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Chronic GPR30 Agonist Therapy Causes Restoration of Normal Cardiac Functional Performance in a Male Mouse Model of Progressive Heart Failure: Insights into Cellular Mechanisms

Xiaowei Zhang[a, b], Tiankai Li[b,c], Heng-Jie Cheng[b], Hao Wang[d], Carlos M Ferrario[e], Leanne Groban[d],

Che Ping Cheng [b☆]

[a] Department of Cardiology, The Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China.

[b] Cardiovascular Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina

[c] Department of Cardiology, The First Affiliated Hospital of Harbin Medical University, Harbin, China

[d] Department of Anesthesiology, Wake Forest School of Medicine, Winston-Salem, North Carolina

[e] Department of Surgery, Wake Forest School of Medicine, Winston-Salem, North Carolina

☆Corresponding author:
Che Ping Cheng, Cardiovascular Medicine
Wake Forest School of Medicine
Medical Center Boulevard, Winston-Salem, NC 27157-1045
Tel: 336-716-2887; Fax: 336-716-5324
E-mail: ccheng@wakehealth.edu

taianrenxiaowei@126.com (Xiaowei Zhang) litiankaidoctor@163.com (Tiankai Li)

hcheng@wakehealth.edu (Heng-Jie Cheng) haowang@wakehealth.edu (Hao Wang)

cferrari@wakehealth.edu (Carlos M Ferrario) lgroban@wakehealth.edu (Leanne Groban)

ccheng@wakehealth.edu(Che Ping Cheng)

Abstract

Aims: G protein-coupled estrogen receptor 30 (GPR30) activation by its agonist, G1, exhibits

beneficial actions in female with heart failure (HF). Recent evidence indicates its cardiovascular benefits may also include male as well. However, whether and how GPR30 activation may limit HF progression and have a salutary role in males is unknown. We hypothesized that chronic G1 treatment improves LV and cardiomyocyte function, $[Ca^{2+}]_i$ regulation and β-adrenergic reserve, thus limiting HF progression in male.

Main Methods: We compared left ventricle (LV) and myocyte function, $[Ca^{2+}]_i$ transient ($[Ca^{2+}]_{iT}$) and β-AR modulation in control male mice (12/group) and isoproterenol-induced HF (150 mg/kg s.c. for 2 days). Two weeks after isoproterenol injection, HF mice received placebo, or G1 (150 μg/kg/day s.c. mini-pump) for 2 weeks.

Key Findings: Isoproterenol-treated mice exhibited HF with preserved ejection fraction (HFpEF) at 2-weeks and progressed to HF with reduced EF (HFrEF) at 4-weeks, manifested by significantly increased LV time constant of relaxation (τ), decreased EF and mitral flow (dV/dt_{max}), which were accompanied by reduced myocyte contraction (dL/dt_{max}), relaxation (dR/dt_{max}) and $[Ca^{2+}]_{iT}$. Acute isoproterenol-superfusion caused significantly smaller increases in dL/dt_{max}, dR/dt_{max} and $[Ca^{2+}]_{iT}$. G1 treatment in HF increased basal and isoproterenol-stimulated increases in EF and LV contractility of E_{ES}. Importantly, G1 improved basal and isoproterenol-stimulated dL/dt_{max}, dR/dt_{max} and $[Ca^{2+}]_{iT}$ to control levels and restored normal cardiac β-AR subtypes modulation.

Significance: Chronic G1 treatment restores normal myocyte basal and β-AR-stimulated contraction, relaxation, and $[Ca^{2+}]_{iT}$, thereby reversing LV dysfunction and playing a rescue role in a male mouse model of HF.

Keywords

G protein-coupled estrogen receptor 30 (GPR30), Heart failure, Pressure-volume relation,

