.2*	1.2 ± 0.2	1.8 ± 0.1*	1.0 ± 0.3	3.1 ± 0.6*	1.3 ± 0.4‡
Procollagen I	1.0 ± 0.2	2.7 ± 0.5*	1.3 ± 0.3	4.4 ± 0.6*	–	–	–
Procollagen III	1.0 ± 0.1	3.8 ± 0.5*	1.3 ± 0.4	8.6 ± 1.0*†	1.0 ± 0.4	5.3 ± 1.5*	1.2 ± 0.4‡

β_2-TG + NAC, β_2-TG mice treated with NAC for 2 weeks. Data are folds of the respective NTG control.
*$P < 0.05$ versus age-matched NTG; †$P < 0.05$ versus 5-month-old β_2-TG; ‡$P < 0.05$ versus 7- to 8-month-old untreated β_2-TG. $n = 6$–11/group.

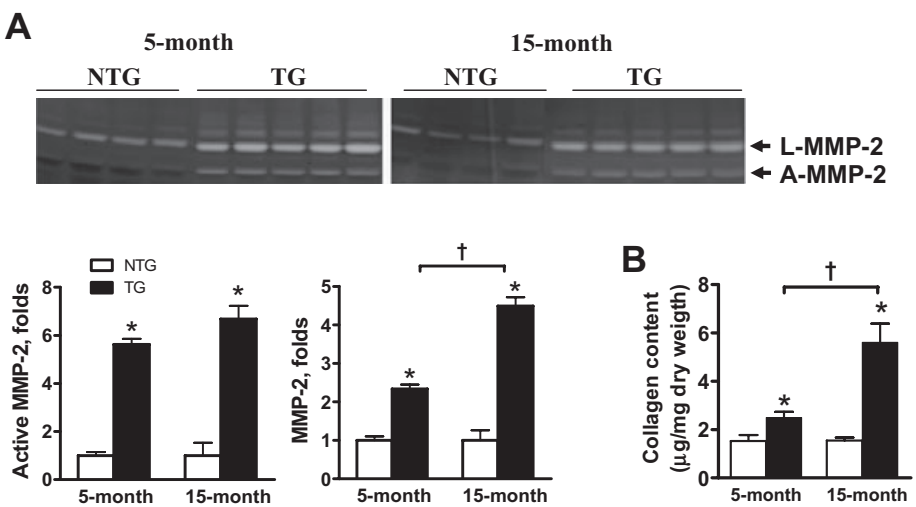

Figure 4

Myocardial fibrosis and extracellular matrix remodelling in the LV of β_2-TG mice. A, Gelatine zymography revealed increased levels of activated and latent forms of MMP-2 (A-MMP-2 and L-MMP-2) in β_2-TG than NTG mice (5- and 15-month-old). Data are presented as changes relative to age-matched NTG mice ($n = 5$–9/group). B, Collagen content in the LV of β_2-TG and NTG mice (5 and 15 months of age, $n = 5$–9/group) was quantified by hydroxyproline assay. *$P < 0.05$ versus respective NTG controls; †$P < 0.05$ versus untreated β_2-TG group.

reduced LV superoxide production and NADPH oxidase activity as quantitated by ESR assay and NADPH-enhanced lucigenin assay (both $P < 0.05$ vs. untreated β_2-TG group, $n = 7$–11/group, Figure 6B). Further, up-regulated mRNA levels of NOX2 and NOX4 isoforms in the LV of β_2-TG mice were eliminated by NAC treatment (both $P < 0.05$, Figure 6C). Administration of NAC for 2 weeks abolished the increased mRNA expression of TNF-α, IL-1β, IL-6, CTGF, α-SMA and procollagen-3 (all $P < 0.05$, Table 1). Administration of the p38 MAPK inhibitor SB202190 (2 mg·kg^{-1}, i.p. twice daily) for 24 h also abolished the mRNA expression of TNF-α (0.4 ± 0.1 vs. 1.4 ± 0.1, $P < 0.05$), IL-1β (1.2 ± 0.2 vs. 2.4 ± 0.4, $P < 0.05$) and IL-6 (4.7 ± 1.1 vs. 10.7 ± 1.9, $P = 0.11$) in the LV of β_2-TG mice.

