Published in final edited form as:

Basic Res Cardiol. 2015 January ; 110(1): 456. doi:10.1007/s00395-014-0456-3.

Chronic β_1 -adrenergic blockade enhances myocardial β_3 -adrenergic coupling with nitric oxide-cGMP signaling in a canine model of chronic volume overload: new insight into mechanisms of cardiac benefit with selective β_1 -blocker therapy

Danielle M. Trappanese,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Yuchuan Liu,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Ryan C. McCormick,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Alessandro Cannavo,

Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA, USA

Gayani Nanayakkara,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Marina M. Baskharoun,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Harish Jarrett,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Felix J. Woitek,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

D. Michael Tillson,

College of Veterinary Medicine, Auburn University, Auburn, AL, USA

A. Ray Dillon,

College of Veterinary Medicine, Auburn University, Auburn, AL, USA

Correspondence to: Emily J. Tsai, emily.tsai@tuhs.temple.edu.

D. M. Trappanese and Y. Liu contributed equally.

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

Fabio A. Recchia,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Department of Physiology, Temple University School of Medicine, Philadelphia, PA, USA

Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa, Italy

Jean-Luc Balligand,

Institute of Experimental and Clinical Research, Catholic University of Louvain, Brussels, Belgium

Steven R. Houser,

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Department of Physiology, Temple University School of Medicine, Philadelphia, PA, USA

Walter J. Koch,

Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA, USA

Louis J. Dell'Italia, and

Division of Cardiovascular Disease, Department of Medicine, University of Alabama, Birmingham, AL, USA

Emily J. Tsai

Cardiovascular Research Center, Temple University School of Medicine, 3500 North Broad Street, MERB 1047, Philadelphia, PA 19140, USA

Department of Physiology, Temple University School of Medicine, Philadelphia, PA, USA

Cardiology Section, Department of Medicine, Temple University School of Medicine, Philadelphia, PA, USA

Emily J. Tsai: emily.tsai@tuhs.temple.edu

Abstract

The β_1 -adrenergic antagonist metoprolol improves cardiac function in animals and patients with chronic heart failure, isolated mitral regurgitation (MR), and ischemic heart disease, though the molecular mechanisms remain incompletely understood. Metoprolol has been reported to upregulate cardiac expression of β_3 -adrenergic receptors (β_3 AR) in animal models. Myocardial β_3 AR signaling via neuronal nitric oxide synthase (nNOS) activation has recently emerged as a cardioprotective pathway. We tested whether chronic β_1 -adrenergic blockade with metoprolol enhances myocardial β_3 AR coupling with nitric oxide-stimulated cyclic guanosine monophosphate (β_3 AR/NO-cGMP) signaling in the MR-induced, volume-overloaded heart. We compared the expression, distribution, and inducible activation of β_3 AR/NO-cGMP signaling proteins within myocardial membrane microdomains in dogs (canines) with surgically induced MR, those also treated with metoprolol succinate (MR+ β B), and unoperated controls. β_3 AR mRNA transcripts, normalized to housekeeping gene RPLP1, increased 4.4×10^3 - and 3.2×10^2 -fold in MR and MR+ β B hearts, respectively, compared to Control. Cardiac β_3 AR expression was increased 1.4- and nearly twofold in MR and MR+ β B, respectively, compared to Control. β_3 AR was detected within caveolae-enriched lipid rafts (Cav3⁺LR) and heavy density, non-lipid raft membrane (NLR) across

all groups. However, in vitro selective β_3 AR stimulation with BRL37344 (BRL) triggered cGMP production within only NLR of MR+ β B. BRL induced *Ser*¹⁴¹² phosphorylation of nNOS within NLR of MR+ β B, but not Control or MR, consistent with detection of NLR-specific β_3 AR/NO-cGMP coupling. Treatment with metoprolol prevented MR-associated oxidation of NO biosensor soluble guanylyl cyclase (sGC) within NLR. Metoprolol therapy also prevented MR-induced relocalization of sGC β_1 subunit away from caveolae, suggesting preserved NO-sGC-cGMP signaling, albeit without coupling to β_3 AR, within MR+ β B caveolae. Chronic β_1 -blockade is associated with myocardial β_3 AR/NO-cGMP coupling in a microdomain-specific fashion. Our canine study suggests that microdomain-targeted enhancement of myocardial β_3 AR/NO-cGMP signaling may explain, in part, β_1 -adrenergic antagonist-mediated preservation of cardiac function in the volume-overloaded heart.

Keywords

β -Blockade; Nitric oxide; Cyclic guanosine monophosphate; Caveolae; β_3 -Adrenoceptor; Soluble guanylyl cyclase; Volume overload

Introduction

β -blockers (β Bs) confer cardiac benefit in disease through multiple mechanisms [4, 6, 16, 19, 27, 31, 38, 53], though many remain incompletely understood. In addition to disrupting the chronic adrenergic toxicity underlying heart failure (HF), β Bs improve β -adrenoceptor (β AR) function largely by reversing the downregulation of myocardial β_1 - and β_2 AR [18, 59] and by countering the upregulation of G protein-coupled receptor kinase 2 (GRK2) expression [21] otherwise induced by chronic sympathetic activation. Thus, chronic β B therapy recouples β AR and G protein activity and restores β AR responsiveness. While studies of the effect of β B on β AR signaling in the diseased heart focus predominantly on β_1 - and β_2 ARs, few have examined its effect on β_3 AR. Myocardial β_2 AR expression is reportedly increased in HF animal models and patients [8, 35] and, in limited studies, even more so with chronic β B therapy [51, 64]. Recently, β_3 AR activation has been shown to induce nitric oxide (NO)-mediated cardioprotection in animal models of cardiac pressure overload, neurohormone-induced hypertrophy, myocardial ischemia/reperfusion injury, and acute myocardial infarction [1, 5, 14, 41, 60]. Chronic β_1 -blockade (β_1 B) might well amplify cardioprotective β_3 AR signaling in the pathologically remodeled heart. Intriguing but little explored, such a mechanism would suggest novel approaches to enhancing innate cardioprotective signaling while assuring compatibility with current guideline-directed medical therapy.

β AR signaling regulates cardiac myocyte contractility and hypertrophic signaling and, under normal physiological conditions, is modulated by NO via both direct [i.e., soluble guanylyl cyclase (sGC) activation] and indirect actions (e.g. S-nitrosylation, tyrosine nitration). NO activation of sGC, with its subsequent production of cyclic guanosine monophosphate (cGMP), blunts β_1 - and β_2 AR-stimulated cardiac contractility [55]. S-nitrosylation of GRK2 (also known as β -adrenergic receptor kinase 1 or β ARK1) inhibits the desensitization and downregulation of β_1 - and β_2 ARs [61]. Conversely, β_2 - and β_3 AR signaling can activate NO

synthase (NOS), thereby coupling β AR signaling with NO signaling. Unlike β_1 - and β_2 ARs, β_3 ARs are stimulated at high catecholamine levels and thus may contribute more significantly to β AR signaling in conditions of catecholamine excess such as HF and mitral regurgitation (MR) [17, 34]. In the healthy, non-failing heart, β_3 AR activation inhibits cardiac myocyte contraction and induces cardiac myocyte relaxation [15]. However, the effect of β_3 AR signaling on cardiac contractility remains incompletely understood, as both negative [40] and positive [7] inotropic effects have been demonstrated with β_3 AR stimulation in diseased, failing hearts. Importantly, transgenic mice with cardiac-specific overexpression of human β_3 AR show enhanced cardiac contractility [26] and attenuated neurohormone-induced hypertrophic remodeling [5].

Gene expression analysis of left ventricular (LV) tissue recently revealed that cardiac β AR and NO signaling pathways are markedly altered in patients with isolated MR compared to control subjects [66]. Yet how the interaction between β AR and NO signaling differs in the stressed and pathologically remodeled heart is not fully understood. We previously demonstrated that NO-sGC-cGMP signaling is dysregulated in animal models of concentric and eccentric cardiac hypertrophy with respect to myocardial expression, subcellular localization, and oxidation of sGC heterodimer subunits [29, 57].

In prior studies, chronic β_1 B therapy preserved normal cardiac myocyte contractility and enhanced β AR responsiveness in the experimental chronic MR canine model after four months of treatment [43]. In the present study, we used a 4-week chronic MR canine model to investigate the early effects of chronic β_1 B therapy on myocardial NO-sGC-cGMP signaling as well as β_3 AR/NO-cGMP coupling. We hypothesized that chronic β_1 B therapy preserves normal NO-sGC-cGMP signaling and enhances myocardial β_3 AR/NO-cGMP coupling in a microdomain-specific fashion, thereby potentially mediating later cardiac myocyte functional benefits. We focused on signaling within and outside of myocardial caveolae—small, flask-shaped, lipid- and protein-rich invaginations of the plasma membrane (PM) that compartmentalize signal transduction. Importantly, all three β AR subtypes [2, 30, 45, 47, 49], eNOS [23], nNOS [9], sGC [57], and calcium channels [32, 33], reside within caveolae; some, exclusively.