Cardiomyocyte, $[Ca^{2+}]_i$ regulation, β-adrenergic reserve

ABBREVIATIONS

GPR30, G protein-coupled receptor 30; HF, heart failure; HFpEF, HF with preserved ejection fraction; HFrEF, HF with reduced ejection fraction; β-AR, β-adrenergic receptor; ISO, isoproterenol; LV, left ventricle; P, Pressure; V, Volume; SV, stroke volume; τ, LV time constant of relaxation; V_{ED}, end-diastolic volume; V_{ES}, end-systolic volume; EF, ejection fraction; dV/dt_{max}, the peak rate of mitral flow; E_A, arterial elastance, E_{ES}, the slope of linear P_{ES}-V_{ES} relation; E_{ES}/E_A, ratio of LV-arterial coupling; LVAC, LV-arterial coupling, M_{SW}, the slope of stroke work-V_{ED} relation; OVX, bilateral ovariectomy; P_{ES}, end-systolic pressure; P_{ED}, end-systolic pressure; P_{min}, LV minimum pressure; FS, fractional shortening; E/e', early transmitral flow velocity-to-mitral annular velocity ratio; SA, myocyte percent shortening; dL/dt_{max}, the maximum rate of myocyte shortening; dR/dt_{max}, the maximum rate of myocyte re-lengthening; $[Ca^{2+}]_{iT}$, calcium transient; ANF, atrial natriuretic factor; PLB, phospholamban; SERCA2a, sarcoplasmic reticulum Ca^{2+} ATPase 2a.

1. Introduction

Heart failure (HF) with preserved ejection fraction (HFpEF) is outpacing other forms of HF because of the expanding elderly population. Large cohort studies reveal that almost 50% of the HF populations have HFpEF with a comparable poor prognosis compared to HF with reduced EF (HFrEF). These worrisome epidemiological trends contrast with the uncertainties concerning the pathophysiological mechanisms underlying HFpEF, diagnostic guidelines and therapeutic strategies [1-3] So far, no randomized controlled trial has shown improved survival of HFpEF. There are no targeted, effective treatments available.

The G protein-coupled receptor 30 (GPR30), functions alongside traditional estrogen receptors, ERα and ERβ. ERα and GPR30 are expressed at similar levels in cardiac tissue from male and female rodents and humans [4-6]. GPR30 plays critical roles in the development of left ventricular (LV) dysfunction and HF [4, 7, 8]. Activation of GPR30 exhibits beneficial actions in ischemia/reperfusion injury, hypertension and HF [4, 7, 9-12]. While reports on GPR30 have highlighted mostly female sex-specific health issues, recent evidence indicates equivalent benefits in males [4, 13, 14]. It was reported that male, but not female, GPR30-deficient mice suffered from impaired cardiac function [8]. Ejection fraction (EF) and fractional shortening (FS) were both decreased in an age-dependent manner only in GPR30-knockout male mice [13]. Also, cardiomyocyte-specific GPR30-knockout leads to LV dysfunction in both sexes, but with more adverse changes in systolic function and LV structural remodeling among male knockout mice [15], which suggests a potential salutary role of GPR30 activation in male HF [8, 13]. However, the changes caused by GPR30 activation during HF progression in males have not been systematically evaluated. The functional effect of chronic GPR30 activation on single-myocyte mechanics, the dynamics of the cytosolic $[Ca^{2+}]_i$ and contractility reserve have not been previously assessed in an integrated fashion in HF. The direct cardiac effects and cellular mechanisms of GPR30 activation-caused cardiac protective effects remain unclear. Moreover, although HFpEF is the most common form of HF, limited studies have explored the roles of GPR30 in the development and progression of diastolic dysfunction. Whether and to what extent chronic GPR30 activation can limit the progression of HFpEF to HFrEF, or even reverse HFpEF, thereby changing the natural history of HF, remains to be critically examined.

The aim of this study was to explore the impact of chronic GPR30 activation on the progression of HFpEF. We simultaneously assessed the direct cardiac effects and underlying

cellular mechanisms on the effects of early initiation of G1, a highly selective GPR30 agonist [9, 16-19], in male mice with isoproterenol-induced HF. Isoproterenol-induced HF, a time-and dose-dependent method, mimics many of the structural, functional, and hormonal changes observed in clinical HF [20-25]. In the current study, isoproterenol-treated mice exhibited HFpEF at 2 weeks, which progressed to HFrEF at 4 weeks following the last isoproterenol injection. We assessed the hypothesis that early initiation of chronic G1 therapy could interrupt the progression of HFpEF to HFrEF and lead to regression of HFpEF in a male mouse model of isoproterenol-induced progressive HF by: (a) restoring normal LV function; and (b) improving intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_i$ transient ($[Ca^{2+}]_{iT}$) and β-adrenergic reserve accompanied with reversing the abnormal cardiac β-adrenergic receptor (β-AR) subtype system modulation.

