Published in final edited form as:

J Mol Cell Cardiol. 2009 November ; 47(5): 576–585. doi:10.1016/j.yjmcc.2009.06.005.

Adverse Ventricular Remodeling and Exacerbated NOS Uncoupling from Pressure-Overload in Mice Lacking the Beta3- Adrenoreceptor

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Abstract

Stimulation of the β -adrenergic system is important in the pathological response to sustained cardiac stress, forming the rationale for the use of β -blockers in heart failure. The β_3 -adrenoreceptor (AR) is thought to couple to the inhibitory G-protein, G_i , with downstream signaling through nitric oxide, although its role in the heart remains controversial. In this study, we tested whether lack of β_3 -AR influences the myocardial response to pressure-overload. Baseline echocardiography in mice lacking β_3 -AR ($\beta_3^{-/-}$) compared to wild type (WT) showed mild LV hypertrophy at 8 weeks that worsened as they aged. $\beta_3^{-/-}$ mice had much greater mortality after transverse aortic constriction (TAC) than WT controls. By 3 weeks of TAC, systolic function was worse. After 9 weeks of TAC, $\beta_3^{-/-}$ mice also had greater LV dilation, myocyte hypertrophy and enhanced fibrosis. NOS activity declined in $\beta_3^{-/-}$ -TAC hearts after 9 weeks, and total and NOS-dependent superoxide rose, indicating heightened oxidative stress and NOS uncoupling. The level of eNOS phosphorylation in $\beta_3^{-/-}$ -TAC hearts was diminished, and nNOS and iNOS expression levels were increased. GTP cyclohydrolase-1 expression was reduced, although total BH4 levels were not depleted. 3 weeks of BH4 treatment rescued $\beta_3^{-/-}$ mice from worsened remodeling after TAC, and lowered NOS-dependent superoxide. Thus, lack of β_3 -AR signaling exacerbates cardiac pressure-overload induced remodeling and enhances NOS uncoupling and consequent oxidant stress, all of which can be rescued with exogenous BH4. These data suggest a cardioprotective role for the β_3 -AR in modulating oxidative stress and adverse remodeling in the failing heart.

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DISCLOSURES

None of the authors has any conflicts of interest related to this manuscript.

Keywords

β 3-adrenoreceptor; hypertrophy; NOS uncoupling; oxidative stress; pressure-overload; tetrahydrobiopterin

INTRODUCTION

β 3-adrenoreceptors (β 3-AR) mediate lipolysis¹ and thermogenesis² in white and brown adipocytes. They are expressed in the heart, where they are thought to act via G_i-protein coupled activation of nitric oxide synthase (NOS).³ While studies have shown they can modulate cardiac contractility in both negative^{4,5} and positive directions,⁶ they have less effect on lusitropy,^{7,8} and their role in cardiac physiology and pathobiology remains unclear.⁹ Interestingly, β 3-ARs are activated at catecholamine levels higher than that required for β 1/ β 2-AR activation, and they lack phosphorylation sites for protein kinase A and β -adrenoreceptor kinase (β ARK), which are important for β 1/ β 2-AR receptor desensitization. Thus, β 3-AR signaling remains active during prolonged sympathetic stimulation even as β 1/ β 2-AR signals are diminished.¹⁰

In human myocardium, the β 3-AR selective agonist BRL 37344 (BRL) results in endothelial NOS activation accompanied by reduced cardiac contractility.¹¹⁻¹⁴ A similar decrease is observed in transgenic mouse with cardiac-specific overexpression of β 3-AR.⁵ The specificity of BRL-induced eNOS activation via β 3-AR is supported by data showing its absence in β 3^{-/-} mice¹⁵. Such cardio-depressant effects of β 3-AR stimulation have been suggested to contribute to impaired function in cardiac failure,^{8,16} particularly given relative downregulation of β 1-AR-signaling versus upregulated β 3-AR expression,¹³ and led to proposals for β 3-AR blockade as a therapeutic intervention to enhance inotropy in the failing heart. However, short term hemodynamic gain from increased inotropy is not necessarily beneficial in the long run. Chronic β 3-AR stimulation may actually provide protective effects¹⁷ via enhanced NOS signaling that are central to myocardial homeostasis. Blunting of NOS stimulation and/or NOS functional uncoupling occurs in disease models such as pressure-overload hypertrophy, contributing to maladaptive remodeling.^{18,19}

To determine which mechanism predominates in chronic disease, we tested the role of β 3-AR signaling in hearts subjected to sustained pressure-overload from transverse aortic constriction. We reveal marked exacerbation of pathologic remodeling, enhanced myocardial oxidant stress, and worsened NOS uncoupling in mice genetically lacking β 3-AR, supporting an important role for β 3-AR signaling in the heart.