Methods

Animal experiments

Mitral regurgitation (MR) was induced in conditioned mongrel dogs (19–26 kg) by rupturing a papillary chord under fluoroscopy, as previously described [25, 29, 46]. Sixteen dogs underwent chordal rupture and ten unoperated dogs served as controls. At 24 h after MR induction, half of the MR dogs were started on metoprolol succinate 100 mg by mouth once daily (MR n = 8, MR+ β B n = 8). Two-dimensional and M-mode echocardiography (2.25-MHz transducer, ATL Ultramark VI) was performed at baseline and at the time of euthanasia (4 weeks after MR induction). Animals were maintained at a deep plane of general anesthesia using isoflurane and were mechanically ventilated. At the end of the in vivo experiments, the heart was arrested with intracardiac injection of KCl and quickly extirpated and placed in phosphate-buffered ice slush. The coronaries were flushed with oxygenated Krebs solution. A portion of the LV was cut and snap-frozen in liquid nitrogen

for subsequent studies. We chose to study 4-week duration of therapy so as to identify early signaling changes that precede and thus underlie subsequent functional benefits already demonstrated with longer term treatment. Animal studies were approved by the Animal Services Committees at the University of Alabama at Birmingham and College of Veterinary Medicine, Auburn University and the Institutional Animal Care and Use Committee of Temple University School of Medicine. All animal protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No.85-23, revised 1996).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from flash-frozen LV myocardium using a spin column chromatography method (Animal Tissue RNA Purification Kit, Norgen Biotek, Ontario, Canada), according to the manufacturer's instructions. Reverse transcription (RT) was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies) and oligodt primers according to the manufacturer's instructions. Real-time PCR (qPCR) was performed using QuantiFast SYBR[®] Green PCR Kit (Qiagen). Data were normalized to large ribosomal protein P1 (RPLP1) RNA expression. The following primer sets were used (forward, reverse): β_3 AR (5'-CGCCTCCAACATACCTACG-3', 5'-CGCGTAGCCACGAGGAAAA-3'); RPLP1 (proprietary primer sequences, Qiagen). Individual samples were run in triplicate.

Isolation of caveolin-3-enriched lipid raft fraction by isopycnic ultracentrifugation

Caveolae-enriched lipid raft fractions (Cav3⁺LR) were prepared from snap-frozen LV tissue, using a discontinuous 35–5 % sucrose density gradient ultracentrifugation method as previously described [29, 57]. LV tissue homogenization was carried out on ice, in detergent-free buffer (50 mmol/L Tris-HCl, pH 7.6, 1 mmol/L EDTA, 1 mmol/L DTT, 2 mmol/L PMSF, 50 mmol/L NaF, 1 mmol/L Na Vanadate) with protease inhibitors (Mammalian Cocktail, Sigma-Aldrich). Following 1-h incubation on ice with intermittent vortex, 0.6 mL of tissue homogenate was mixed with 1.4 mL of 60 % (w/w) sucrose in 20 mmol/L KCl, 0.5 mmol/L MgCl₂ and placed at the bottom of an ultracentrifuge tube. A discontinuous 35–5 % sucrose gradient was formed by overlaying each sample with 1.3 mL of 35 % sucrose and then with 1.3 mL of 5 % sucrose. The sucrose density gradient was topped off with 0.5 mL of 200 mmol/L KCl. Each sample was then centrifuged at [180,000g for 16–18 h at 4 °C in a swinging bucket rotor (Beckman Instruments, Palo Alto, CA) without any brake. The top KCl layer was discarded and fractions were collected every 400 μ l from the top sucrose layer corresponding to F1 (top, most buoyant) to F11 (bottom, least buoyant/heaviest).

A light-scattering band confined to the 35–5 % sucrose interface, typically F3–F5, corresponds to Cav3⁺LR fractions. Ponceau staining and protein concentrations determined by bicinchoninic acid (BCA) assay confirmed that total protein distribution was weighted toward heavier sucrose density gradient fractions (F7 through F11) lacking caveolin-3 (Cav3) in all hearts. Proteins were precipitated using 0.1 % w/v deoxycholic acid in 100 % w/v trichloroacetic acid. Protein concentrations were determined by BCA protein assay (Pierce). Non-lipid raft (NLR, F11) and Cav3⁺LR fractions (F3–F5) without TCA

precipitation were also collected for BCA and subsequent cGMP assays. Cav3⁺LR fractions were selected based upon greatest enrichment of Cav3 as detected by Western.

Cardiomyocyte caveolae-immunoaffinity isolation

Cardiomyocyte caveolae organelles were isolated using an immunoaffinity protocol [42]. In brief, sheep anti-mouse IgG-coated magnetic beads (Dynabeads, Life Technologies, Carlsbad, CA) were pre-incubated with a monoclonal antibody specific for caveolin-3 (BD transduction) for 2 h at room temperature. Control beads were pre-incubated in PBS only. Both anti-Cav3 Dynabeads and control IgG Dynabeads were subsequently washed with PBS for 1 h at 4 °C on a rotator.

Plasmalemmal membrane rafts were fractionated from ventricular myocardial tissue as previously reported [44]. Canine LV (100 mg) was dounce homogenized in 1 mL ice cold, detergent-free Tricine buffer (20 mM Tricine, 1 mM EDTA, 250 mM sucrose, pH 7.4) and centrifuged at 1,000g for 10 min at 4 °C. Cell pellets were resuspended in 1 mL Tricine buffer, dounce homogenized, and centrifuged again at 1,000g for 10 min at 4 °C. An aliquot of pooled supernatant (30 µl) was set aside as the homogenate (H) sample. The pooled supernatant was then mixed with 30 % Percoll (Sigma) in Tricine buffer and subjected to ultracentrifugation at [116,000g for 30 min at 4 °C (Beckman MLS50 rotor, 33,000 rpm, no break). The separated PM was collected, mixed with ice-cold MBS buffer (20 mM 2-N-morpholinoethane-sulfonic acid, 20 mM KCl, 135 mM NaCl), and microcentrifuged at approximately 21,000g for 30 min at 4 °C to pellet the membrane. The supernatant was discarded. The membrane pellet was resuspended in ice cold 1 mL PBS and sonicated on ice (30 s bursts × 3). An aliquot (30 µl) was set aside as the PM for subsequent Western blotting.

Remaining PM was incubated with the anti-Cav3-coated Dynabeads for 1 h at 4 °C, using a rotator. Bound material (B), representative of cardiomyocyte caveolae vesicles, was separated magnetically from unbound (U), non-caveolae membranes. An aliquot (30 µl) of the unbound fraction (U) was set aside for subsequent Western blotting. Using the magnetic rack, beads were washed five times with PBS, resuspended in 2× loading buffer (100 mM Tris, pH 6.8, 2 % SDS, 0.02 % bromophenol blue, 20 % glycerol, 100 mM DTT), and boiled at 95 °C for 5 min to dissociate the protein from the beads. Homogenate (H), PM, bound material representative of cardiomyocyte caveolae (B), and unbound material representative of non-caveolae plasmalemmal membrane (U) were subjected to SDS-PAGE and immunoblotted using the indicated antibodies.

Western analysis

Protein extracts were run on SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted using primary antibody probes as follows: β_3 AR (1:1,000, Abnova); sGC α_1 (1:1,000, Abcam); sGC β_1 (1:4,000, Cayman Chemicals); Cav3 (1:10,000, BD Transduction); total nNOS (1:1,000, BD Transduction); phospho-nNOS (*Ser*¹⁴¹² p-nNOS, 1:1,000, gifted by K.J. Hurt, University of Colorado) [20]; GAPDH (1:10,000, cell signaling). Specificity of anti-sGC α_1 and - β_1 antibodies was confirmed using protein extracts from sGC $\alpha_1^{-/-}$ and sGC $\beta_1^{-/-}$ mouse hearts as previously published [57]. Primary

antibody binding was visualized by either horseradish peroxidase-conjugated secondary antibodies with enhanced chemiluminescence (GE Healthcare) or Alexa Fluor 680 or 800-conjugated secondary antibodies (molecular probes) with an Odyssey infrared imager (LI-COR). Densitometry analysis of immunoblots was performed using Image J Software (NIH) or Image Studio Software (Odyssey CLx v2.1, LI-COR).

sGC activity assay and redox state determination

Baseline and agonist-stimulated cGMP levels of total LV, Cav3⁺LR, and NLR from Control, MR, and MR+ β B hearts were measured by direct cGMP ELISA kit (New East Biosciences, Malvern, PA), as previously described [29, 57]. LV homogenates, Cav3⁺LR and NLR membrane fractions were pre-incubated at room temperature for 15 min in a solution for final concentrations of Tris 50 mM, pH 7.6, IBMX (3-isobutyl-1-methylxanthine) 0.75 mmol/L, creatine phosphate 3.5 mmol/L, creatinine phosphokinase 1 unit, GTP 1 mmol/L, and MgCl₂ 3 mmol/L. Samples were then incubated with or without DEA/NO (1 μ mol/L) or BAY 60-2770 (0.01 μ mol/L) at 37 °C for 10 min and subjected to diethyl ether extraction. cGMP levels of ether-extracted samples were measured by EIA according to kit manufacturer protocol. BAY60 compound was provided by J-P Stasch (Bayer AG, Wuppertal, Germany).