2. Materials and Methods

2.1. Animal Model

This study was approved by the Animal Care and Use Committee of Wake Forest School of Medicine and conformed to the National Guide for the Care and Use of Laboratory Animals (NIH Publication 8th Edition, update 2011). The experimental procedures are illustrated in **Figure 1**. Briefly, total 47 age-matched C57BL/6 male mice (~4 months old) weighing 26~34 g (Charles River Laboratories International, Inc.) were randomly divided into control (n=12) and HF groups (n=35). HF was induced by two subcutaneous injections of isoproterenol (ISO), spaced 24 hours apart, at a dose of 150 mg/kg as documented elsewhere [22-25]. Thirty isoproterenol-treated mice survived after ISO-induction (~12% mortality within 48 hours); these included in the HF group. High doses of isoproterenol cause time and dose-dependent structural remodeling and cardiac dysfunction that results in HF. Based

on our laboratory and others previously published serial time course studies in this model [20-25], and well-characterized histology and altered LV structure and function in isoproterenol-induced HF, we designed a 4-week study. Cardiac function was assessed at the beginning of the study and weekly via transthoracic echocardiography during light isoflurane anesthesia [26]. ISO-treated animals had HFpEF onset by 2 weeks and this progressed to mid-range HFrEF by 4 weeks. To further establish the validity of early diastolic functional impairments by ISO, with maintained systolic function, 6 animals were studied 2 weeks after receiving ISO injections. Other animals were studied during a 4-week period after administration of ISO. Two weeks after receiving ISO injections, the HFpEF animals were divided into 2 subgroups (12/group) without or with 2-weeks therapeutic intervention: 1) HF, received placebo; and 2) HF/G1, received G1 treatment 150ug/kg/day s.c. via osmotic mini-pumps [Alzet®, model 1004]. The dosing protocol of G1 used was based on our initial concentration-response studies and past reports [16, 27, 28], which had been shown to activate GPR30 *in vivo, but* no marked effects on heart rate and end-systolic pressure. All animals were maintained in the same environment, including temperature and humidity, and had free access to chow and water.

2.2. Experimental Protocol

Three sets of experiments were conducted. **First,** studies were performed in the intact mice to examine systemic hemodynamics, LV contractility, LV diastolic filling dynamic, and LV arterial coupling. In addition, as previously described [27], echocardiographic evaluation was also performed in a subgroup animals before and 2 weeks after receiving ISO injections under light anesthesia (Figure 1). **Second,** to assess the cellular basis of LV functional responses to chronic G1 treatment, we measured cell contraction, relaxation, and $[Ca^{2+}]_{iT}$ responses and β-adrenergic reserve (measured as response to acute β-adrenergic stimulation by isoproterenol) as well as β-

AR subtypes (β_1- and β_3-ARs) responsiveness in the freshly isolated LV myocytes from the same mice. **Third**, analysis of gene expressions of LV myocytes from each group was performed by quantitative real-time PCR.

2.3. Intact Mice Study

Echocardiographic evaluation. As described previously [27], LV function and dimensions were assessed only in a subgroup animals before and 2 weeks after receiving ISO injections under light anesthesia using a Philips 5500 echocardiography (Philips Medical Systems, Andover, MA, USA) and a 12 MHz phased array probe (Figure 1).

Determination of Hemodynamic and LV Functional Responses. As we and others described previously [23, 24, 29, 30], all the mice were anesthetized with intraperitoneal xylazine (10 mg/kg) and ketamine (50 mg/kg), then intubated. To maintain anesthesia and arterial oxygen tension, animals were mechanically-ventilated with a positive-pressure respirator (Model RSP1002, Kent Scientific Corp, Litchfield, CT) using oxygen-enriched room air with isoflurane (0.5-2%). For drug administration, a polyethylene catheter was inserted into the left external jugular vein. With adequate calibration, using our well-established closed-chest approach, a 1.2 F microtip P-V catheter (SPR-839, Millar Instruments, Austin, TX) was inserted into the right carotid artery and advanced into the LV apex. After stabilization, signals were continuously recorded at a sampling rate of 1000 samples/s using a Pressure (P)-Volume (V) conductance system (MPCU-200, Millar Instruments) with BioBench software (National Instruments, Inc). Steady-state and transient inferior vena cava occlusion at baseline were collected. As previously described, with the use of a special P-V analysis program (PVAN, Millar Instruments), standard steady-state hemodynamic parameters such as heart rate, LV pressure (P), LV relaxation time constant of (τ), LV volume (V) and the peak rate of mitral flow