METHODS

Experimental Model

Baseline echocardiography was performed on 8-week and 4-month-old homozygous β 3^{-/-} mice (n=57 total, original breeding pairs kindly provided by Dr. Bradford Lowell²⁰) and age-matched FVB background WT controls (n=13, Jackson Laboratories, Bar Harbor, Maine). Transverse aortic constriction (TAC) was performed on 8-week-old β 3^{-/-} mice (n=21) and age-matched male WT controls (n=24) as previously described.¹⁸ Briefly, after anesthesia with isoflurane (2%), the chest was opened through a small thoracic window between ribs 2 and 4, and a 25G-needle placed on the transverse aorta. This needle size was chosen to elicit a milder response as initial studies using our standard TAC model (27G needle) led to pulmonary edema and 100% early mortality in β 3^{-/-} mice. The band was secured using a 7.0 prolene suture, the needle was then removed and the chest closed. Twelve animals per strain underwent sham surgery. To measure pressure changes after TAC, pressure volume loops were obtained using

a Millar micromanometer catheter as previously described.¹⁴ Animals were sacrificed 3 or 9 weeks after TAC and myocardial tissue preserved in 10% formalin or snap-frozen in liquid nitrogen for subsequent analysis. To determine whether there were any differences in proximal pressures after TAC between strains, a 1.4 F pressure catheter (Millar Instruments, Houston, TX) was advanced into the ascending aorta from the LV, and pressures recorded before and after TAC. Mice were housed in a university animal facility with a 12-hour light-dark cycle and allowed water and food *ad libitum*. For tetrahydrobiopterin (BH4) treated mice, 200 mg/kg/day (Schircks Laboratories, Jona, Switzerland) or vehicle was mixed in soft diet. Animal treatment and care was provided in accordance with institutional guidelines. The Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all protocols and experimental procedures.

Echocardiographic evaluation

In vivo cardiac geometry and function were serially assessed by transthoracic echocardiography (Acuson Sequoia C256, 13 MHz transducer; Siemens) in conscious mice. M-mode LV end-systolic and end-diastolic cross-sectional diameter (LVESD, LVEDD), and the mean of septal and posterior wall thicknesses were determined from an average of 3-5 cardiac cycles. LV fractional shortening (%FS) and LV mass were determined using a cylindrical model as previously described.²¹

Histological evaluation and cellular morphometry

Myocyte cross sectional diameter was determined from 3-4 different hearts in each group, averaging results from >20 cells per heart. Digitized hematoxylin and eosin stained images were analyzed with Adobe Photoshop 7.0.1. Myocardial fibrosis was determined from Masson trichrome and picosirius red stained paraffin-embedded myocardial sections, the latter examined using standard as well as polarized light illumination. All slides were scored by a pathologist blinded as to tissue source using a semi-quantitative scale (0=absent; 3=marked fibrosis).¹⁹

Measurement of NOS activity

NOS calcium-dependent activity was determined from myocardial homogenates by measuring C¹⁴ arginine to citrulline conversion (assay kits from Stratagene, La Jolla, CA or Cayman Chemical, Ann Arbor, MI) as previously described.¹⁸

Measurement of total and NOS-dependent superoxide generation

Myocardial superoxide was assayed by lucigenin-enhanced chemiluminescence in snap frozen LV myocardium. Tissue was homogenized and equilibrated in Krebs-Hepes solution, and after sonification and centrifugation to remove cell debris and nuclei, the supernatant was added to a 5 μ M lucigenin-solution, containing 150 μ M NADPH. Baseline and maximum lucigenin-enhanced chemiluminescent signal were detected by a liquid scintillation counter (LS6000IC, Beckman Instruments, Fullerton, CA), with data reported as counts per minute per milligrams of tissue after background subtraction (cpm/mg).²² In the same experiment, N (G)-nitro-L-arginine methyl ester (L-NAME, 100 μ M) was added to another sample from each heart and the results subtracted from the total to determine NOS-dependent O₂⁻ generation.²³

Western blot analysis

Snap frozen heart tissues were homogenized in cell lysis buffer (Cell Signaling Technology, Danvers, MA) with 0.01% phosphatase inhibitor cocktails (Sigma, St. Louis, MO) and protease inhibitor PMSF (10 mM, Roche, Nutley, NJ). 60 μ g protein was loaded onto 8-16% Tris-Glycine Novex mini-gels (Invitrogen, Carlsbad, CA), electrophoreses and transferred to nitrocellulose or PDVF membranes. 10% SDS/PAGE gels and semi-dry transfer cell (Bio-Rad,