Interestingly, NAC treatment for 2 weeks also increased the mRNA expression of β_2-adrenoceptor transgene by 31% and restored that of α-MHC in the LV of β_2-TG mice, and the changes in

Table 2

Haemodynamic parameters of NTG and β_2-TG mice at 5 and 15 months of age

	5 months of age		15 months of age	
	NTG	TG	NTG	TG
N	8	6	4	3
Heart rate, bpm	348 ± 5	497 ± 15*	344 ± 4	527 ± 41*
LV end-systolic pressure, mmHg	123 ± 7	117 ± 4	122 ± 11	129 ± 10†
LV end-diastolic pressure, mmHg	3 ± 1	4 ± 1	4.8 ± 1.1	21.3 ± 4.8*†
LV EDV, μL	44 ± 5	46 ± 6	35 ± 2	61 ± 8*†
LV ESV, μL	28 ± 4	29 ± 5	20 ± 2	50 ± 9*†
Ea, mmHg·μL^{-1}	7 ± 1	6 ± 1	7 ± 1	11 ± 1*†
Ejection Fraction, %	43.0 ± 2.3	45.8 ± 5.0	46.3 ± 3.0	20.9 ± 4.5*†
Stroke Volume, μL	19 ± 2	20 ± 1	17 ± 1	12 ± 1*†
dP/dt_{max}, mmHg·s^{-1}	7784 ± 524	14 082 ± 869*	7405 ± 257	9557 ± 1052†
dP/dt_{min}, mmHg·s^{-1}	−6066 ± 339	−9489 ± 482*	−5836 ± 403	−5926 ± 589†
dV/dt_{max}, μL·s^{-1}	673 ± 75	1013 ± 122*	646 ± 37	818 ± 114
dV/dt_{min}, μL·s^{-1}	−685 ± 72	−1032 ± 89**	−700 ± 50	−634 ± 44†
τ, ms	9.01 ± 0.65	5.41 ± 0.20*	9.1 ± 0.9	8.8 ± 2.0†
Ees, mmHg·μL^{-1}	3.62 ± 0.38	6.63 ± 1.12*	4.8 ± 0.5	7.5 ± 2.5
Preload adjusted maximal power, mW·mL^{-2}	49.8 ± 8.3	94.0 ± 15.5*	67.4 ± 6.8	25.3 ± 7.4*†
dPdt – EDV, mmHg·s^{-1}·μL^{-1}	157 ± 29	448 ± 84*	183 ± 33	265 ± 83
dP/dt_{max}/IP, s^{-1}	105 ± 7	172 ± 7*	113 ± 8	116 ± 9
M_w, erg·cm^{-3}·10^3	105 ± 7	172 ± 7*	95 ± 8	26 ± 6*†
Peak filling rate/EDV, s^{-1}	15.5 ± 0.7	23.5 ± 2.5*	18.5 ± 1.6	15.6 ± 5.0
EDPVR, mmHg·μL^{-1}	0.28 ± 0.04	0.46 ± 0.06*	0.24 ± 0.04	1.66 ± 0.52*†

*P < 0.05 versus NTG; †P < 0.05 versus 5-month-old TG.
Ea, effective arterial elastance; EDPVR, end-diastolic P–V relationship; EDV, end-diastolic volume; Ees, ventricular end-systolic elastance; ESV, end-systolic volume; IP, instantaneous pressure; M_w, preload recruitable stroke work relationship; LV, left ventricle.

the latter two variable were well correlated (Figure 6D). Further, NAC treatment restored the density of LV β_2-adrenoceptor in TG mice to 2062 ± 204 fmol·mg^{-1} membrane protein (Figure 6D), which is similar to that in 2- to 3-month-old TG mice free of cardimyopathy (1943 ± 291 fmol·mg^{-1}).

Antioxidant therapy arrested progression of cardiomyopathy in β_2-TG mice

Based on the above effects obtained by 2-week NAC treatment, we then examined the long-term efficacy of NAC therapy (500 mg·kg^{-1}·day^{-1}) by treating β_2-TG mice for a period of 3 months from 7 to 10 months of age, when they display a rapid progression in cardiomyopathy and HF (Du *et al.*, 2000). By serial echocardiography, LV chamber size and function remained stable in NTG littermates (n = 11) during this period. β_2-TG mice at 7 months of age had similar LV chamber size and enhanced LV contractile function compared with NTG mice. However, untreated β_2-TG mice (n = 17) showed LV dilatation and impaired LV function at 10 months of age (Figure 7, Table 3). Treatment with NAC (n = 6) did not alter the tachycardia phenotype in β_2-TG mice, but significantly attenuated the progression of cardiomyopathy, evidenced by prevention of LV dilatation (LVDd, LVDs, end-diastolic volume and end-systolic volume) and preserved LV function (fractional shortening, EF and preload recruitable stroke work, Figure 7, Table 3, all P < 0.05 vs. untreated β_2-TG group). LV stiffening, estimated from end-diastolic pressure-volume relationship, was also attenuated by NAC treatment (P < 0.05).