(dV/dt_{max}) were measured. LV P-V relations and the slopes were derived. Effective arterial elastance (E_A) was calculated as the ratio of end-systolic pressure (P_{ES}) and stroke volume (SV), and LV-arterial coupling (LVAC) was calculated as the ratio of the slope of linear-P_{ES}-end-systolic volume (V_{ES}) relation (E_{ES}) to E_A [31].

2.4. Studies in Isolated LV Myocytes

2.4.1. Myocyte Isolation

After the hemodynamic study, the animals were deeply anesthetized and the hearts were excised and placed in ice-cold calcium-free HEPES buffer solution. By using techniques well-established in our laboratory [23, 24, 29, 32], calcium-tolerant, high-yield myocytes were obtained from each animal. Finally, cells were suspended in the modified HEPES solution ("the study buffer") with 1.2 mM $CaCl_2$ and stored at room temperature until ready for use. After stabilization (~ 2 hours), as we described previously, LV myocytes were counted, viability and morphology were evaluated. Within 12-14 hours, about 60-70 rod-shaped cells were randomly selected for measurement of myocyte dimensions from each experiment.

2.4.2. Myocyte Function Evaluation

2.4.2.1. Myocyte Function at Baseline and Response to a Non-Selective β-AR Agonist

As we reported previously [24, 29], freshly isolated LV myocytes were placed in superfused culture dishes. Myocyte contraction was elicited by field-stimulation (0.5 Hz) and measured with the Fluorescence and Contractility System (IonOptix, Milton, MA). First, baseline data were recorded. Then data were acquired during superfusion of a non-selective β-AR Agonist**,** isoproterenol (10^{-8} M) for 10 min and after drug washout. Changes between baseline and post-superfusion function were defined as myocyte β-adrenergic reserve.

2.4.2.2. Myocyte Functional Response to Selective β_1- and β_3-AR Agonists

Previously, we have shown that HF is associated with a selective cardiac β_1-AR downregulation and β_3-AR upregulation (unchanged β_2-AR expression) with resultant a decreased β_1-AR-mediated positive inotropic action and an enhanced β_3-AR-coupled negative inotropic effect in the heart. This restructuring of β-AR system plays a crucial role in the decline of β-adrenergic reserve [32, 33]. To determine the contribution of the subtypes of β-AR stimulation on β-AR reserve with chronic G1 in HF, the above protocol was repeated in subsets of myocytes after myocytes were randomly exposed to a selective β_1-agonist, Norepinephrine (NE, 10^{-7} M) or a selective β_3-agonist, BRL-37,344 (BRL, 10^{-8} M), respectively [32, 33]. Data were acquired for 8 to 10 minutes during drug exposure. The percent shortening (SA), the peak velocity of shortening (dL/dt_{max}), the peak velocity of re-lengthening (dR/dt_{max}) and the peak systolic $[Ca^{2+}]_i$ transient ($[Ca^{2+}]_{iT}$) were obtained.

2.4.3. Simultaneous Measurement of LV Myocyte Contractile and Calcium Transient Responses

Myocytes were incubated with 10 mM indo-1-AM (Molecular Probes, Eugene, OR) and then placed in a flow-through dish. Contractile and $[Ca^{2+}]_{iT}$ responses in a single cell were measured simultaneously with a dual excitation fluorescence photo-multiplier system (IonOptix) [24, 29, 32, 33]. As described above, contractile protocol was repeated in the subsets of myocytes. Myocytes were randomly exposed to isoproterenol or a selective β_1-, or β_3-agonist. After stabilization, both the steady-state baseline data and the responses to β-AR and β-AR Subtype stimulation data were recorded, respectively. After myocytes were loaded with indo-1-AM, compartmentalization of the indicator in mitochondria might have occurred, thus the absolute value of $[Ca^{2+}]_i$ was not used. Instead, we calculated relative changes in peak $[Ca^{2+}]_{iT}$ before and after interventions as the ratio of the emitted fluorescence [24, 33].