In vitro functional assays of β_3 AR/NO-cGMP coupling

Selective β_3 AR agonist BRL 37344 (BRL, 0.1 and 1.0 μ mol/L, Tocris Bioscience) was used to assess β_3 AR induction of NO-sGC-cGMP signaling. cGMP levels were measured as above.

Statistical analysis

All values are expressed as mean $\pm$ SEM. Statistical analyses were performed using: two-way ANOVA when determining interaction of conditions or the source of variance; one-way ANOVA followed by Tukey or Holm–Sidak’s multiple comparison test as appropriate; Brown-Forsythe test of variance, if relevant; and two-tailed paired, ratio paired, or one-tailed unpaired Student’s *t* test, as appropriate. Statistical significance was defined as *P* < 0.05. GraphPad Prism 6.0 was used for statistical and graphical analysis.

Results

MR-induced early eccentric remodeling persists and normal LV function remains preserved following 4 weeks of metoprolol therapy

As expected, dogs developed increased LV chamber dilation and decreased LV wall thickness to end-diastolic dimension, by 4 weeks post-MR induction, irrespective of metoprolol therapy (Table 1). LV fractional shortening remained normal in Control, MR, and MR+ β B hearts at 4 weeks.

Chronic metoprolol therapy increases myocardial β_3 AR expression without altering membrane microdomain distribution

Although expressed at low levels in the normal adult heart, β_3 AR is upregulated in the concentric hypertrophied and failing heart [8, 35, 60, 65]. We induced early stage eccentric hypertrophy via MR-mediated chronic volume overload in dogs, as previously described [29], treated some MR dogs with β_1 B metoprolol succinate (MR+ β B), and measured β_3 AR mRNA and protein expression as well as β_3 AR membrane microdomain distribution, comparing control, MR, and MR+ β B animals. β_3 AR mRNA expression, normalized to housekeeping gene RPLP1, increased 4.4×10^3 - and 3.2×10^2 -fold in MR and MR+ β B, respectively, relative to control (Fig. 1a). We detected β_3 AR by Western immunoblot at its predicted molecular weight of 43 kDa in both isolated adult canine cardiac myocytes and canine LV myocardium (Fig. 1b). Sensitivity and specificity of our β_3 AR antibody was demonstrated by immunoblotting various tissue and fractions known to have differential β_3 AR expression. As expected, β_3 AR expression was enriched in the PM fraction of murine LV relative to LV total protein. Our immunoblot likewise confirmed greater hepatic than myocardial β_3 AR expression [62]. β_3 AR expression was also greater in LV of human β_3 AR expressing transgenic mice than of wild type mice [5]. Chronic β_1 AR-blockade potentiated the increase in myocardial β_3 AR expression in MR, with 1.4 ± 0.1 -fold and 1.9 ± 0.2 -fold higher expression in MR and MR+ β B, respectively, than in Control (Fig. 1c, d). We also examined the distribution of β_3 AR in myocardial membrane microdomains as resolved by isopycnic ultracentrifugation. β_3 AR was detected within caveolae microdomains (Cav3⁺LR, F4–F5) and heavy density, non-lipid raft membrane fractions (NLR, F11) of all hearts (Fig. 1e). While overall expression varied amongst the study groups, distribution of β_3 AR across myocardial membrane fractions was similar (Fig. 1f). Caveolae localization of β_3 AR within cardiomyocytes was further verified by plasmalemmal caveolae-immunoaffinity isolation and immunoblot analysis (Fig. 1g). Since only myocytes exclusively express Cav3, caveolae-immunoaffinity isolation using anti-Cav3-antibody-coated magnetic beads ensured selection of only myocyte caveolae, and not that of endothelial cells, fibrocytes, or adipocytes.

Selective β_3 AR stimulation with BRL37344 (BRL) increases cGMP production within heavy density, NLR fractions

To assess β_3 AR/NO-cGMP coupling within membrane microdomains, we measured cGMP levels within Cav3⁺LR and NLR fractions at baseline and in response to the selective β_3 AR agonist BRL, all in the presence of non-selective phosphodiesterase inhibitor IBMX. While basal cGMP levels did not vary amongst Control, MR, and MR+ β B within respective membrane microdomains, basal cGMP levels within Cav3⁺LR exceeded that within corresponding NLR of Control and MR+ β B hearts (Fig. 2a). β_3 AR stimulation at increasing concentrations of BRL did not induce cGMP production within Cav3⁺LR of any study group (data not shown). Conversely, BRL did induce significant cGMP production within NLR of only MR+ β B (Table 2; Fig. 2b). To verify that NO-inducible sGC cyclase activity was intact in NLR of all groups and not solely MR+ β B, NO-donor DEA/NO-induced cGMP responses are shown for comparison. Thus, coupling of β_3 AR and NO-sGC-cGMP signaling was uniquely detected in NLR of MR+ β B.

β_3 AR couples with NO-sGC-cGMP signaling via nNOS activation

β_3 AR stimulation has been demonstrated to modulate the phosphorylation and activity of NOS isoforms [1, 5, 41, 60]. While eNOS activation by β_3 AR is inconsistently reported amongst various animal and human models [1, 40, 41, 60], recent in vitro studies of isolated neonatal rat ventricular cardiac myocytes and in vivo mouse studies demonstrated cardioprotective β_3 AR signaling mediated via nNOS activation, specifically through *Ser*¹⁴¹² phosphorylation of nNOS [5, 41, 60]. To corroborate the role of this mechanism in BRL-induced cGMP production within MR+ β B NLR, we assessed *Ser*¹⁴¹² p-nNOS and total nNOS by Western blot in BRL-stimulated NLR of Control, MR, and MR+ β B hearts (Fig. 3a). Increased phosphorylation of nNOS upon BRL stimulation was detected in NLR of MR+ β B but not MR or Control (Fig. 3b). These results are consistent with β_3 AR/NO-cGMP coupling in the NLR domain in a β_1 B therapy-dependent manner.

Effect of β_1 AR-blockade on myocardial membrane microdomain distribution of sGC subunits

As we have previously shown, α_1 and β_1 subunits of the sGC heterodimer localize to both Cav3⁺LR and NLR but differentially relocalize in the hemodynamically stressed heart. Under pressure- and volume-overload cardiac stress, sGC β_1 relocalizes away from Cav3⁺LR [29, 57]. Here, the distribution of sGC α_1 was unperturbed by either volume overload or chronic β_1 AR-blockade at this 4 week time point (Fig. 4a). Interestingly, chronic β_1 AR-blockade prevented the shift of sGC β_1 towards NLR that was otherwise observed in MR hearts. In MR+ β B, Cav3⁺LR localization of sGC β_1 was preserved and relocalization of sGC β_1 to heavy density, NLR was abated (Fig. 4b).

Chronic β_1 AR-blockade is associated with less oxidized and more reduced sGC

Given the observed membrane distribution of the sGC subunits and β_3 AR, physical colocalization of these proteins alone would not explain the functional coupling of β_3 AR and NO-sGC-cGMP signaling detected exclusively within NLR of MR+ β B. We hypothesized that chronic β_1 AR-blockade may have impacted the redox state of sGC within the various membrane microdomains. Dependent upon its ferrous heme moiety, inducible cyclase activity of the sGC heterodimer varies with its redox state. Oxidized sGC does not respond normally to NO. Heme-free sGC is unresponsive to NO stimulation. sGC activators (e.g. BAY60) can stimulate sGC cyclase activity independent of NO or heme; oxidized sGC has a relatively potentiated response to sGC activators [24]. Thus, differential cyclase response of sGC to BAY60 versus NO-donor DEA/NO can reveal its redox state [48]. We previously showed that while NLR-localized sGC becomes oxidized in the volume-overloaded heart, caveolae-localized sGC does not [29]. We measured cGMP levels of LV total protein extracts, Cav3⁺LR, and NLR at baseline and following stimulation with DEA/NO and BAY60, all in the presence of IBMX (Fig. 5). BAY60-induced cGMP production exceeded DEA/NO-induced cGMP production in MR and MR+ β B, suggesting an overall predominance of oxidized sGC in the volume-overloaded heart. However, BAY60-induced cGMP production in MR+ β B was similar to that in Control, both lower than in MR, suggesting that chronic β_1 AR-blockade blunts oxidation of sGC in the volume-overloaded heart (Fig. 5a). Alternatively, differential cyclase activity could reflect differential

expression of sGC. Immunoblots of sGC subunits confirmed that sGC β_1 expression did not vary amongst Control, MR, and MR+ β B (Fig. 6). While sGC α_1 levels fell slightly in MR + β B compared to either Control or MR, there was no impact on either basal or NO-inducible sGC activity. Baseline and DEA/NO-stimulated cGMP levels were similar for total protein extracts of all hearts.