Hercules, CA) were used for NOS protein analysis. Primary antibodies were Akt: 1:1000, p-Akt: 1:250 (Cell Signaling, Danvers, MA); GTPCH-1: 1:500 (gift from Dr. Shimizu, Showa University, Japan); GAPDH: 1:10,000 (Imgenex, San Diego, CA) or 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); eNOS: 1:500 (BD Transduction Laboratories, San Diego, CA) or 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA); and p-eNOS (Serine 1177) 1/500 (Cell Signaling Technology, Danvers, MA); iNOS: 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); nNOS: 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were developed on film using enhanced chemiluminescence (SuperSignal West Pico and Femto, Pierce, Rockford, IL). Controls included: eNOS+: Bovine Aortic Endothelial cells treated with VEGF; eNOS-: eNOS^{-/-} heart tissue (Jackson Laboratories, Bar Harbor, Maine); nNOS+: rat brain lysate (Santa Cruz Biotechnology, Santa Cruz, CA); nNOS-: nNOS^{-/-} heart tissue (Jackson Laboratories, Bar Harbor, Maine); iNOS+: iNOS electrophoresis standard (Cayman Chemical, Ann Arbor, MI); iNOS-: iNOS^{-/-} heart tissue (Jackson Laboratories, Bar Harbor, ME).

BH4 Measurement

HPLC analysis with fluorescent detection after differential iodine oxidation of tissue extracts in either acidic or alkaline conditions, respectively measured total biopterins (BH4, BH2, and biopterin) and biopterins excluding BH4 (BH2 + biopterin). BH4 was calculated as the difference between the two measurements as previously described.²⁴

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Echocardiographic data were compared using repeated measures analysis of variance (RM-ANOVA), excluding data from the 9 week time point due to survival bias. A Huynh-Feldt correction was chosen since the Mauchly test for sphericity was significant. Kaplan-Meier survival curves were compared using the log rank test. Other data were analyzed using a one-way (or two-way in the case of BH4 treatment group comparisons) ANOVA with a Bonferroni post hoc test for multiple comparisons, or a Kruskal-Wallis test followed by a Mann-Whitney test for non-parametric data. P-values less than 0.05 were considered to be statistically significant. We used SPSS version 14.0, Sigmatat 3.0, and GraphPad Prism 5.0 for statistical analysis.

RESULTS

Baseline characteristics and age-related hypertrophy in $\beta 3^{-/-}$

$\beta 3^{-/-}$ mice develop mildly increased body weight, LV wall thickness, and LV mass by echocardiography compared to WT mice by 8 weeks of age. Heart rate, LV dimensions and systolic function are similar between strains (Table 1). The degree of hypertrophy is similar at 8 weeks of age (Fig. 1A). In older age (14-18 months old), WT mice develop mild hypertrophy ($P < 0.05$ vs. young WT), however the $\beta 3^{-/-}$ animals have markedly increased LV wall thickness (1.30 ± 0.04 vs. 0.86 ± 0.07 mm, $P < 0.001$) and mass (196 ± 12 vs. 129 ± 20 mg, $P < 0.05$) compared to old WT (Fig. 1A-B).

Mild pressure-overload increases mortality, hypertrophy, and fibrosis in $\beta 3^{-/-}$ mice

Baseline LV systolic pressures were similar between WT (95 ± 5 mmHg) and $\beta 3^{-/-}$ (96 ± 4 mmHg) mice and were increased with mild (25G) transverse aortic constriction to similar levels (WT-TAC 137 ± 15 mmHg; $\beta 3^{-/-}$ -TAC 130 ± 4 mmHg) (Table 1). Figure 2A shows Kaplan-Meier survival curves for both mouse strains following mild TAC. With TAC, 85% of WT animals survived the full 9 week protocol, whereas only 38% of the $\beta 3^{-/-}$ animals did (Fig. 2A, $\chi^2 = 10.78$, $P = 0.001$). The worsened mortality in $\beta 3^{-/-}$ mice was coupled to exacerbated cardiac remodeling, which was mild in WT-TAC versus WT-sham controls (heart weight/tibia

length ratio, 123.3 ± 4.0 mg/cm vs. 84.6 ± 2.0 mg/cm, $P=0.004$), but much greater in $\beta 3^{-/-}$ mice (175.2 ± 17.8 mg/cm vs. 89.0 ± 4.6 mg/cm, $P=0.017$ vs. $\beta 3^{-/-}$ -sham; $P=0.003$ vs. WT-TAC; Fig. 2B). These findings were paralleled by calculated LV mass based on echocardiography ($P=0.001$ for WT vs. $\beta 3^{-/-}$ response; Fig. 3B). Although there were baseline differences in calculated LV mass by echocardiography, there was no difference in sham heart weight/ tibia length ratio due to larger size of the $\beta 3^{-/-}$ mice. Myocyte width was significantly greater in $\beta 3^{-/-}$ -TAC vs. WT-TAC (39.3 ± 0.9 μ m vs. 31.3 ± 0.9 μ m, $P<0.001$), and myocardial fibrosis was also far more pronounced (2.7 ± 0.3 vs. 1.2 ± 0.1 , $P=0.014$; Fig. 2C).