Further, NAC treatment for 3 months significantly suppressed the levels of active and latent forms of MMP-2 (both P < 0.05 vs. untreated TG group, Figure 8A), alleviated cardiomyocyte hypertrophy (P < 0.05) and lowered LV fibrosis, as quantified by Picrosirius staining (P < 0.05) or hydroxyproline assay (P = 0.20, Figure 8B). Myocyte apoptosis was detected by the presence of TUNEL-positive cardiomyocyte nuclei in the LV of untreated

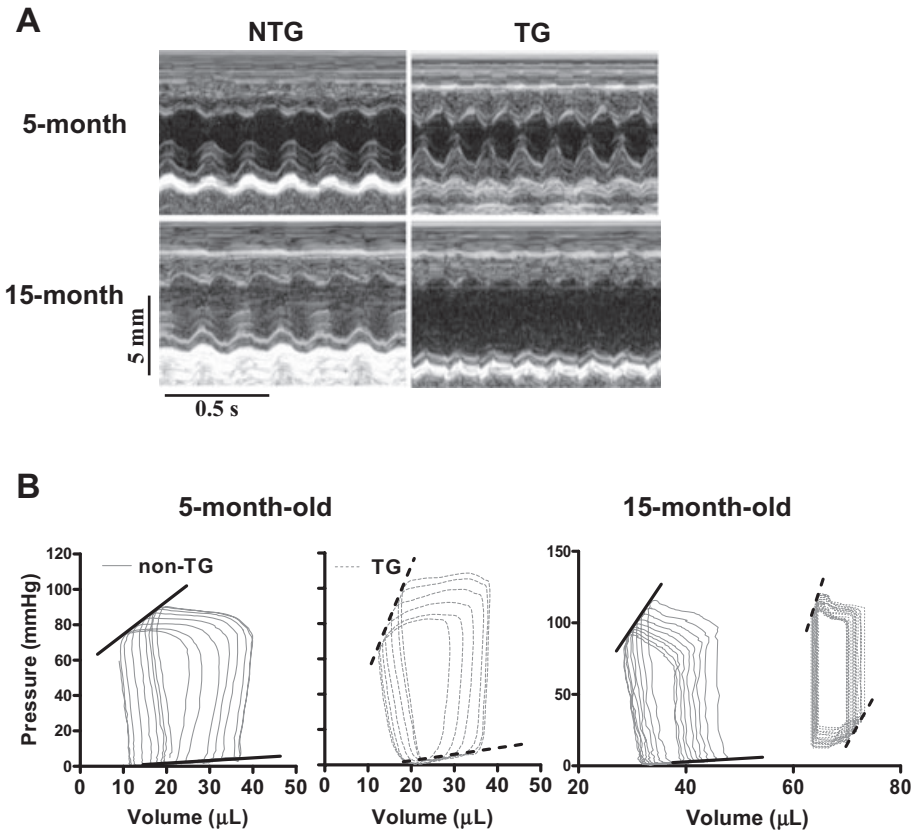

Figure 5

β_2-TG mice developed progressive LV dysfunction with aging. Representative M-mode echocardiographic tracings from short-axis LV 2-D images (A) and pressure-volume loops (B) from NTG and β_2-TG mice (5- and 15-month-old), β_2-TG mice at 5 months of age manifested similar chamber size and enhanced contractility, while they displayed impaired LV compliance versus their NTG counterparts. Older β_2-TG mice (15-month-old) showed stiffened LV, impaired systolic and diastolic function and dilated LV.

β_2-TG mice, and this was significantly reduced by NAC treatment (Figure 8C, $P < 0.05$).

Discussion

In the current study, we have shown that production of NADPH oxidase-derived ROS is fundamental to cardiac β_2-adrenoceptor adverse signalling. Transgenic activation of β_2-adrenoceptor in cardiomyocytes leads to a sustained elevation of NADPH oxidase activity, which is accompanied by a greater ROS production as well as phosphorylation of p38 MAPK. Inhibition of NADPH oxidase or ROS significantly reduced the p38 MAPK signalling cascade. Further, we provided novel findings that upon chronic β_2-adrenoceptor activation, ROS up-regulation represents an early event contributing to the initiation and progression of the cardiomyopathy and HF phenotype. Chronic β_2-adrenoceptor activation *in vivo* is associated with greater extent of cardiac dilatation and dysfunction as well as augmented pro-inflammatory and pro-fibrotic signalling, while antioxidant treatment protected hearts against these abnormalities, indicating ROS production to be central to the detrimental signalling of β_2-adrenoceptors. Collectively, these results provide new insight to the non-classical signal pathway of β_2-adrenoceptor through NADPH oxidase/ROS/p38 MAPK in contributing to the pathogenesis of cardiomyopathy and HF.

Enhanced β-adrenoceptor stimulation exacerbates a range of pathological abnormalities, including loss of cardiomyocyte, inflammation, fibroblast proliferation and extracellular matrix (ECM) remodelling (Bristow, 2000). In hypertrophic and failing hearts, desensitization and down-regulation of β_1-adrenoceptors together with a reduced β_1/β_2 ratio have been well documented both clinically and experimentally (Bristow *et al.*, 1986; Feldman *et al.*, 2008), implying a more important role of β_2-adrenoceptor in failing hearts. To date, the role of β_2-adrenoceptor in the progression of HF remains controversial. Whereas *in vitro* and *in vivo*