We again found that caveolae localization protected sGC from oxidation in all hearts. In Cav3⁺LR, inducible sGC cyclase activity was similar for all study groups (Fig. 5b). Notably, the response to BAY60 was not potentiated within Cav3⁺LR, relative to control or DEA/NO. In fact, DEA/NO-induced cGMP production exceeded that of BAY60 within Cav3⁺LR, most significantly for MR and MR+ β B, suggesting a predominance of reduced sGC within caveolae. As we previously reported oxidation of sGC within MR NLR, we hypothesized that chronic β_1 -blockade might prevent this stress-induced change in NLR-localized sGC. Indeed, we found that NLR-localized sGC was not oxidized in MR+ β B (Fig. 5c). The BAY60 response of MR+ β B NLR was markedly blunted compared to that of MR NLR and similar to that of Control NLR.

Discussion

We demonstrated that chronic β_1 B in MR is associated with enhanced myocardial NO-sGC-cGMP signaling and β_3 AR/NO-cGMP coupling in specific membrane microdomains. Although myocardial β_3 AR expression was slightly increased, β_3 AR/NO-cGMP coupling could not be detected in untreated MR hearts. Increased oxidation of sGC in MR may have accounted for the lack of β_3 AR/NO-cGMP coupling. In contrast, MR+ β B hearts had greater upregulation of myocardial β_3 AR expression, improved sGC redox state, and detectable β_3 AR/NO-cGMP coupling, specifically in heavy-density membrane microdomains. These early changes in myocardial β_3 AR signaling at 4 weeks of metoprolol therapy in MR may contribute to the subsequent potentiated β AR responsiveness and improved cardiac myocyte and LV function seen after more prolonged 4-month metoprolol therapy [43].

Although the functional role of β_3 AR upregulation in HF had been widely debated, several recent studies have clearly shown a cardioprotective role for cardiac β_3 AR signaling. Chronic BRL treatment blunted pathologic cardiac remodeling and improved cardiac function, via a proposed restoration of the nitroso-redox balance, in mice subjected to pressure-overload cardiac stress. [41]. Similarly, transgenic mice with cardiac myocyte-specific expression of human β_3 AR had attenuated hypertrophic response to chronic isoproterenol and angiotensin II stimulation [5]. Cardiac-specific β_3 AR transgenic mice have also been shown to have enhanced cardiac contractility [26]. Furthermore, improved cardiac function and metabolism in a diabetic rat model was associated with metoprolol-induced upregulation of β_3 AR signaling [50, 51].

In the studies reported here, we interrogated a presumed myocardial β_3 AR/NO-cGMP signaling pathway to investigate this as a potential mechanism of cardiac benefit for metoprolol. We previously demonstrated that volume- overload cardiac stress disturbs the nitroso-redox balance as reflected by increased oxidation of sGC in the myocardium [29]. Chronic β_1 AR-blockade not only prevented the nitroso-redox imbalance in volume-

overloaded hearts, as reflected by decreased oxidation of sGC in MR+ β B hearts, but also induced coupling of β_3 AR and NO-sGC-cGMP signaling in a membrane microdomain specific fashion.

Using the selective β_3 AR agonist BRL, we tested the capacity for β_3 AR triggered cGMP production within LV myocardium, caveolae, and heavy-density, NLR. We compared the BRL response to the DEA/NO response to assess the relative coupling between β_3 AR and NO-sGC-cGMP signaling. Given reports of caveolae localization of a β_3 AR isoform in mice and of human β_3 AR in a cardiac-specific transgenic mouse [5, 47], along with overall upregulation of β_3 AR in metoprolol-treated diseased hearts [50–52], we expected to find enhanced β_3 AR/NO-cGMP coupling specifically within caveolae of MR+ β B hearts. Strikingly, chronic metoprolol therapy enhanced β_3 AR/NO-cGMP coupling within NLR, not Cav3⁺LR, of the eccentric hypertrophied heart. In MR hearts, β_3 AR did not increase cGMP production in any myocardial micro-domain despite upregulation of β_3 AR. This discrepancy between β_3 AR upregulation and coupling with NO-sGC-cGMP may be explained by the relocalization of sGC β_1 away from caveolae and the oxidation of sGC within NLR of MR hearts. While the potential signaling partners may be within a shared membrane microdomain, it appears that inducible sGC cyclase activity was compromised by oxidation.

Alternatively, coupling of β_3 AR and NO-sGC-cGMP within MR+ β B NLR might reflect local coupling of β_3 AR and nNOS. Recent studies in rodents demonstrated that β_3 AR signaling activates nNOS, as the effects of β_3 AR-mediated cardioprotection, NO production, and NOS signaling were blunted in mice with genetically deleted nNOS [41]. Moreover, β_3 AR activation of nNOS was found to occur via phosphorylation of the nNOS positive regulatory site, Ser¹⁴¹² [5, 41, 60]. While levels of Ser¹⁴¹²-phosphorylated nNOS (p-nNOS) are low under physiological conditions, nNOS activation, as reflected by elevated Ser¹⁴¹² p-nNOS, may play a critical counter-regulatory role in stress induced, pathophysiological conditions. In our canine model, selective β_3 AR agonist BRL induced an increase in Ser¹⁴¹² p-nNOS within NLR of MR+ β B but not in MR or Control, which is consistent with our findings of BRL-induced cGMP production within NLR of MR+ β B alone. These findings as a whole suggest that β_1 AR-blockade within the volume-overloaded heart induces β_3 AR/NO-cGMP coupling via enhanced β_3 AR activation of nNOS.

Our findings regarding β_3 AR and NO-sGC-cGMP signaling are novel and significant on several fronts. We are the first to demonstrate active and inducible coupling of β_3 AR and NO-sGC-cGMP signaling within the heart, notably in the β_1 B-treated, volume-overloaded heart and in a microdomain specific fashion. Recent studies of a double transgenic mouse with cardiac myocyte-specific expression of both the human β_3 AR and a cGMP-specific FRET sensor revealed increased basal sGC activity [5]. However, inducible or active coupling between β_3 AR and NO-sGC-cGMP was not interrogated. Our findings suggest that chronic β_1 B therapy is associated with a beneficial inducible and active β_3 AR/NO-cGMP coupling outside of myocardial caveolae. This is consistent with reports of (a) differential subcellular localization and G protein coupling of murine β_3 AR isoforms [47]; and (b) G_i mediation of GPCR activation of NOS [3, 10]. Moreover, previously reported nuclear β_3 AR regulation of gene transcription depends upon local downstream NO-sGC-cGMP activation [58]. While more studies are warranted to elucidate the mechanistic details, such reports of

nuclear β_3 AR activation of NO-sGC-cGMP signaling raise the possibility that the NLR microdomain may encompass nuclear envelope.

Interestingly, chronic metoprolol treatment was associated with the retention of sGC β_1 within caveolae. As the exact mechanism of caveolae localization of sGC β_1 remains to be determined, we can only hypothesize at this time that chronic β_1 AR-blockade either directly or indirectly impacts upon protein–protein interactions or reversible post-translational modifications (e.g. S-palmitoylation) that are generally believed to mediate caveolae localization [11, 22, 63]. Such studies are beyond the scope of this manuscript.

Myocardial β_3 AR expression is challenging to assess given the low expression of β_3 AR relative to β_1 AR and β_2 AR in the heart [36]. While radioligand binding assays are commonly used to determine receptor expression, high-affinity and high-specificity radiolabeled β AR subtype antagonists are necessary to differentiate the relative expression of the three β AR subtypes. In adipose tissue, which predominantly expresses β_3 AR over either β_1 AR or β_2 AR, radioligand binding assays are feasible for determining β_3 AR expression. However, the lack of high-affinity, selective β_3 AR antagonists precludes the use of radioligand binding assays for determining β_3 AR expression and distribution in the heart [12, 37, 39]. The exceedingly high concentration of radioligand β_3 AR antagonist needed would lead to high non-specific binding and inaccurate characterization of β_3 AR expression. Hence, in lieu of radioligand binding assay, we selected a highly sensitive and specific β_3 AR antibody that recognizes an epitope conserved within both canine and human β_3 AR amino acid sequences and has only 60–80 % identity, at high E values, with β_1 AR or β_2 AR amino acid sequences. We confirmed our Western analysis with qRT-PCR. Most importantly, we successfully demonstrated functional coupling of β_3 AR/NO-cGMP signaling with our in vitro BRL stimulation studies.