Pressure-overload induces LV dilation and dysfunction in $\beta 3^{-/-}$ mice

$\beta 3^{-/-}$ mice also developed exacerbated LV chamber dilation and systolic dysfunction, assessed by echocardiography (Fig. 3A) in response to pressure-overload. After 9 weeks of TAC, LVEDD was unchanged in WT mice but increased in $\beta 3^{-/-}$ (3.90 ± 0.26 vs. 2.91 ± 0.04 mm, $P=0.001$). Similarly, LVESD was increased vs. baseline (2.47 ± 0.36 mm vs. 1.02 ± 0.05 mm, P -interaction <0.001), with a net decline in fractional shortening (38.2 ± 5.0 vs. 64.9 ± 1.8 %, $P=0.002$) in $\beta 3^{-/-}$ -TAC but not WT-TAC. Average wall thickness increased in both WT-TAC (1.30 ± 0.02 vs. 0.83 ± 0.01 mm, $P<0.001$) and $\beta 3^{-/-}$ -TAC mice, but was higher in $\beta 3^{-/-}$ -TAC (1.43 ± 0.03 vs. 1.02 ± 0.03 mm, $P<0.001$ vs. $\beta 3^{-/-}$ -sham, $P<0.01$ vs. WT-TAC; Fig. 3C), although percent increase in wall thickness was similar between strains due to the baseline hypertrophy in the $\beta 3^{-/-}$ mice. Likewise, percent increase in LV mass was similar in $\beta 3^{-/-}$ and WT (Fig. 3D).

Lack of $\beta 3$ -AR alters NOS isoform expression and decreases NOS activity with TAC

Since $\beta 3$ cardiac modulation is coupled to nitric oxide synthase (NOS), we examined whether mice lacking the receptor had decreased NOS activity. After 3 weeks of TAC, there were no significant differences in arginine-citrulline conversion (Fig. 4A) from baseline in either WT-TAC (10.6 ± 2.0 vs. 6.7 ± 2.1 ; arbitrary units (A.U.), $P=NS$) or $\beta 3^{-/-}$ -TAC (10.3 ± 1.4 vs. 8.8 ± 0.6 ; A.U., $P=NS$). At 9 weeks, NOS activity was similar between $\beta 3^{-/-}$ and WT (26.9 ± 0.4 vs. 27.6 ± 0.4 ; A.U., $P=NS$). Mild pressure-overload did not alter NOS activity in WT-TAC (27.7 ± 0.3 ; A.U.) but it decreased activity in $\beta 3^{-/-}$ -TAC (19.3 ± 1.2 ; A.U., $P<0.001$; Fig. 4B) after 9 weeks. This decline was not associated with reduced eNOS protein expression (Fig. 4C). However, S1177 phosphorylation, an indication of eNOS activation, was increased in WT-TAC. In contrast, $\beta 3^{-/-}$ mice showed no increase in p-eNOS with TAC (Fig. 4D). Furthermore, we noticed an increase in total nNOS expression in the $\beta 3^{-/-}$ -TAC hearts compared to baseline levels ($P<0.05$, Fig. 4E) and levels seen in WT ($P<0.05$). iNOS protein levels also increased in $\beta 3^{-/-}$ -TAC above baseline ($P<0.01$, Fig. 4F), though this was not significantly different from levels in WT controls.

Lack of $\beta 3$ -AR results in greater NOS uncoupling with TAC

Reduced NOS activity can also be due to its functional uncoupling, wherein the enzyme shifts to generate superoxide rather than NO. To test for this, we examined lucigenin-enhanced chemiluminescence in myocardium in the presence and absence of the NOS inhibitor L-NAME. Superoxide was similar in both genotypes 9 weeks following sham surgery (1145 ± 146 vs. 1106 ± 109 cpm/mg, $P=NS$) but rose almost twice as much in $\beta 3^{-/-}$ compared to WT mice after 9 weeks of TAC (2730 ± 121 vs. 1719 ± 52 cpm/mg; $P<0.05$ vs. baseline, $P<0.001$ between groups; Fig. 5A). Importantly, the dominant component of enhanced O_2^- in $\beta 3^{-/-}$ -TAC could be attributed to NOS uncoupling, although both NOS-dependent and NOS-independent superoxide were increased in $\beta 3^{-/-}$ -TAC hearts. NOS-dependent superoxide was similar between $\beta 3^{-/-}$ and WT at baseline, although there was a trend toward higher levels in the $\beta 3^{-/-}$; however, levels rose nearly 300% in $\beta 3^{-/-}$ -TAC mice vs. $\beta 3^{-/-}$ at baseline, compared

with <200% in WT-TAC vs. WT at baseline; $P < 0.05$ for both (Fig. 5B). In addition, NOS dependent superoxide was higher in $\beta 3^{-/-}$ -TAC vs. WT-TAC ($P < 0.01$).