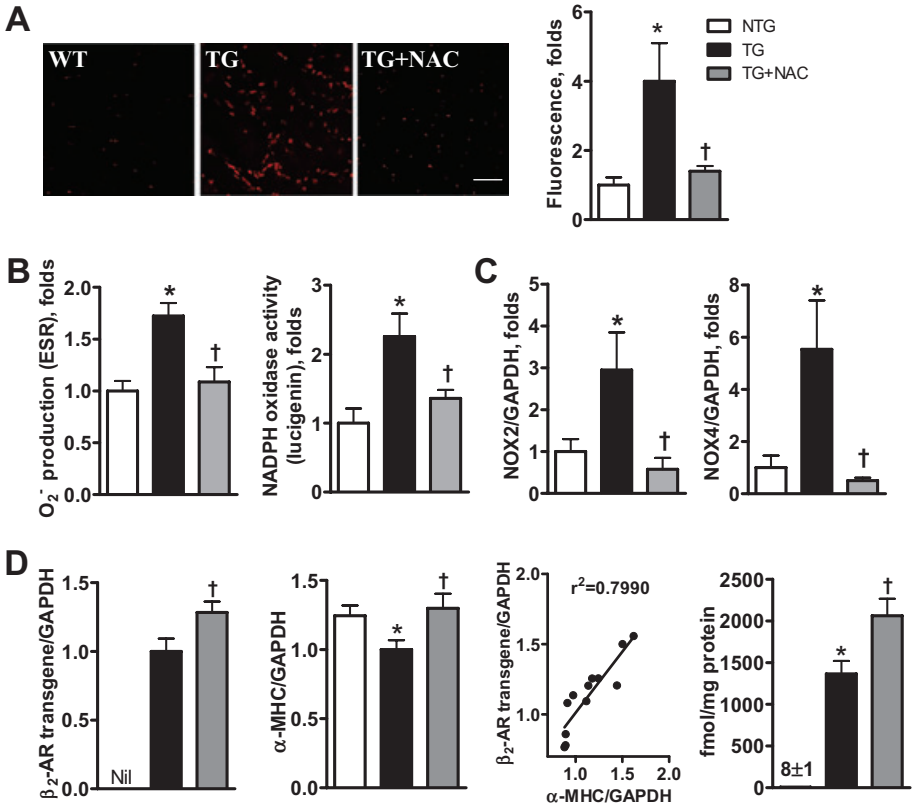

Figure 6

The antioxidant NAC significantly reduced oxidative stress and restored gene expression in the LV of β_2-TG mice. β_2-TG mice (7-month-old) were treated with NAC (500 mg·kg^{-1}·day^{-1} in drinking water) for 2 weeks. ROS production was determined by DHE fluorescence, Bar = 50 μm (A), electron spin resonance assay using superoxide probe, CMH, or lucigenin-enhanced chemiluminescence assay (B). C, quantitative real-time PCR for mRNA expression of NOX2 and NOX4 with data presented as changes relative to NTG (5–10/group). D, mRNA expression of β_2-adrenoceptor (β_2-AR) transgene and endogenous α-MHC was assayed by quantitative real-time PCR, and expressed as -fold changes relative to untreated β_2-TG mice. Changes in the expression level of human β_2-adrenoceptor were plotted against that of α-MHC. β_2-adrenoceptor density was assayed by binding assay (n = 6/group). *P < 0.05 versus NTG mice; †P < 0.05 versus untreated β_2-TG group.

studies indicate that β_2-adrenoceptor activation may be beneficial (Zhu *et al.*, 2001; Ahmet *et al.*, 2008), long-term β_2-adrenoceptor stimulation has been shown to provoke detrimental consequences (Hirono *et al.*, 2001; Cazzola *et al.*, 2005; Molenaar *et al.*, 2006; Ryall *et al.*, 2008). Moreover, Nikolaev *et al.* recently observed that in failing cardiomyocyte β_2-adrenoceptors acquire a similar distribution and cAMP signalling pattern as β_1-adrenoceptors. β_2-Adrenoceptors redistribute from T-tubules, their normal location, to cell crests, which is accompanied by a transition from compartmented cAMP signalling to diffused cAMP activation (Nikolaev *et al.*, 2010). Although it is premature to link the redistribution of β_2-adrenoceptors causally to HF progression, β_2-adrenoceptors become widely distributed across the cell surface and mediate widespread signalling. We postulate that such changes in the distribution and signalling of β_2-adrenoceptor in the failing versus normal heart might also increase its 'non-classic' signalling mechanisms, such as the NADPH oxidase/ROS/p38 MAPK pathway. So far the membrane localization of β_2-adrenoceptors in this TG model remains undetermined. However, we speculate that with the marked transgenic overexpression, β_2-adrenoceptors are likely to be similarly distributed extensively over the cell surface, and are coupled to machinery leading to global and adverse intracellular signalling. Indeed, the β_2-TG mice manifested transgene number-dependent premature death, ventricular dysfunction and dilatation (Du *et al.*, 2000; Liggett *et al.*, 2000; Peter *et al.*, 2007). Although low level overexpression of β_2-adrenoceptor does not result in overt cardiomyopathy phenotype up to 12 months of age, it remains unknown whether those TG mice will develop HF with aging (Liggett *et al.*, 2000). In sharp contrast, knockout of both β_1- and β_2-adrenoceptors largely abolished pressure overload-induced hypertrophy and fibrosis and preserved LV function (Kiriazis *et al.*, 2008), together