Our assessment of myocardial microdomain signaling is, by necessity, synthetic in nature, in that cyclase activity assays are performed on myocardial membrane fractions, as opposed to live, intact cardiac myocytes. Ideally, subcellular cGMP signaling within caveolae- and heavy-density NLR microdomains could be visualized in live cells with a FRET-based cGMP sensor. While transgenic mice with cardiac myocyte-specific expression of FRET-based cGMP sensors have recently been reported [5, 56], none are microdomain-targeted cGMP sensors. Thus, our combinatorial approach of cGMP enzyme immunoassays and Western analyses effectively allow us to interrogate microdomain signaling.

This study shows for the first time that chronic β_1 B with metoprolol therapy preserves and even enhances NO-cGMP signaling within specific myocardial microdomains in the volume-overloaded heart, in particular inducing coupling of β_3 AR/NO-cGMP signaling in the heavy-density non-lipid raft fraction. The present analysis raises the possibility that the antioxidant action shared by β Bs may be an indirect restoration of nitroso-redox balance in myocardial NLR membrane microdomain, mediated by enhanced β_3 AR signaling coupled with nNOS activation and sGC stimulation (Fig. 7). This indirect antioxidant mechanism may be particular to selective β_1 -blockers, or even metoprolol succinate for that matter. Whereas metoprolol upregulates myocardial β_3 AR expression, the non-selective β -blocker carvedilol does not [64]. Whereas carvedilol can directly scavenge free radicals, selective

β_1 -blockers do not [54]. In fact, carvedilol has been shown to block oxidative stress-mediated signaling via various mechanisms [27]. Thus, the comparative cardioprotection and cardioprotective mechanism of β_1 B metoprolol versus non-selective β Bs may depend upon the etiology of HF and thereby the effect of the specific cardiac injury upon myocardial NO and/or β_3 AR signaling [28]. Regardless, the enhancement of myocardial β_3 AR/NO-cGMP coupling with chronic β_1 AR-blockade in the volume-overloaded heart suggests new approaches to β AR modulation to optimize cardiac function. β AR modulation, with initial chronic β_1 AR antagonism followed by additional β_3 AR agonism, may offer further functional and clinical benefit to HF and MR patients. Moreover, direct stimulation of myocardial sGC on a background of neurohormonal blockade may similarly confer additional clinical benefit [13]. Such potential therapeutic implications warrant further mechanistic and eventual clinical studies.

Acknowledgments

We thank K. Joseph Hurt for providing the p-nNOS antibody; Johannes-Peter Stasch for providing BAY 60-2770; and Doug Tilley for reviewing the manuscript. This research was supported by the American Heart Association (Post-Doctoral Fellowship to D.M. Trappanese, Scientist Development Grant to E.J. Tsai), NIH (T32-HL091804 to S.R. Houser; R37-HL061690, R01-HL085503, P01-HL075443, and P01-HL108806 to W.J. Koch; P01-HL74237 and R01-HL108213 to F.A. Recchia; P50-HL077100 to L. Dell'Italia; K08-HL109159 to E.J. Tsai), and the Pennsylvania Department of Health (E.J. Tsai).

References

1. Aragon JP, Condit ME, Bhushan S, Predmore BL, Patel SS, Grinsfelder DB, Gundewar S, Jha S, Calvert JW, Barouch LA, Lavu M, Wright HM, Lefer DJ. Beta3-adrenoreceptor stimulation ameliorates myocardial ischemia-reperfusion injury via endothelial nitric oxide synthase and neuronal nitric oxide synthase activation. *J Am Coll Cardiol*. 2011; 58:2683–2691.10.1016/j.jacc.2011.09.033 [PubMed: 22152956]
2. Balijepalli RC, Foell JD, Hall DD, Hell JW, Kamp TJ. Localization of cardiac L-type Ca(2+) channels to a caveolar macromolecular signaling complex is required for beta(2)-adrenergic regulation. *Proc Natl Acad Sci USA*. 2006; 103:7500–7505. [PubMed: 16648270]
3. Banquet S, Delannoy E, Agouni A, Dessy C, Lacomme S, Hubert F, Richard V, Muller B, Leblais V. Role of G(i/o)-Src kinase-PI3K/Akt pathway and caveolin-1 in beta(2)-adrenoceptor coupling to endothelial NO synthase in mouse pulmonary artery. *Cell Signal*. 2011; 23:1136–1143.10.1016/j.cellsig.2011.02.008 [PubMed: 21385608]
4. Bartholomeu JB, Vanzelli AS, Rolim NP, Ferreira JC, Bechara LR, Tanaka LY, Rosa KT, Alves MM, Medeiros A, Mattos KC, Coelho MA, Irigoyen MC, Krieger EM, Krieger JE, Negrao CE, Ramires PR, Guatimosim S, Brum PC. Intracellular mechanisms of specific beta-adrenoceptor antagonists involved in improved cardiac function and survival in a genetic model of heart failure. *J Mol Cell Cardiol*. 2008; 45:240–249.10.1016/j.yjmcc.2008.05.011 [PubMed: 18632114]
5. Belge C, Hammond J, Dubois-Deruy E, Manoury B, Hamelet J, Beauloye C, Markl A, Pouleur AC, Bertrand L, Esfahani H, Jnaoui K, Gotz KR, Nikolaev VO, Vanderper A, Herijgers P, Lobysheva I, Iaccarino G, Hilfiker-Kleiner D, Tavernier G, Langin D, Dessy C, Balligand JL. Enhanced expression of beta3-adrenoceptors in cardiac myocytes attenuates neurohormone-induced hypertrophic remodeling through nitric oxide synthase. *Circulation*. 2013; 129:451–462.10.1161/CIRCULATIONAHA.113.004940 [PubMed: 24190960]
6. Bohm M, Deutsch HJ, Hartmann D, Rosee KL, Stablein A. Improvement of postreceptor events by metoprolol treatment in patients with chronic heart failure. *J Am Coll Cardiol*. 1997; 30:992–996. [PubMed: 9316529]
7. Bundgaard H, Liu CC, Garcia A, Hamilton EJ, Huang Y, Chia KK, Hunyor SN, Figtree GA, Rasmussen HH. beta(3) adrenergic stimulation of the cardiac Na⁺-K⁺ pump by reversal of an