2.5. Analysis of LV Myocyte Gene Expression by Quantitative Real-Time PCR

As described in our previous report [27], total RNA was extracted from the subsets of LV myocytes in each group. The quality and quantity of RNA samples were determined by spectrometry and agarose gel electrophoresis. Relative quantification of mRNA levels by real-time qPCR was performed using a SYBR Green PCR kit (Qiagen Inc). Amplification and detection were performed with the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA). Sequence-specific oligonucleotide primers were designed according to published GenBank sequences and confirmed with OligoAnalyzer 3.0. The relative target mRNA levels in each sample were normalized to S16 ribosomal RNA. Expression levels are reported relative to the geometric mean of the control group.

2.6. Drugs

Isoproterenol hydrochloride, a non-selective βAR agonist; Norepinephrine, a selective β_1-agonist; and BRL-37,344, a selective β_3-agonist were purchased from Tocris Bioscience (Minneapolis, MN, USA). G1, a highly selective GPR30 activator was purchased from Tocris Bioscience (Ellisville, MO, USA). Indo-1-AM, fluorescent Ca^{2+} indicator was purchased from Molecular Probes, Eugene, OR.

2.7. Statistical Analysis

Data are presented as mean ± SD or mean ± SE as indicated. Indices of LV function, hemodynamics, and myocyte function were compared among the treatment groups by ANOVA for the repeated measures. When the ANOVA showed significant differences, a Bonferroni adjustment was used to compare pairwise tests among each group. Treatment effects were determined by ANOVA on the outcome measures adjusted for baseline values. In each mouse, LV myocyte contraction, relaxation, and $[Ca^{2+}]_{iT}$ values were averaged and treated as a single data point. The mean differences in cell dynamics and the indo-1-AM fluorescence ratios

between groups were calculated. Statistical significance was considered at $p<0.05$.

3. Results

3.1. Animal Follow-Up and Verification of Experimental HF

Consistent with past reports by our laboratory and others [20, 23-25, 34], in the current study, following the last isoproterenol injection, mice exhibited a time-dependent cardiac dysfunction and adverse structural remodeling. Four weeks after isoproterenol injection, these mice showed clear signs of clinical features of HF, including significant increases in heart weight and the ratio of heart weight/body weight (Table 1).

HFpEF. Two weeks after receiving the initial isoproterenol injection, mice had HFpEF onset. Hemodynamic analysis showed significant increases in τ and end-diastolic pressure of LV (P_{ED}), but reduced dV/dt_{max} (Table 2). Examples of LV P-V loops are presented in Figure 2 A-D. Compared with control (A), in HFpEF (B) and HF (C), there were clearly upward shifts of the diastolic portion of LV P-V loops, so that early diastolic LV P were much higher. LV minimum pressure (P_{min}) and LV diastolic operating stiffness of K_{LV} (measured as the average slope of the diastolic P V trajectories) were significantly increased. Examples of LV P_{ES}-V_{ES} relations are shown in Figure 2 E-H. Compared with control (E), in HFpEF (F), LV contractility of E_{ES} and M_{SW} (the slope of stroke work-V_{ED} relation), LV EF, and SV had no significant changes (Table 2). These hemodynamic findings of HFpEF are supported by echocardiographic (Echo) evaluation in the subgroup animals before (baseline) and 2 weeks after isoproterenol injection. As displayed in Figure 3 A-C, compared with baseline, 2 weeks after isoproterenol injection caused no changes in LV systolic function, as determined by percent fractional shortening (A) (33.8±5.6 vs 32.4±4.2 %). Myocardial relaxation, defined by tissue Doppler-derived myocardial annular descent, or e´ (B) (2.7±0.3 vs 3.2±0.2 cm/s) had a trend for reduced, but did not reach

statistical significance. LV filling pressure, defined by early transmitral flow velocity-to-mitral annular velocity ratio (or E/e') (C) trended to increase (28.8±5.2 vs 18.6±2.2), and was very close to be statistical significance (p=0.05).

Consistently, as shown in Table 3, compared with control, in HFpEF, LV myocyte relaxation measured as dR/dt_{max} was significantly reduced 34%. There were no significant changes in myocyte contractility measured as SA and dL/dt_{max}.