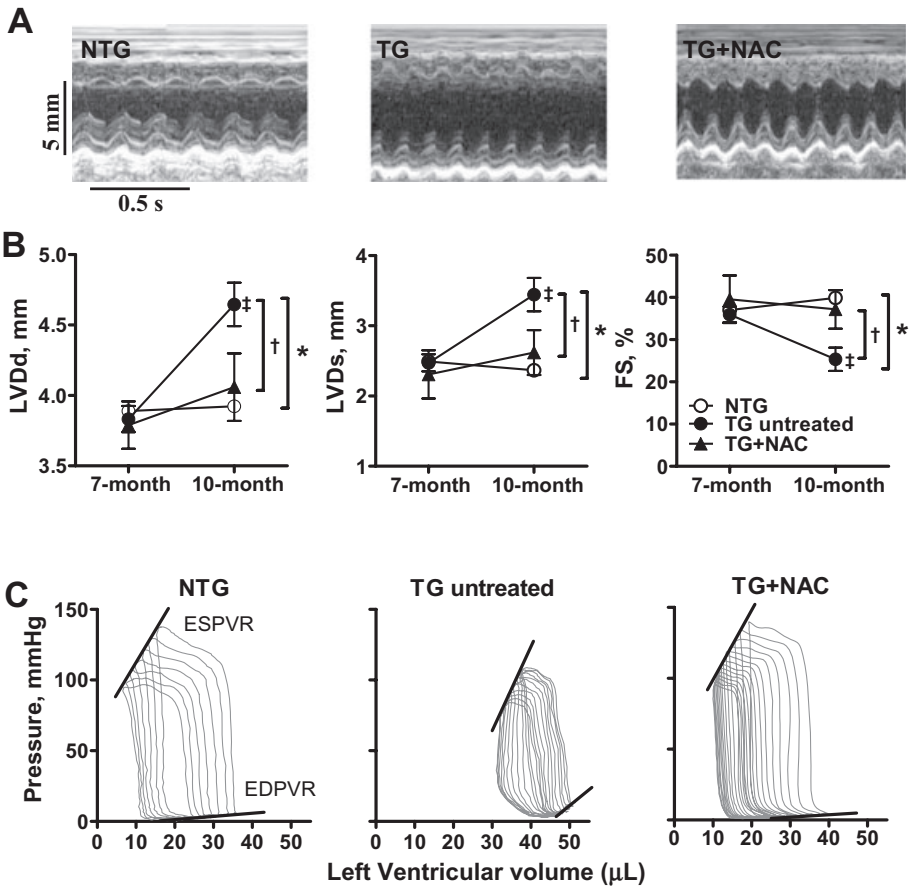

Figure 7

Chronic treatment with NAC prevented the progression of cardiomyopathy. β_2-TG and NTG mice (7-month-old) were treated with NAC (500 mg·kg^{-1}·day^{-1} in drinking water) for 3 months. LV function was assessed by echocardiography (A, B) and pressure-volume catheterization (C). LVDd, LV dimension at end-diastole; LVDs, LV dimension at end-systole; FS, fractional shortening; EDPVR, end-diastolic pressure-volume relationship; ESPVR, end-systolic pressure-volume relationship. Data (n = 6–17/group) were analysed by two-way repeated measures ANOVA followed by *post hoc* tests. *P < 0.05 versus NTG mice; $^{\dagger}P$ < 0.05 versus untreated β_2-TG mice; $^{\ddagger}P$ < 0.05 versus same mice at 7-month-old.

with the prevention of enhanced ROS formation (Xu *et al.*, unpubl. data).

Our study reveals that ROS production is pivotal for the age-dependent progression of cardiomyopathy seen with prolonged myocardial β_2-adrenoceptor activation. We have shown that β_2-TG mice had an augmented cardiac ROS production and p38 MAPK activation, changes accompanied by cardiomyocyte apoptosis, inflammation, interstitial fibrosis and enhanced MMP-2 expression, as well as *in vivo* findings of marked LV dysfunction and remodelling with aging (Du *et al.*, 2000; Peter *et al.*, 2007). The antioxidant NAC abolished all the morphological abnormalities and prevented progressive LV remodelling and dysfunction in this model.