- inhibitory oxidative modification. *Circulation*. 2010; 122:2699–2708.10.1161/CIRCULATIONAHA.110.964619 [PubMed: 21135361]
8. Cheng HJ, Zhang ZS, Onishi K, Ukai T, Sane DC, Cheng CP. Upregulation of functional beta(3)-adrenergic receptor in the failing canine myocardium. *Circ Res*. 2001; 89:599–606. [PubMed: 11577025]
 9. Damy T, Ratajczak P, Shah AM, Camors E, Marty I, Hasenfuss G, Marotte F, Samuel JL, Heymes C. Increased neuronal nitric oxide synthase-derived NO production in the failing human heart. *Lancet*. 2004; 363:1365–1367.10.1016/S0140-6736(04)16048-0 [PubMed: 15110495]
 10. Datar R, Kaesemeyer WH, Chandra S, Fulton DJ, Caldwell RW. Acute activation of eNOS by statins involves scavenger receptor-B1, G protein subunit Gi, phospholipase C and calcium influx. *Br J Pharmacol*. 2010; 160:1765–1772.10.1111/j.1476-5381.2010.00817.x [PubMed: 20649578]
 11. Feron O, Michel JB, Sase K, Michel T. Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. *Biochemistry*. 1998; 37:193–200.10.1021/bi972307p [PubMed: 9425039]
 12. Feve B, Emorine LJ, Lasnier F, Blin N, Baude B, Nahmias C, Strosberg AD, Pairault J. Atypical beta-adrenergic receptor in 3T3-F442A adipocytes. Pharmacological and molecular relationship with the human beta 3-adrenergic receptor. *J Biol Chem*. 1991; 266:20329–20336. [PubMed: 1682311]
 13. Fraccarollo D, Galuppo P, Schmidt I, Ertl G, Bauersachs J. Additive amelioration of left ventricular remodeling and molecular alterations by combined aldosterone and angiotensin receptor blockade after myocardial infarction. *Cardiovasc Res*. 2005; 67:97–105.10.1016/j.cardiores.2005.03.001 [PubMed: 15949473]
 14. Garcia-Prieto J, Garcia-Ruiz JM, Sanz-Rosa D, Pun A, Garcia-Alvarez A, Davidson SM, Fernandez-Friera L, Nuno-Ayala M, Fernandez-Jimenez R, Bernal JA, Izquierdo-Garcia JL, Jimenez-Borreguero J, Pizarro G, Ruiz-Cabello J, Macaya C, Fuster V, Yellon DM, Ibanez B. beta3 adrenergic receptor selective stimulation during ischemia/reperfusion improves cardiac function in translational models through inhibition of mPTP opening in cardiomyocytes. *Basic Res Cardiol*. 2014; 109:422.10.1007/s00395-014-0422-0 [PubMed: 24951958]
 15. Gauthier C, Leblais V, Kobzik L, Trochu JN, Khandoudi N, Bril A, Balligand JL, Le Marec H. The negative inotropic effect of beta3-adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. *J Clin Invest*. 1998; 102:1377–1384.10.1172/JCI2191 [PubMed: 9769330]
 16. George I, Sabbah HN, Xu K, Wang N, Wang J. beta-adrenergic receptor blockade reduces endoplasmic reticulum stress and normalizes calcium handling in a coronary embolization model of heart failure in canines. *Cardiovasc Res*. 2011; 91:447–455.10.1093/cvr/cvr106 [PubMed: 21493701]
 17. Hanks GH, Ardell JL, Tallaj J, Wei CC, Aban I, Holland M, Rynders P, Dillon R, Cardinal R, Hoover DB, Armour JA, Husain A, Dell'Italia LJ. Beta1-adrenoceptor blockade mitigates excessive norepinephrine release into cardiac interstitium in mitral regurgitation in dog. *Am J Physiol Heart Circ Physiol*. 2006; 291:H147–H151.10.1152/ajpheart.00951.2005 [PubMed: 16772522]
 18. Heilbrunn SM, Shah P, Bristow MR, Valantine HA, Ginsburg R, Fowler MB. Increased beta-receptor density and improved hemodynamic response to catecholamine stimulation during long-term metoprolol therapy in heart failure from dilated cardiomyopathy. *Circulation*. 1989; 79:483–490.10.1161/01.CIR.79.3.483 [PubMed: 2537158]
 19. Heusch G. Beta3-adrenoceptor activation just says NO to myocardial reperfusion injury. *J Am Coll Cardiol*. 2011; 58:2692–2694.10.1016/j.jacc.2011.09.034 [PubMed: 22152957]
 20. Hurt KJ, Sezen SF, Lagoda GF, Musicki B, Rameau GA, Snyder SH, Burnett AL. Cyclic AMP-dependent phosphorylation of neuronal nitric oxide synthase mediates penile erection. *Proc Natl Acad Sci USA*. 2012; 109:16624–16629.10.1073/Pnas.1213790109 [PubMed: 23012472]
 21. Iaccarino G, Tomhave ED, Lefkowitz RJ, Koch WJ. Reciprocal in vivo regulation of myocardial G protein-coupled receptor kinase expression by beta-adrenergic receptor stimulation and blockade. *Circulation*. 1998; 98:1783–1789.10.1161/01.CIR.98.17.1783 [PubMed: 9788834]

22. Ishizaka N, Griendling KK, Lassegue B, Alexander RW. Angiotensin II type 1 receptor: relationship with caveolae and caveolin after initial agonist stimulation. *Hypertension*. 1998; 32:459–466. [PubMed: 9740611]
23. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Groszmann RJ, Shah VH, Sessa WC. Nitric oxide synthase generates nitric oxide locally to regulate com-partmentalized protein S-nitrosylation and protein trafficking. *Proc Natl Acad Sci USA*. 2006; 103:19777–19782.10.1073/pnas.0605907103 [PubMed: 17170139]
24. Jones AW, Durante W, Korthuis RJ. Heme oxygenase-1 deficiency leads to alteration of soluble guanylate cyclase redox regulation. *J Pharmacol Exp Ther*. 2010; 335:85–91. doi:10.1124/jpet.110.169755. [PubMed: 20605906]
25. Kleaveland JP, Kussmaul WG, Vinciguerra T, Deters R, Cara-bello BA. Volume overload hypertrophy in a closed-chest model of mitral regurgitation. *Am J Physiol*. 1988; 254:H1034–H1041. [PubMed: 3381891]
26. Kohout TA, Takaoka H, McDonald PH, Perry SJ, Mao L, Lef-kowitz RJ, Rockman HA. Augmentation of cardiac contractility mediated by the human beta(3)-adrenergic receptor overexpressed in the hearts of transgenic mice. *Circulation*. 2001; 104:2485–2491. [PubMed: 11705829]
27. Koitabashi N, Arai M, Tomaru K, Takizawa T, Watanabe A, Niwano K, Yokoyama T, Wuytack F, Periasamy M, Nagai R, Kurabayashi M. Carvedilol effectively blocks oxidative stress-mediated downregulation of sarcoplasmic reticulum Ca²⁺-ATPase 2 gene transcription through modification of Sp1 binding. *Biochem Biophys Res Commun*. 2005; 328:116–124.10.1016/j.bbrc.2004.12.139 [PubMed: 15670758]
28. Le DE, Pascotto M, Leong-Poi H, Sari I, Micari A, Kaul S. Anti-inflammatory and pro-angiogenic effects of beta blockers in a canine model of chronic ischemic cardiomyopathy: comparison between carvedilol and metoprolol. *Basic Res Cardiol*. 2013; 108:384.10.1007/s00395-013-0384-7 [PubMed: 24072434]
29. Liu Y, Dillon AR, Tillson M, Makarewich C, Nguyen V, Dell'Italia L, Sabri AK, Rizzo V, Tsai EJ. Volume overload induces differential spatiotemporal regulation of myocardial soluble guanylyl cyclase in eccentric hypertrophy and heart failure. *J Mol Cell Cardiol*. 2013; 60:72–83.10.1016/j.yjmcc.2013.03.019 [PubMed: 23567617]
30. Macdougall DA, Agarwal SR, Stopford EA, Chu H, Collins JA, Longster AL, Colyer J, Harvey RD, Calaghan S. Caveolae compartmentalise beta2-adrenoceptor signals by curtailing cAMP production and maintaining phosphatase activity in the sarcoplasmic reticulum of the adult ventricular myocyte. *J Mol Cell Cardiol*. 2012; 52:388–400.10.1016/j.yjmcc.2011.06.014 [PubMed: 21740911]
31. Maczewski M, Mackiewicz U. Effect of metoprolol and ivabradine on left ventricular remodelling and Ca²⁺ handling in the post-infarction rat heart. *Cardiovasc Res*. 2008; 79:42–51.10.1093/cvr/cvn057 [PubMed: 18310678]
32. Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Berretta RM, Rizzo V, Molkentin JD, Houser SR. A caveolae-targeted L-type Ca(2)+ channel antagonist inhibits hypertrophic signaling without reducing cardiac contractility. *Circ Res*. 2012; 110:669–674.10.1161/CIRCRESAHA.111.264028 [PubMed: 22302787]
33. Markandeya YS, Fahey JM, Pluteanu F, Cribbs LL, Balijepalli RC. Caveolin-3 regulates protein kinase A modulation of the Ca(V)3.2 (alpha1H) T-type Ca²⁺ channels. *J Biol Chem*. 2011; 286:2433–2444.10.1074/jbc.M110.182550 [PubMed: 21084288]
34. Mehta RH, Supiano MA, Oral H, Grossman PM, Montgomery DS, Smith MJ, Starling MR. Compared with control subjects, the systemic sympathetic nervous system is activated in patients with mitral regurgitation. *Am Heart J*. 2003; 145:1078–1085.10.1016/S0002-8703(03)00111-X [PubMed: 12796766]
35. Moniotte S, Kobzik L, Feron O, Trochu JN, Gauthier C, Balligand JL. Upregulation of beta(3)-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation*. 2001; 103:1649–1655. [PubMed: 11273992]
36. Monto F, Oliver E, Vicente D, Rueda J, Aguero J, Almenar L, Ivorra MD, Baretino D, D'Ocon P. Different expression of adrenoceptors and GRKs in the human myocardium depends on heart