Since Akt can modulate eNOS phosphorylation, we examined if it was differentially phosphorylated (S476). Although basal Akt phosphorylation was reduced in $\beta 3^{-/-}$ mice, it rose with 9 weeks of TAC to similar levels in both genotypes (Fig 5C), indicating that p-eNOS and NOS activity must be regulated by a non-Akt dependent mechanism.

NOS coupling depends directly upon levels of tetrahydrobiopterin (BH4) whose rate-limiting synthetic enzyme is guanosine triphosphate cyclohydrolase 1 (GTPCH-1). We therefore tested whether GTPCH-1 expression was altered in the $\beta 3^{-/-}$ model. GTPCH-1 expression was similar at baseline but declined significantly after 9 weeks of TAC in $\beta 3^{-/-}$ -TAC vs. $\beta 3^{-/-}$ ($P < 0.05$; Fig. 5D).

Tetrahydrobiopterin (BH4) treatment rescues $\beta 3^{-/-}$ mice from LV hypertrophy and dysfunction following pressure-overload

Given the decrease in GTPCH-1 protein levels, we considered whether BH4 levels might differ in the $\beta 3^{-/-}$ mice, either at baseline or in response to TAC. Using HPLC to fraction biopterins, total BH4 levels did not differ significantly between strains, although there was a slight increase ($P < 0.01$, Fig. 6A) in $\beta 3^{-/-}$ -TAC (35.6 ± 1.9 pmol/mg protein) above baseline (27.0 ± 0.9 pmol/mg protein). The ratio of BH4 to other biopterins (BH2+Biopterin) was decreased by approximately 25% ($P = 0.03$) in $\beta 3^{-/-}$ mice at baseline (1.49 ± 0.2) compared to WT (1.91 ± 0.3), yet was unchanged after TAC (Fig. 6B).

Given the greater amounts of NOS-dependent O_2^- generated in $\beta 3^{-/-}$ -TAC, we tested whether exogenously adding BH4 might be a viable therapeutic strategy, as has been reported previously in systems of uncoupled NOS.¹⁹ We therefore supplemented BH4 or vehicle to the feed of $\beta 3^{-/-}$ and WT mice and performed TAC or sham surgery. This cohort of mice was sacrificed after 3 weeks of TAC, in order to minimize any survival bias or secondary pathway activation that might be more significant by at later time points. After 3 weeks of TAC, $\beta 3^{-/-}$ -TAC mice experienced a decrease in fractional shortening ($-16.1 \pm 4.9\%$, Fig. 6C) and increase in LV mass ($81.8 \pm 13.7\%$, Fig. 6D) as estimated by echocardiography. BH4 treatment completely rescued the impairment in function, with no change in fractional shortening in $\beta 3^{-/-}$ -TAC/BH4 ($-0.4 \pm 0.2\%$, $P < 0.05$), similar to WT-TAC ($2.5 \pm 1.2\%$) and WT-TAC/BH4 ($-1.8 \pm 3.0\%$) controls ($P = \text{NS}$ for both). Similarly, the change in calculated LV mass was significantly lower in $\beta 3^{-/-}$ -TAC/BH4 ($15.0 \pm 6.8\%$, $P < 0.01$) to a level not significantly different from WT (Fig. 6D).

Recognizing the dramatic protection of BH4 treatment from pathological hypertrophy and impaired systolic function induced by TAC, we hypothesized that this protection might correlate with a decrease in NOS-dependent superoxide production. Indeed, after 3 weeks of TAC, BH4 treatment reduced NOS-dependent superoxide production in whole heart homogenates ($P < 0.05$) to a level similar to baseline and WT-TAC controls (Fig. 6E).

DISCUSSION

In this study, we demonstrated that the absence of the $\beta 3$ -AR exacerbates pressure-overload induced NOS uncoupling and subsequent increased NOS-dependent superoxide generation. Consequently, $\beta 3^{-/-}$ mice developed marked adverse remodeling, reflected by increased gross and cellular hypertrophy, fibrosis, LV dilation and depressed LV systolic function.