HFrEF (HF). Four weeks after receiving the last isoproterenol injection, the cardiac indices of HFpEF were extended into a hemodynamic pattern consistent with the presence of HFrEF, with both LV systolic and diastolic dysfunction. As summarized in Table 2, compared with control, τ and P_{ED} were further increased with about 30% decreases in dV/dt_{max} and SV, respectively. There was upward and rightward shift of the LV P-V loop with significantly increased LV end-diastolic volume. Importantly, LV contractility of E_{ES} was reduced ~31%. EF decreased from 57% (observed in controls) to 35% (4 weeks after isoproterenol injection) (Table 2 and Figure 2). As shown in Table 1, there were no significant differences in body weight among the groups. In HF group, the surrogates of symptomatic HF, including heart weight, calculated ratio of heart weight/body weight (Control: 5.6 ± 0.5 vs. HF: 8.0 ± 0.6 mg/g), and LV myocyte Length were all significantly increased (113 ± 5 vs. 125 ± 9 μm). As shown in Table 3 and Figures 4 and 5, these LV abnormalities in HF were accompanied by intrinsic defects in LV myocyte force-generating capacity and relaxation. There were ~ 31% decreases in dL/dt_{max} and 35% decreases in dR/dt_{max} with significantly reduced the peak systolic $[Ca^{2+}]_{iT}$.

3.2. LV Systolic and Diastolic Function in HF: Effects of Chronic GPR30 Activation

As summarized in Table 2 and displayed in Figure 2, there were no differences in heart rate and P_{ES} among the groups. In HF animals, P_{ED}, V_{ES} and V_{ED} were all significantly increased and

accompanied by significant reductions in LV dV/dt_{max} and SV. The slopes of LV P-V relations of E_{ES}, load-insensitive measures of LV contractile performance, were decreased by 31%. The P-V relations shifted to the right and ratio of LV arterial coupling (E_{ES}/E_A) was reduced by ~53 %, indicating significant systolic function impairment. On the contrary, LV systolic and diastolic function and general hemodynamics were similar between the control and HF/G1 groups. Chronic treatment with G1 prevented HF-caused decreased LV contractility of E_{ES}, EF, SV, and the abnormal upward and rightward shifts of LV P-V loops (Figure 2 D and H). τ, P_{ED}, V_{ED}, SV, dV/dt_{max} and E_{ES}/E_A as well as the ratio of heart weight/body weight were all close to the control values.

3.3. Myocyte Function, β-AR Reserve, and $[Ca^{2+}]_{iT}$ in HF: Effects of Chronic GPR30 Activation

3.3.1. Cardiomyocyte Contraction, Relaxation, and $[Ca^{2+}]_{iT}$ at Baseline

Basal cell contractile function and $[Ca^{2+}]_{iT}$ responses in LV myocytes are summarized in Table 3 and displayed in Figures 4 and 5 A-C. Compared with controls, in HFpEF, only LV myocyte dR/dt_{max} was significantly reduced. In contrast, in HF, LV myocyte dL/dt_{max}, dR/dt_{max} and $[Ca^{2+}]_{iT}$ were significantly decreased 31%, 35% and 24%, respectively (Table 3). After G1 treatment, LV myocyte SA, dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$ recovered to control values. In addition, myocyte length and the length-width ratio were normalized.

3.3.2. Myocyte Functional Responses to β-AR, and β-AR Subtype Stimulation

3.3.2.1. Effects of Isoproterenol. Compared with controls, functional performance of LV myocyte in HF mice was impaired at baseline. Further, the ability of acute β-adrenergic agonist isoproterenol to increase myocyte contractility was also significantly reduced. As presented in Table 3 and Figures 4 and 5 D-F, compared with control myocytes, in HF myocytes, acute

isoproterenol-superfusion-induced increases in SA, dL/dt_{max} and dR/dt_{max}, as well as $[Ca^{2+}]_{iT}$ were all significantly lower, demonstrating a decline in β-adrenergic reserve. G1 treatment restored normal β-adrenergic reserve in HF myocytes.