- failure etiology and correlates to clinical variables. *Am J Physiol Heart Circ Physiol.* 2012; 303:H368–H376.10.1152/ajpheart.01061.2011 [PubMed: 22685168]
37. Muzzin P, Revelli JP, Fraser CM, Giacobino JP. Radioligand binding studies of the atypical beta 3-adrenergic receptor in rat brown adipose tissue using [3H]CGP 12177. *FEBS Lett.* 1992; 298:162–164.10.1016/0014-5793(92)80046-J [PubMed: 1347503]
 38. Nagatomo Y, Yoshikawa T, Kohno T, Yoshizawa A, Anzai T, Meguro T, Satoh T, Ogawa S. Effects of beta-blocker therapy on high sensitivity c-reactive protein, oxidative stress, and cardiac function in patients with congestive heart failure. *J Card Fail.* 2007; 13:365–371.10.1016/j.cardfail.2007.02.004 [PubMed: 17602983]
 39. Nahmias C, Blin N, Elalouf JM, Mattei MG, Strosberg AD, Emorine LJ. Molecular characterization of the mouse beta 3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *EMBO J.* 1991; 10:3721–3727. [PubMed: 1718744]
 40. Napp A, Brixius K, Pott C, Ziskoven C, Boelck B, Mehlhorn U, Schwinger RH, Bloch W. Effects of the beta3-adrenergic agonist BRL 37344 on endothelial nitric oxide synthase phosphorylation and force of contraction in human failing myocardium. *J Card Fail.* 2009; 15:57–67.10.1016/j.cardfail.2008.08.006 [PubMed: 19181295]
 41. Niu X, Watts VL, Cingolani OH, Sivakumaran V, Leyton-Mange JS, Ellis CL, Miller KL, Vandegaer K, Bedja D, Gabrielson KL, Paolucci N, Kass DA, Barouch LA. Cardioprotective effect of beta-3 adrenergic receptor agonism: role of neuronal nitric oxide synthase. *J Am Coll Cardiol.* 2012; 59:1979–1987.10.1016/j.jacc.2011.12.046 [PubMed: 22624839]
 42. Oh P, Schnitzer JE. Immunolocalization of caveolae with high affinity antibody binding to the oligomeric caveolin cage. Toward understanding the basis of purification. *J Biol Chem.* 1999; 274:23144–23154.10.1074/jbc.274.33.23144 [PubMed: 10438484]
 43. Pat B, Killingsworth C, Denney T, Zheng J, Powell P, Tillson M, Dillon AR, Dell'Italia LJ. Dissociation between cardiomyocyte function and remodeling with beta-adrenergic receptor blockade in isolated canine mitral regurgitation. *Am J Physiol Heart Circ Physiol.* 2008; 295:H2321–H2327.10.1152/ajpheart.00746.2008 [PubMed: 18849331]
 44. Rizzo V, Morton C, DePaola N, Schnitzer JE, Davies PF. Recruitment of endothelial caveolae into mechanotransduction pathways by flow conditioning in vitro. *Am J Physiol Heart Circ Physiol.* 2003; 285:H1720–H1729.10.1152/ajpheart.00344.2002 [PubMed: 12816751]
 45. Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of beta-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem.* 2000; 275:41447–41457. [PubMed: 11006286]
 46. Sabri A, Rafiq K, Seqqat R, Kolpakov MA, Dillon R, Dell'Italia LJ. Sympathetic activation causes focal adhesion signaling alteration in early compensated volume overload attributable to isolated mitral regurgitation in the dog. *Circ Res.* 2008; 102:1127–1136. [PubMed: 18356543]
 47. Sato M, Hutchinson DS, Halls ML, Furness SG, Bengtsson T, Evans BA, Summers RJ. Interaction with caveolin-1 modulates G protein coupling of mouse beta3-adrenoceptor. *J Biol Chem.* 2012; 287:20674–20688.10.1074/jbc.M111.280651 [PubMed: 22535965]
 48. Schmidt K, Neubauer A, Kolesnik B, Stasch JP, Werner ER, Gorren AC, Mayer B. Tetrahydrobiopterin protects soluble guanylate cyclase against oxidative inactivation. *Mol Pharmacol.* 2012; 82:420–427.10.1124/mol.112.079855 [PubMed: 22648973]
 49. Schwencke C, Okumura S, Yamamoto M, Geng YJ, Ishikawa Y. Colocalization of beta-adrenergic receptors and caveolin within the plasma membrane. *J Cell Biochem.* 1999; 75:64–72. [PubMed: 10462705]
 50. Sharma V, Dhillon P, Wambolt R, Parsons H, Brownsey R, Al-lard MF, McNeill JH. Metoprolol improves cardiac function and modulates cardiac metabolism in the streptozotocin-diabetic rat. *Am J Physiol Heart Circ Physiol.* 2008; 294:H1609–H1620.10.1152/ajpheart.00949.2007 [PubMed: 18203848]
 51. Sharma V, Parsons H, Allard MF, McNeill JH. Metoprolol increases the expression of beta(3)-adrenoceptors in the diabetic heart: effects on nitric oxide signaling and forkhead transcription factor-3. *Eur J Pharmacol.* 2008; 595:44–51.10.1016/j.ejphar.2008.07.042 [PubMed: 18703049]
 52. Sharma V, Sharma A, Saran V, Bernatchez PN, Allard MF, McNeill JH. beta-receptor antagonist treatment prevents activation of cell death signaling in the diabetic heart independent of its

- metabolic actions. *Eur J Pharmacol.* 2011; 657:117–125.10.1016/j.ejphar.2011.01.044 [PubMed: 21296063]
53. Spoladore R, Fragasso G, Perseghin G, De Cobelli F, Esposito A, Maranta F, Calori G, Locatelli M, Lattuada G, Scifo P, Del Maschio A, Margonato A. Beneficial effects of beta-blockers on left ventricular function and cellular energy reserve in patients with heart failure. *Fundam Clin Pharmacol.* 2013; 27:455–464.10.1111/j.1472-8206.2012.01029.x [PubMed: 22320703]
 54. Szajerski P, Zielonka J, Sikora A, Adamus J, Marcinek A, Gebicki J, Kozlovski VI, Drelicharz L, Chlopicki S. Radical scavenging and NO-releasing properties of selected beta-adrenoreceptor antagonists. *Free Radic Res.* 2006; 40:741–752. [PubMed: 16984001]
 55. Takimoto E, Champion HC, Belardi D, Moslehi J, Mongillo M, Mergia E, Montrose DC, Isoda T, Aufiero K, Zaccolo M, Dostmann WR, Smith CJ, Kass DA. cGMP catabolism by phosphodiesterase 5A regulates cardiac adrenergic stimulation by NOS3-dependent mechanism. *Circ Res.* 2005; 96:100–109.10.1161/01.RES.0000152262.22968.72 [PubMed: 15576651]
 56. Thunemann M, Wen L, Hillenbrand M, Vachavios A, Feil S, Ott T, Han X, Fukumura D, Jain RK, Russwurm M, de Wit C, Feil R. Transgenic mice for cGMP imaging. *Circ Res.* 2013; 113:365–371.10.1161/CIRCRESAHA.113.301063 [PubMed: 23801067]
 57. Tsai EJ, Liu Y, Koitabashi N, Bedja D, Danner T, Jasmin JF, Lisanti MP, Friebe A, Takimoto E, Kass DA. Pressure-overload-induced subcellular relocalization/oxidation of soluble guanylyl cyclase in the heart modulates enzyme stimulation. *Circ Res.* 2012; 110:295–303. [PubMed: 22095726]
 58. Vaniotis G, Glazkova I, Merlen C, Smith C, Villeneuve LR, Chatenet D, Therien M, Fournier A, Tadevosyan A, Trieu P, Nattel S, Hebert TE, Allen BG. Regulation of cardiac nitric oxide signaling by nuclear beta-adrenergic and endothelin receptors. *J Mol Cell Cardiol.* 2013; 62:58–68.10.1016/j.yjmcc.2013.05.003 [PubMed: 23684854]
 59. Waagstein F, Caidahl K, Wallentin I, Bergh CH, Hjalmarson A. Long-term beta-blockade in dilated cardiomyopathy. Effects of short- and long-term metoprolol treatment followed by withdrawal and readministration of metoprolol. *Circulation.* 1989; 80:551–563.10.1161/01.CIR.80.3.551 [PubMed: 2548768]
 60. Watts VL, Sepulveda FM, Cingolani OH, Ho AS, Niu X, Kim R, Miller KL, Vandegaer K, Bedja D, Gabrielson KL, Rameau G, O'Rourke B, Kass DA, Barouch LA. Anti-hypertrophic and anti-oxidant effect of beta3-adrenergic stimulation in myocytes requires differential neuronal NOS phosphorylation. *J Mol Cell Cardiol.* 2013; 62:8–17.10.1016/j.yjmcc.2013.04.025 [PubMed: 23643588]
 61. Whalen EJ, Foster MW, Matsumoto A, Ozawa K, Violin JD, Que LG, Nelson CD, Benhar M, Keys JR, Rockman HA, Koch WJ, Daaka Y, Lefkowitz RJ, Stamler JS. Regulation of beta-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2. *Cell.* 2007; 129:511–522.10.1016/j.cell.2007.02.046 [PubMed: 17482545]
 62. Wu JL, Liu WZ, Liu JH, Qiao LY, Yuan YN. Distribution and quantification of beta-3 adrenergic receptor in tissues of sheep. *Animal.* 2011; 5:88–93.10.1017/S1751731110001564 [PubMed: 22440706]
 63. Yeh DC, Duncan JA, Yamashita S, Michel T. Depalmitoylation of endothelial nitric-oxide synthase by acyl-protein thioesterase 1 is potentiated by Ca(2+)-calmodulin. *J Biol Chem.* 1999; 274:33148–33154. [PubMed: 10551886]
 64. Zhao Q, Wu TG, Jiang ZF, Chen GW, Lin Y, Wang LX. Effect of beta-blockers on beta3-adrenoceptor expression in chronic heart failure. *Cardiovasc Drugs Ther.* 2007; 21:85–90.10.1007/s10557-007-6016-4 [PubMed: 17440824]
 65. Zhao Q, Zeng F, Liu JB, He Y, Li B, Jiang ZF, Wu TG, Wang LX. Upregulation of beta3-adrenergic receptor expression in the atrium of rats with chronic heart failure. *J Cardiovasc Pharmacol Ther.* 2013; 18:133–137.10.1177/1074248412460123 [PubMed: 23008154]
 66. Zheng J, Yancey DM, Ahmed MI, Wei CC, Powell PC, Shanmugam M, Gupta H, Lloyd SG, McGiffin DC, Schiros CG, Denney TS Jr, Babu GJ, Dell'Italia LJ. Increased sarcolipin expression and adrenergic drive in humans with preserved left ventricular ejection fraction and chronic isolated mitral regurgitation. *Circ Heart Fail.* 2014; 7:194–202.10.1161/CIRCHEARTFAILURE.113.000519 [PubMed: 24297688]

Fig. 1.