Further, we showed NADPH oxidase is an important source of ROS subsequent to β_2-adrenoceptor activation, and that p38 MAPK serves as a downstream target of NADPH oxidase/ROS in both intact hearts and cultured cardiomyocytes. Of several biochemical sources for ROS, our recent work in HEK293 cells identified NADPH oxidase as a key mediator in β_2-adrenoceptor/p38 MAPK signalling (Gong *et al.*, 2008). NADPH oxidases are known to be involved in redox signalling and cardiac remodelling evoked by diverse hormonal or mechanical stimuli (Takimoto and Kass, 2007). In addition, ROS derived from NADPH oxidase initiate oxidative stress that is amplified further by mechanism such as eNOS uncoupling following oxidation of tetrahydrobiopterin or activation of xanthine oxidase (Takimoto and Kass, 2007). Here, we have attempted to generate *in vivo* evidence for a role of NADPH oxidase-derived ROS in the adverse signalling and detrimental cardiac effects of β_2-adrenoceptors. In β_2-TG hearts, expression of two major NADPH oxidase isoforms (Byrne *et al.*, 2003), NOX2 and NOX4, together with overall NADPH oxidase activity, are markedly increased. Treatment with either the antioxidant (NAC) or NADPH oxidase inhibitors

Table 3

Haemodynamic parameters of 10-month-old NTG and β_2-TG mice following a 3-month period of treatment with NAC

	NTG	β_2-TG	β_2-TG + NAC
number	6	5	6
Heart rate, beats/min	348 ± 5	522 ± 29*	526 ± 22*
LV systolic pressure, mmHg	120 ± 8	115 ± 11	104 ± 5
LV end-diastolic pressure, mmHg	3.3 ± 0.9	11.0 ± 2.3*	6.5 ± 1.1†
LV end-diastolic volume, μL	36 ± 2	58 ± 6*	33 ± 4†
LV end-systolic volume, μL	21 ± 1	40 ± 8*	18 ± 3†
Ea, mmHg·μL^{-1}	7.2 ± 0.7	6.6 ± 1.5	6.0 ± 0.6
Ejection Fraction, %	46.5 ± 2.3	31.8 ± 4.0*	52.3 ± 4.8†
Stroke Volume, μL	15 ± 1	18 ± 3	15 ± 2
dP/dt_{max}, mmHg·s^{-1}	7212 ± 348	11 078 ± 900*	10 760 ± 969*
dP/dt_{min}, mmHg·s^{-1}	5828 ± 363	7463 ± 499	8228 ± 770
dV/dt_{max}, μL·s^{-1}	618 ± 38	1029 ± 119*	796 ± 108
dV/dt_{min}, μL·s^{-1}	630 ± 49	871 ± 141	917 ± 104
τ, ms	8.6 ± 0.7	5.3 ± 0.5*	5.2 ± 0.1*
Ees, mmHg·μL^{-1}	4.3 ± 0.3	5.4 ± 1.9	7.9 ± 1.9
Preload adjusted maximal power, mW·mL^{-2}	61.5 ± 6.7	55.8 ± 12.0	106.3 ± 20.5
dP/dt – EDV, mmHg·s^{-1}·μL^{-1}	155 ± 14	266 ± 63	584 ± 134
dP/dt_{max}/IP, s^{-1}	104 ± 7	154 ± 13*	161 ± 8
M_w, erg·cm^{-3}·10^3	134 ± 18	36 ± 8*	98 ± 11†
Peak filling rate/EDV, s^{-1}	17.4 ± 1.1	19.6 ± 3.8	24.8 ± 3.5
EDPVR, mmHg·μL^{-1}	0.28 ± 0.04	0.79 ± 0.11*	0.40 ± 0.11†

*$P < 0.05$ versus NTG; †$P < 0.05$ versus untreated β_2-TG.
Ea, effective arterial elastance; EDPVR, end-diastolic P-V relationship; EDV, end-diastolic volume; Ees, ventricular end-systolic elastance; IP, instantaneous pressure; LV, left ventricle; M_w, preload recruitable stroke work relationship.

(apocynin, DPI) blunted the hyper-phosphorylation of p38 MAPK and HSP27 in β_2-TG hearts, suggesting that p38 MAPK and HSP27 are downstream targets of ROS, and therefore might negatively affect myocardial contractility (Liao *et al.*, 2002). Interestingly, NAC treatment abolished NOX2 and NOX4 up-regulation in β_2-TG hearts. It implies that there exists a positive feedback amplifying the ROS/p-38 signalling evoked by β_2-adrenoceptor activation. We speculate that enhanced inflammatory (Anrather *et al.*, 2006; St Hilaire *et al.*, 2008) and fibrogenic (Hecker *et al.*, 2009) signalling is responsible for the up-regulated NOX expression and that this involves the interaction of cardiomyocytes with non-cardiomyocytes.