The upstream regulation of the $\beta 3$ -AR in cardiac myocytes is relatively well established, although its physiological role in the heart and level of interspecies variation remain

controversial.³ Gauthier et al. demonstrated that β_3 -AR stimulation decreases cardiac contractility through activation of a NOS pathway,⁴ and studies have suggested this may play a role in cardiodepression observed in cardiac failure and sepsis.^{13,25} The negative effect is blunted by NOS inhibitors and reversed by an excess of the NOS substrate, L-arginine.⁴ Imbrogno et al. showed the negative inotropic effect of BRL37344 in isolated hearts from fresh water eels is abolished by exposure to the $G_{i/o}$ inhibitor pertussis toxin,²⁶ and that pre-treatment with inhibitors of soluble guanylate cyclase or cGMP-activated protein kinase G (PKG) abolished β_3 -AR negative inotropy as well. This supports a central role of G_i -eNOS-NO-cGMP-PKG signaling.^{4,12} Similarly, mice lacking β_3 -AR and/or myocytes with the receptor pharmacologically acutely inhibited display enhanced contractile responses to isoproterenol.^{11,27} *In vivo*, stimulation of the β_3 -AR receptor occurs concurrent with β_1 and β_2 stimulation, so this mechanism can provide a physiologic “brake” to sympathetic stimulation.

Removal of this regulatory “brake” in the $\beta_3^{-/-}$ mouse results in an exaggerated response to pressure-overload. Our study supports a major role for NOS as a source of both protective NO and damaging myocardial ROS induced by pressure-overload. At present, it is unknown which NOS isoform is responsible for generating the enhanced levels of ROS in the $\beta_3^{-/-}$ heart. eNOS dysfunction has been demonstrated to play a substantial role in adverse cardiac remodeling, and thus is an attractive candidate.¹⁸ eNOS normally generates NO to stimulate cGMP and PKG, which protect the heart from hypertrophy and remodeling via transcriptional regulation, phosphorylation, and suppression of targeted signaling, such as from G_{α_q} stimulation.²⁸ eNOS activity is generally modulated by either translocation or phosphorylation. Phosphorylation at Ser¹¹⁷⁹ (or Ser¹¹⁷⁷ in mouse) activates eNOS, whereas phosphorylation at Thr⁴⁹⁷ or Ser¹¹⁶ is associated with inhibition.²⁹ The increase in eNOS¹¹⁷⁷ phosphorylation we see in WT mice with TAC was blunted in the $\beta_3^{-/-}$ mice, which had no augmentation of eNOS phosphorylation after TAC, indicating an inability of these mice to mount the normal response to pressure-overload. In the normal heart, exposure to severe TAC ($\geq 100\%$ rise in LV mass after 3 weeks) leads to marked eNOS uncoupling, and mice lacking eNOS are protected, developing compensated concentric hypertrophy instead.¹⁸ Others have found that the lack of eNOS exacerbates pathological remodeling if mice are exposed to lower severity banding stress,³⁰ perhaps due to less ROS stimulation. In the current study, we used a milder TAC model as severe pressure-overload proved fatal in all $\beta_3^{-/-}$ hearts. Although, eNOS remained present and loss of normal NOS activation in the $\beta_3^{-/-}$ hearts may have contributed to a ROS/NOS imbalance favoring subsequent NOS uncoupling.

Despite the potential role of eNOS uncoupling, the increases in nNOS and iNOS expression in $\beta_3^{-/-}$ hearts after TAC are intriguing. Both nNOS and iNOS derived NO production has been shown to increase in failing human hearts,^{31,32} whereas eNOS activity is depressed.³³ iNOS may also be cardioprotective in some situations, without causing overt myocyte injury or dysfunction.³⁴ Following coronary occlusion and reperfusion, iNOS expression in cardiomyocytes was associated with a decrease in oxygen radicals and mitochondrial swelling and permeability transition. Interestingly, β_3 -AR mediated decrease in cardiac contractility in the diabetic rat heart has shown to be nNOS-dependent.³⁵ Furthermore, nNOS, which is normally localized to the sarcoplasmic reticulum, is found at the sarcolemma after MI or in failing hearts where it serves to decrease $\beta_1/2$ -AR responsiveness in a fashion analogous to β_3 -AR stimulation.^{31,36} Intriguing recent data produced by Idigo et al. reveals that β_3 -AR agonist stimulation failed to decrease Ca^{2+} transients and cardiomyocyte shortening in nNOS^{-/-} mice, or in WT cardiomyocytes with nNOS inhibition. This is associated with an increase in eNOS-derived superoxide production in nNOS^{-/-} mice, which was abolished by xanthine oxidase inhibition with oxypurinol.³⁷ This may support a role for nNOS activity in maintaining eNOS coupling by constraining XOR activity, with both isoforms potentially acting through β_3 -AR mediated pathways.