3.3.2.2. Effects of Norepinephrine or BRL-37344. As shown in Figure 6, compared with control myocytes, in HF myocytes, β_1-AR stimulation with norepinephrine caused significantly less increases in the normalized percent changes of myocyte contractility dL/dt_{max} (Control: 61% ± 4% vs. HF: 24% ± 2%), dR/dt_{max}, and $[Ca^{2+}]_{iT}$, indicating β_1-AR desensitization. In contrast, β_3-AR stimulation with BRL-37344 produced significantly greater decreases in the normalized percent changes of dL/dt_{max} (Control: 8% ± 1% vs. HF: 15% ± 2%), dR/dt_{max}, and $[Ca^{2+}]_{iT}$, demonstrating enhanced β_3-AR-mediated negative modulation. Importantly, G1 treatment reversed HF produced contract changes of β_1-AR and β_3-AR-stimulated inotropic responses. As presented in Figure 6, in G1-treated myocytes, Norepinephrine or BRL-37344 caused changes in dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$ were similar as that in normal control myocytes, respectively, indicating restoration of cardiac β-AR subtypes (β_1- and β_3-ARs) normal responsiveness.

3.4. Gene mRNA levels of ANF and PLB/SERCA2a in HF: Effects of Chronic GPR30 Activation

Figure 7 presents the gene mRNA levels of LV myocyte atrial natriuretic factor (ANF), sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) and phospholamban (PLB), (two key proteins involved in intracellular Ca^{2+} handling) and the PLB-to-SERCA2a ratio in the Control, HF and HF/G1 animals. Compare with control group, LV myocyte ANF mRNA, a marker of cardiac wall stress, was markedly increased in HF (A). LV myocyte SERCA2a mRNA expression reduced (B), but PLB mRNA level trended to be elevated (C). The PLB-to-SERCA2a ratio, an index of SERCA2a inhibition (D) was not significant, but clearly trended to increase. In

HF/G1, these LV myocyte gene mRNA expression changes were reversed to control levels.

4. Discussion

We show here, for the first time, that chronic GPR30 activation reverses HFpEF LV diastolic dysfunction and prevents the progression of HFpEF to HFrEF. The normalization of LV systolic and diastolic function performance in G1-treated HF is associated with preservation of normal intrinsic LV myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$ and β-adrenergic reserve accompanied by the restoration of normal cardiac β-AR subtypes (β_1- and β_3-AR) modulation. These data provide evidence and important insights that chronic GPR30 activation is able to rescue or reverse HFpEF, suggesting GPR30 agonist may provide significant benefits in HF therapy.

4.1. LV Functional Performance and Chronic GPR30 Activation

In the current study, 2-weeks after isoproterenol injection, animals had HFpEF characterized by significantly impaired diastolic function but without systolic dysfunction (Figures 2 and 3). These observations are consistent with previously well-characterized histology, LV function and structure alterations in isoproterenol induced HF by others and our serial time course studies in this model [20-22, 24, 25]. Grimm et al [21] reported that myocardial injury was associated with increases in right atrial pressure, LV filling pressure and LV hypertrophy two weeks after isoproterenol (150 mg/kg) application. Teerlink et al [20] clearly described the histology, and LV structure and function alterations in male Wistar rats receiving two subcutaneous isoproterenol injections of either 85 mg/kg or 170 mg/kg at 2 and 6 weeks after injection. They reported that histological resolution of the damage by both doses to the myocardium occurred by 2 weeks; there was no evidence for any further increase in this pathology score with respect to time (2 to 16 weeks). Further, Brooks and Conrad [22] showed myocardial injury and LV diastolic dysfunction associated with hypertrophy of surviving myocytes and increased myocardial

fibrosis 14 days after the last isoproterenol injection. LV diastolic dysfunction was evidenced by decreased LV compliance, an upward shift in the diastolic relationship, with normal LV systolic function. Isoproterenol produces a time-and dose-dependent impairment of cardiac function and structural remolding that results primarily in diastolic dysfunction within two weeks. In agreement, in the present study, without treatment, at 4 weeks after receiving isoproterenol, HFpEF was further preceded to HFrEF, with both LV systolic and diastolic dysfunction.