p38 MAPK mediates a variety of adverse cardiac events (Liao *et al.*, 2002; Chen *et al.*, 2003; Li *et al.*, 2005; Ren *et al.*, 2005). Because p38 MAPK activity was elevated by β_2-adrenoceptor activation, we therefore selected p38 MAPK as the key molecule in mediating the adverse signalling of β_2-adrenoceptor/ROS. Loss of cardiomyocytes is a key contributor to the progression of LV remodelling and dysfunction. In cultured adult cardiomyocytes, ROS, p38 MAPK, jun N-terminal kinase and Ca^{2+}/calmodulin-dependent protein kinase II are involved in cardiomyocyte apoptosis induced by β-adrenoceptor stimulation (Remondino *et al.*, 2003; Zhu *et al.*, 2003; Ren *et al.*, 2005; Peter *et al.*, 2007). Based on our findings, we propose that the pro-apoptotic effect of ROS in the β_2-TG heart is likely mediated by the activation of p38 MAPK. This view is also supported by recent findings that gene silencing of NADPH in cells effectively suppressed p38 MAPK activation (Gong *et al.*, 2008) and that while jun N-terminal kinase is not activated in this β_2-TG model, inhibiting p38α MAPK by gene complementation rescued HF and cardiomyopathy, and reduced apoptosis in the same β_2-TG heart *in vivo* (Peter *et al.*, 2007).

Inflammatory cytokines contribute significantly to the progression of HF and activation of p38 MAPK is known to promote cardiac inflammation, interstitial fibrosis and MMP abundance (Li *et al.*, 2005). In the β_2-TG model, up-regulation of pro-inflammatory cytokines is evident at 5 months