NOS coupling depends upon the bioavailability of the essential NOS cofactor BH₄, which in turn depends on expression and activity of the rate-limiting synthetic enzyme GTPCH-1.³⁸ GTPCH-1 expression decreased in $\beta 3^{-/-}$ -TAC in the current study. However, endogenous BH₄ levels were not depleted in $\beta 3^{-/-}$ hearts at baseline or following TAC, thereby arguing against this mechanism as being dominant in inducing NOS uncoupling in the $\beta 3^{-/-}$ model. Nevertheless, BH₄ treatment did rescue $\beta 3^{-/-}$ mice from adverse remodeling after TAC, with reduced preserved systolic function, while lowering NOS-dependent superoxide generation. It is unknown whether BH₄ requirements increase under conditions of increased stress to protect against damaging ROS production and maintain NOS coupling. The underlying protective effects of exogenous BH₄ may have also been due to the direct scavenging of ROS. In addition to decreased BH₄ bioavailability, another possibility is enhanced adrenergic stimulation and consequent ROS generation. Stimulation of $\beta 3$ -AR increases intracellular cGMP, activating PDE2 to enhance its hydrolysis of cAMP.³⁹ $\beta 3^{-/-}$ myocardium had blunted eNOS activation, which suppresses cGMP generation,¹¹ and possibly reducing cAMP hydrolysis by PDE2. Such sustained stimulation can result in calcium mediated injury and myocardial oxidant stress. Lastly, the PI3K/Akt pathway has been proposed as a mechanism for eNOS activation by $\beta 3$ -AR in nonfailing hearts.⁴⁰ However, we did not observe differential Akt activation consistent with NOS activity data in $\beta 3^{-/-}$ myocardium subjected to this model of pressure-overload.

$\beta 3$ -AR are upregulated in human heart failure and animal models.^{13,41} Some groups have hypothesized that the negative inotropic effects of $\beta 3$ -AR are detrimental,^{3,42} and that diminishing $\beta 3$ -AR activity could be beneficial in the treatment of heart failure.^{8,16} Our data, on the other hand, support the idea that $\beta 3$ -AR serves a chiefly protective role in the heart rather than one depressing contraction, and that blocking this pathway maybe disadvantageous in the stressed or aged myocardium. These data are consistent with the protective effect of $\beta 3$ -AR overexpression reported in a mouse model of isoproterenol-induced heart failure.⁴³

To our knowledge, this is the first time that the role of $\beta 3$ -AR in maintaining NOS coupling has been described. Based on these results, we propose that $\beta 3$ -AR protects the heart from the long term adverse effects of adrenergic overstimulation, in part by preserving eNOS in its coupled state, despite the fact that acute stimulation of the $\beta 3$ -AR can itself decrease contractility. Additional studies are needed to test the clinical importance of $\beta 3$ -AR in protecting the heart from adverse cardiac remodeling and cardiac hypertrophy.

ACKNOWLEDGEMENTS

The authors wish to thank Karen Miller and Konrad Vandegaer for their expert technical assistance as well as Solomon H. Snyder for generously allowing the authors access to his laboratory facilities.

SOURCES OF FUNDING

This research was supported by the Grant Program in Heart Research from the W.W. Smith Charitable Trust (LAB), the Belgian-American Education Foundation (Collen) Grant (ALM), the PhD program of the University of Antwerp (AAP), Mid-Atlantic American Heart Association Postdoctoral Fellowship (ALM) and Scientist Development Grant (HCC), NIH grants K08-HL076220 (LAB), HL31069, HL43023 and HL66331 (MSW), R01-AG18324, HL47511, and P01-HL59408 (DAK), R01-HL77575 and R01-HL86965 (YX).

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Figure 1. Cardiac hypertrophy at baseline in the absence of β3-AR

A, Echocardiographic data showing increased LV wall thickness and mass at baseline in β3^{-/-} compared to age-matched WT at 8 weeks, 4 months, and 14-18 months. Differences are further accentuated with age. B, Photographic example of old (14-18 months) WT and β3^{-/-} hearts demonstrating increased hypertrophy in β3^{-/-}. **P*<0.0001 vs. 8 weeks, †*P*<0.05 vs. WT.

Figure 2. Effect of TAC on mortality and hypertrophy in the absence of $\beta 3$ -AR

A, Increased mortality in $\beta 3^{-/-}$ mice after mild TAC ($P=0.001$). B, Heart weight/tibia length ratio and photographic examples of hearts demonstrating a markedly increased hypertrophic response in $\beta 3^{-/-}$ compared to WT mice with mild TAC. C, Increased fibrosis in $\beta 3^{-/-}$ -TAC vs. WT-TAC hearts as demonstrated by Masson trichrome stain (left panel, 400x) and tabulated using a semi-quantitative scoring system (0=none, 3=marked fibrosis; top right panel). Greater myocyte width in $\beta 3^{-/-}$ -TAC vs. WT-TAC by H&E (bottom right). * $P<0.05$ vs. baseline, † $P<0.01$ vs. WT-TAC by post-hoc analysis.