Volume-overload-induced β_3 AR expression is potentiated by chronic β_1 AR-blockade. **a** LV β_3 AR mRNA levels normalized to RPLP1 mRNA levels (mean $\pm$ SEM) were determined in Control, MR, and MR+ β B. $n = 5$ animals per group; one-way ANOVA $P < 0.0001$ **b** Western of β_3 AR in LV plasma membrane fraction (PM) and total protein from myocardium (LV), isolated cardiac myocytes (CM), and liver (Li) of C57Bl/6 mice (WT), human β_3 AR-expressing transgenic mice (Tg), and dogs. **c** Representative Westerns of β_3 AR in LV total protein extracts from study dogs. **d** Summary densitometry analysis of total β_3 AR expression normalized to GAPDH and Control levels. $n = 4-6$ animals per study group. one-way ANOVA $P = 0.002$. * $P < 0.05$, $^\dagger P < 0.01$ on Holm-Sidak's multiple comparison test. **e** Representative Westerns of Cav3 and β_3 AR across sucrose density gradient fractions (F1–F11) of Control, MR, and MR+ β B. **f** Cav3 and β_3 AR distribution is presented for each fraction as a percentage of the sum signal across all fractions. For Cav3, $n = 4-7$ per study group. For β_3 AR, $n = 3-6$ per study group. **g** Cardiac myocyte plasmalemmal caveolae organelles were immunoaffinity-purified with anti-Cav3. β_3 AR was detected LV homogenate (H), LV plasmalemmal membrane fraction (PM), cardiomyocyte plasmalemmal caveolae (B bound fraction), and in LV plasmalemmal membrane depleted of caveolae (U unbound fraction)

Fig. 2.

Coupling of β_3 AR and NO-sGC-cGMP signaling is detectable within NLR of MR+ β B. cGMP levels of Control, MR, and MR+ β B Cav3⁺LR and NLR membrane fractions were measured by EIA at **a** baseline and **b** upon stimulation with selective β_3 AR agonist BRL (0.1 and 1.0 μ M) or NO-donor DEA/NO (1.0 μ M). No significant BRL response was detected for Cav3⁺LR in any study group (not shown). All measurements were in the presence of IBMX. Averaged baseline cGMP levels included 10–12 measurements, including replicates, per study group. For summary analysis of BRL stimulated cGMP production, only paired baseline and BRL-induced cGMP levels were included, $n = 4$ –6 per study group. For each BRL dose, one-way ANOVA $P = 0.1$, $*P < 0.05$, and $^\dagger P = 0.1$ on unpaired student t test. For BRL 0.1 μ M dose, Brown-Forsythe $P < 0.05$.

Fig. 3. Selective β_3 AR agonist BRL induces *Ser*¹⁴¹²-phosphorylation of nNOS within MR+βB NLR. **a** Representative Westerns of *Ser*¹⁴¹² p-nNOS and total nNOS in Control, MR, and MR+βB NLR. Samples were stimulated with BRL 0.1 μ M for 5, 10, and 15 min. **b** Summary densitometry analysis shows the ratio of *Ser*¹⁴¹² p-nNOS to total nNOS as normalized to unstimulated levels of respective samples, $n = 3-4$ per study group. Two-way ANOVA $P < 0.01$ for study group. Tukey's multiple comparison testing $^*P < 0.05$ vs. control, $^\dagger P < 0.05$ vs. MR, $^\ddagger P < 0.01$ vs. MR

Fig. 4. sGC β_1 relocates away from caveolae in MR whereas chronic β_1 B therapy preserves Cav3⁺LR localization in MR+ β B heart. **a** Representative Westerns of sucrose density gradient fractions of Control, MR, and MR+ β B. sGC α_1 and sGC β_1 are enriched in Cav3⁺LR of Control. sGC β_1 shifts away from F4–F5 in MR, with concomitant relocation to NLR (F11). sGC β_1 remains within in Cav3⁺LR in MR+ β B. **b** Summary densitometry analysis of all hearts analyzed ($n = 4–7$ per study group). One-way ANOVA for sGC β_1 $P = 0.012$ for F4, $P = 0.007$ for F9, $P = 0.031$ for F11. Unpaired student t test $*P < 0.05$ for MR vs. control, $^{\dagger}P < 0.05$ for MR+ β B vs. MR, $^{\ddagger}P < 0.10$ for MR+ β B vs. MR.

Fig. 5.

Chronic β_1 AR blockade protects sGC from oxidation. **a** BAY60-induced cGMP production markedly exceeds NO-induced cGMP production of MR, indicating predominance of oxidized sGC in MR hearts. Levels of cGMP were measured at baseline and following stimulation with DEA/NO and BAY60 in the presence of IBMX in **a** LV total protein extract, **b** Cav3⁺LR and **c** NLR fractions. Induced cGMP production is shown as the increment above baseline cGMP levels expressed in fmol cGMP/lg protein. * $P < 0.01$, $^\dagger P < 0.001$ on ratio paired t test; $^\ddagger P < 0.05$ vs. control and MR+ β B on Tukey multiple comparison test; $^\P P < 0.1$ on ratio paired t test; $^\S P = 0.05$ on ratio paired t test; $^\# P < 0.01$ vs. control, $P < 0.001$ vs. MR+ β B on Tukey multiple comparison test

Fig. 6.

Chronic β_1 AR-blockade differentially impacts sGC subunit expression. **a** Representative Western blot of total protein extracts from LV myocardium. **b** Summary densitometry analysis of all Westerns. Signals were standardized to respective GAPDH and then normalized to Control. Mean of replicate measures was used for each heart sample. *Bar graph* represents group mean $\pm$ SEM. Total dogs analyzed: Control $n = 6$, MR $n = 5$, and MR+ β B $n = 4$. One-way ANOVA $P = 0.007$ for sGC α_1 ; $P = 0.095$ for sGC β_1 .

Fig. 7.

β_1 AR-blockade enhances coupling of β_3 AR and NO-sGC-cGMP signaling within myocardial NLR. Increased myocardial β_3 AR expression, colocalization of β_3 AR, sGC, and nNOS, and protection of sGC from oxidation contribute to the enhanced β_3 AR/NO-cGMP coupling in chronic β_1 B treated, volume-overloaded hearts

Table 1

LV structure and function assessed by echocardiography in canine chronic MR model

	Control	MR	MR+βB
LVEDD (cm)	3.7 ± 0.1	4.5 ± 0.2 *	4.9 ± 0.2 *
LVEDS (cm)	2.1 ± 0.1	2.5 ± 0.1 *	2.8 ± 0.1 *
LVWT/LVEDD	0.28 ± 0.03	0.19 ± 0.02 *	0.18 ± 0.01 *
FS (%)	43 ± 3	44 ± 3	43 ± 3

* $P < 0.05$ vs. control on two-tailed, unpaired Student's t test

Table 2

cGMP levels at baseline and following BRL stimulation within NLR

	<i>n</i>	Baseline	BRL-stimulated 0.1 μ M	BRL-stimulated 1.0 μ M
Control	4	418.8 $\pm$ 166.2	438.5 $\pm$ 193.3	445.9 $\pm$ 167.3
MR	6	1,191.0 $\pm$ 678.4	2,052.0 $\pm$ 1,595.0	3,345.0 $\pm$ 2,965.0
MR+ β B	5	503.4 $\pm$ 256.8	622.3 $\pm$ 267.3 [*]	753.4 $\pm$ 314.4 [†]

^{*} $P = 0.10$,

[†] $P < 0.05$ on two-tailed, paired student *t* test