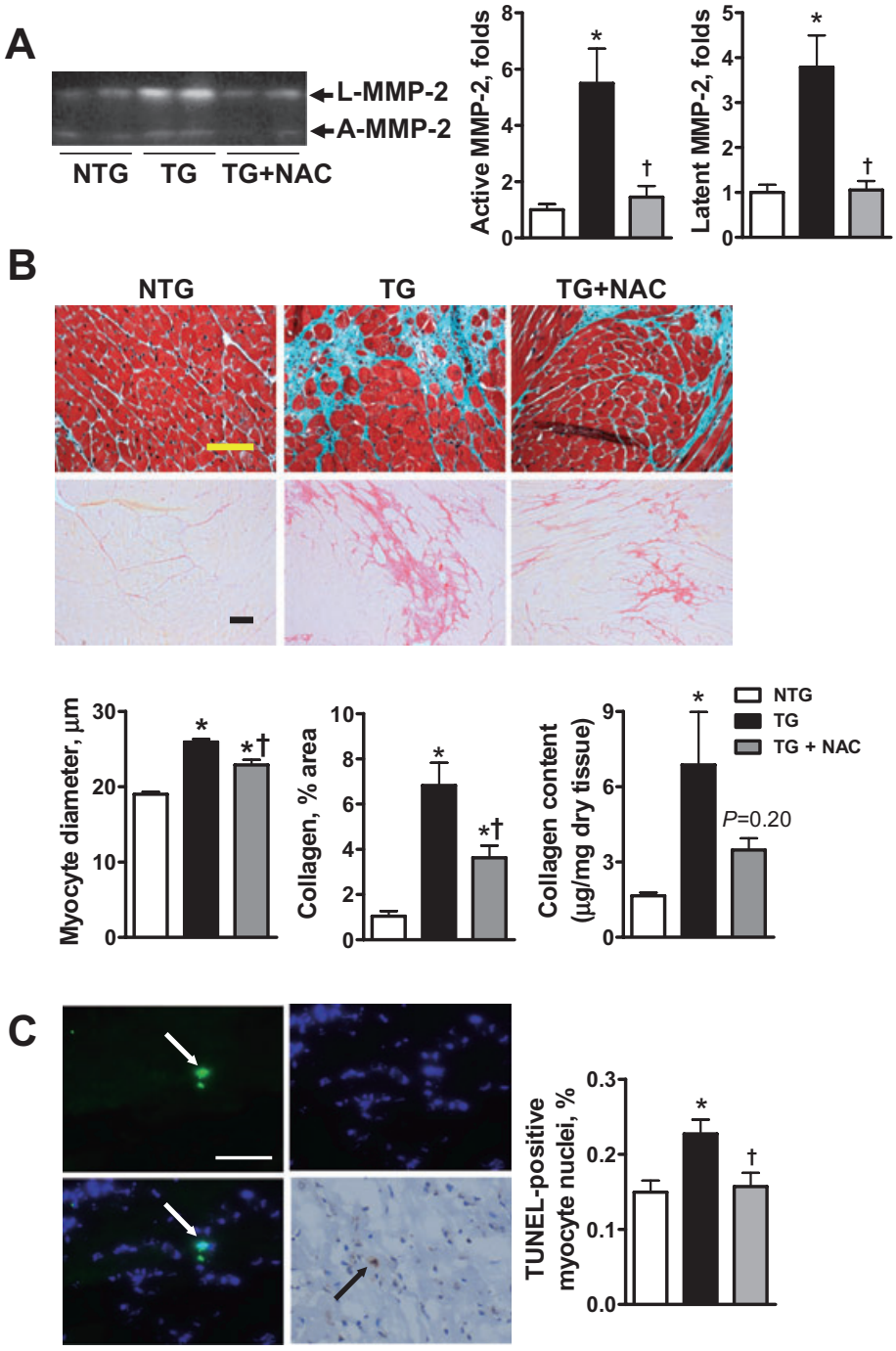

Figure 8

Chronic treatment with NAC halted extracellular matrix remodelling and cardiomyocyte apoptosis. A, gelatine zymography was performed to determine the level of active and latent form of MMP-2 (A-MMP-2 and L-MMP-2) in the LV of β_2-TG and NTG mice after 3 months of NAC treatment. Data are presented as relative changes to NTG mice (n = 6–12/group). B, LV sections of NTG, untreated and NAC-treated β_2-TG mice were stained with Masson's trichrome or Picrosirius red (bar = 100 μm). Cardiomyocyte diameter and LV collagen content were measured (n = 3/group). LV collagen content was quantified by hydroxyproline assay (n = 6–12/group). C, TUNEL-positive LV cardiomyocyte nuclei (arrows) were counted under fluorescent or light microscopy (left panels) counterstained with DAPI (top right) or haematoxylin (bottom right). Results are expressed as a percentage of total cardiomyocytes (4000–6000/heart). *P < 0.05 versus NTG mice; †P < 0.05 versus untreated β_2-TG mice. n = 4/group.

of age when a cardiomyopathy phenotype is not evident. NAC treatment of β_2-TG mice during 7–10 months of age, when cardiomypathy undergoes rapid deterioration (Du *et al.*, 2000), reduced cytokine expression and normalized ROS levels. The link between β_2-adrenoceptor/ROS and the inflammatory signalling is further supported by our findings that deletion of both β_1- and β_2-adrenoceptor eliminated the enhanced oxidative and cytokine expression evoked by pressure overload (Xu *et al.*, unpubl. data) and inhibition of p38 MAPK by SB202190 in β_2-TG mice attenuated the increased pro-inflammatory cytokines.

In β_2-TG mice, β_2-adrenoceptor activation is restricted to cardiomyocytes (Milano *et al.*, 1994). Interestingly, one of the earliest signs of cardiomyopathy in this model is interstitial fibrosis, apparent as early as 5 months of age and progressing with age. In fact, genetic modifications resulting in tonic activation of the sympatho/β-adrenoceptor signalling all lead to a fibrotic cardiomyopathy (Engelhardt *et al.*, 1999; Du *et al.*, 2000; Liggett *et al.*, 2000; Antos *et al.*, 2001; Brum *et al.*, 2002; Hardt *et al.*, 2002). The mechanism mediating sympatho/β-adrenoceptor activation and ECM remodelling, however, remains less defined. In our study, NAC treatment effectively inhibited transformation of fibroblasts into myofibroblasts, measured by α-SMA, and collagen expression. Therefore, in this model, ECM remodelling is also one of the adverse consequences of ROS generated by cardiomyocyte with transgenic β_2-adrenoceptor activation and involves cardiomyocyte-fibroblast interaction.

We showed that NAC treatment restored β_2-adrenoceptor density in the LV of β_2-TG mice. Our previous studies on the β_2-TG mice (Du *et al.*, 2000; Sheridan *et al.*, 2000; Gao *et al.*, 2003) have shown a down-regulation of β_2-adrenoceptor density as well as β_2-adrenoceptor transgene expression in settings of age-dependent development of cardiomyopathy or chronic pressure overload. In this model, human β_2-adrenoceptor transgene expression is driven by the same promoter as for endogenous α-MHC (Milano *et al.*, 1994; Du *et al.*, 2000). Interestingly, NAC treatment restored the expression of the transgene as well as endogenous α-MHC equally well. Therefore, in the failing heart, the increase of transgenic human β_2-adrenoceptor density by NAC treatment is attributable to a reinstallation of transcriptional activity of the α-MHC promoter. Further, as previous studies showed that higher β_2-adrenoceptor overexpression levels are associated with a more rapid progression of cardiomyopathy and HF (Liggett *et al.*, 2000), our findings of preserved cardiac function in the presence of a restored β_2-adrenoceptor density further emphasized the significance of arresting the adverse β_2-adrenoceptor signalling in HF therapy.

One limitation of this study is the use of a HF model due to transgenic β_2-adrenoceptor overexpression, which may not be a close simulation of HF in clinical settings. However, the model meets with our objective of exploring the adverse signalling pathway of β_2-adrenoceptors in cardiomyocytes. Future studies may use conventional HF models to clarify the beneficial effects of blocking the adverse signalling identified in this study.

In conclusion, our study links transgenic β_2-adrenoceptor activation to sustained enhancement of NADPH oxidase activity and ROS production in the heart *in vivo*, and demonstrates that ROS mediates p38 MAPK activation and subsequent abnormalities following chronic β_2-adrenoceptor activation. These findings highlight that the coupling of β_2-adrenoceptors with NADPH oxidase-derived ROS/p38 MAPK is pivotal to the adverse signalling mechanism, and thus forms a potential therapeutic target.

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Conflict of interest

The authors state no conflict of interest.

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