Figure 3. Effect of TAC on LV dilation and dysfunction in the absence of β_3 -AR

A, Examples of M-mode echocardiograms demonstrating decreased systolic function, LV dilation and increased wall thickness in $\beta_3^{-/-}$ -TAC vs. WT-TAC. B, More rapid increase in calculated LV mass in $\beta_3^{-/-}$ -TAC vs. WT-TAC by serial echocardiography. C, Increased LV cavity size by both end-diastolic (LVEDD) and end-systolic (LVESD) dimensions, with concomitant decreases in fractional shortening (FS) and increases in LV wall thickness. D, Percent changes in wall thickness and calculated LV mass are similar in $\beta_3^{-/-}$ -TAC vs. WT-TAC. * $P < 0.01$ vs. $\beta_3^{-/-}$ baseline, † $P < 0.01$ vs. WT-TAC, ‡ $P < 0.05$ vs. WT-baseline by post-hoc analysis.

Figure 4. Changes in NOS activity and NOS isoform expression with TAC

A, Similar levels of NOS activity after 3 weeks of TAC between $\beta 3^{-/-}$ -TAC and WT-TAC by arginine-citrulline conversion. B, Decreased total NOS activity in $\beta 3^{-/-}$ -TAC vs. WT-TAC after 9 weeks of TAC. C, No differences in eNOS expression levels. D, Enhanced eNOS activation shown by increased peNOS/eNOS ratio in WT-TAC, but no response in $\beta 3^{-/-}$ -TAC. E, nNOS is elevated in $\beta 3^{-/-}$ -TAC vs. $\beta 3^{-/-}$ and WT-TAC. F, iNOS is elevated in $\beta 3^{-/-}$ -TAC vs. $\beta 3^{-/-}$ ($P < 0.01$), although no different than WT-TAC. $^*P < 0.05$ vs. baseline, $^{\dagger}P < 0.05$ vs. WT-TAC by post-hoc analysis.

Figure 5. Elevated NOS-dependent superoxide after TAC in the absence of $\beta 3$ -AR

A, Increased total superoxide generation in $\beta 3^{-/-}$ -TAC vs. $\beta 3^{-/-}$ and WT-TAC. B, Increased NOS-dependent superoxide in $\beta 3^{-/-}$ -TAC vs. WT-TAC calculated by subtracting superoxide production in the presence of L-NAME from total superoxide. C, Decreased baseline p-Akt/Akt ratio in $\beta 3^{-/-}$, but no difference between strains after TAC. D, Reduced GTPCH-1 levels in $\beta 3^{-/-}$ -TAC vs. $\beta 3^{-/-}$. * $P < 0.05$ vs. baseline, † $P < 0.01$ vs. corresponding WT or WT-TAC by post-hoc analysis.

Figure 6. BH4 treatment rescues $\beta 3^{-/-}$ mice from pathological hypertrophy and fibrosis
 A, Increased BH4 levels in $\beta 3^{-/-}$ -TAC vs. $\beta 3^{-/-}$ ($P<0.01$), but no difference between strains.
 B, Lower BH4/(BH2+Biopterin) ratio in $\beta 3^{-/-}$ hearts compared to WT ($P=0.03$), but no change with TAC. C, % change in fractional shortening is lower in $\beta 3^{-/-}$ -TAC BH4 vs. $\beta 3^{-/-}$ -TAC ($P<0.05$). D, % change in LV mass is lower in $\beta 3^{-/-}$ -TAC BH4 vs. $\beta 3^{-/-}$ -TAC ($P<0.01$). E, BH4 treatment lowers NOS-dependent superoxide levels in $\beta 3^{-/-}$ -TAC BH4 vs. $\beta 3^{-/-}$ -TAC ($P<0.05$). * $P<0.01$ vs. baseline, † $P<0.05$ vs. corresponding WT or WT-TAC, # $P<0.05$ $\beta 3^{-/-}$ -TAC BH4 vs. $\beta 3^{-/-}$ -TAC by post-hoc analysis.

Table 1

Baseline characteristics

	WT	$\beta 3^{-/-}$
Body weight (g)	24.8±0.3	29.3±0.7 [*]
Heart rate (bpm)	696±16	696±10
LV End Diastolic Diameter (mm)	2.80±0.06	2.81±0.06
LV End Systolic Diameter (mm)	1.07±0.02	1.12±0.03
Wall thickness (mm)	0.94±0.02	1.05±0.03 [*]
Fractional shortening (%)	61.7±0.7	60.2±0.9
Calculated LV mass (mg)	90±3	106±4 [*]
LV systolic pressure before TAC (mmHg)	95±5	96±4
LV systolic pressure after TAC (mmHg)	137±15	130±4

LV, left ventricular; TAC, transverse aortic constriction.

^{*}
 $P < 0.05$