Of importance, chronic administration of G1 completely reversed the HFpEF progression in this mouse model of progressive HF. In HF/G1 group, the major indices of LV systolic and diastolic functional performance and general hemodynamics (EF, P_{ED}, □, SV and dV/dt_{max}) were all restored to control values (Table 2). To avoid the potentially confounding effects of G1-induced changes in loading conditions on conventional measures of LV performance, LV contractile performance was evaluated in the pressure-volume plane. Chronic G1 significantly increased LV contractility (measured as E_{ES} and M_{SW}) and the E_{ES}/E_A ratio (Table 2 and Figure 2). Chronic G1 prevented the classic systolic HF-induced rightward and upward shifts of LV P-V loops (Figure 2) and increased heart weight/body weight ratios.

Although the effects of G1 on HF progress in males have not been previously investigated, our present observation of chronic G1 restoration of normal LV systolic and diastolic function in HF is supported by previous studies in GPR30-deficient male mice [8, 13, 15]. Chronic G1 treatment attenuates salt-induced diastolic dysfunction and myocyte hypertrophy without changes in blood pressure [35]. G1 showed the ability to attenuate HF in female bilateral ovariectomy (OVX) Sprague-Dawley rats [16]. G1 pretreatment reduces infarct size and preserves cardiac function in isolated hearts from male and female rats exposed to ischemia-reperfusion [35].

4.2. Myocyte Function, $[Ca^{2+}]_i$ Regulation, β-Adrenergic Reserve, Gene mRNA levels and Chronic GPR30 Activation

What is the mechanism of the restoration of normal LV systolic and diastolic functional performance in HF after chronic G1 treatment? Since the protective effects are present in the freshly-isolated single LV myocytes, this beneficial action is not due to alterations of heart rate, and loading conditions, but it is directly attributable to changes of LV myocytes. However, this does not rule out additional protective effects that might occur in non-cardiomyocytes, for example, in vivo. Although our dose of G1 had no overt effect on blood pressure, we cannot exclude the possibility that G-1 may have some other systemic action that could indirectly alter vascular hemodynamics and subsequently improve cardiac structure and function.

We found that in isoproterenol-induced HF, LV chamber abnormalities were paralleled with progressive LV myocyte dysfunction with significantly depressed dL/dt_{max}, dR/dt_{max} and $[Ca^{2+}]_{iT}$. There was a maladaptive remodeling of LV myocyte shape. These changes were normalized and myocyte β-AR desensitization reversed after G1 treatment. Normalization of basal and β-AR stimulated Ca^{2+} handling may be the primary driver for reversal of HF-caused intrinsic defects of myocyte force-generating capacity and relaxation after chronic G1 treatment. Recovering normal $[Ca^{2+}]_i$ regulation by chronic G1 may be the key mechanism for reversal of HF-caused intrinsic defects of myocytes and rescuing HFpEF. Growing evidence has shown that estrogen alters cardiovascular gene expression of β-adrenergic receptors (AR) and calcium (Ca^{2+})-handling proteins [36]. We have shown previously that in HF, increased sympathetic nervous system was associated with cardiac β_1-AR downregulation, but β_3-AR upregulation. β_1-AR-stimulated-G_S-coupled positive inotropic effects reduced, while β_3-AR-stimulated-G_i-coupled negative modulation on LV, and myocyte contraction, relaxation as well as $[Ca^{2+}]_i$ regulation were enhanced. These contract changes between cardiac β_1-AR and β_3-AR are responsible for the β-AR desensitization in HF [32-34]. Notably, Kang et al.[16] showed that the ability of GPR30-

agonist G1 to reduce isoproterenol-induced HF in female OVX Sprague-Dawley rats was achieved through the expression of β_1- and β_2-ARs. In the current study, G1 treatment caused improved β-adrenergic reserve is likely due to the restoration of normal β-AR subtypes modulation. However, whether this is due to reversing HF-induced the contrast changes of cardiac β_1- and β_3-AR expressions by chronic G1 remain to be determined. We and others have reported that HF, as well as GPR30-deficiency, decreased LV SERCA2a expression and activity with increased sarcolemmal Na^+- Ca^{2+} exchange expression accompanied by increased SR Ca^{2+} leak with defective Ca^{2+} removal [3, 4, 7]**.** Of note, GPR30 improves LV lusitropy in models of hypertension and aging. This is largely attributable to GPR30-related increase in cardiac Ca^{2+} mobilization by increasing the expression and activity of SERCA2a [4, 37]. In the current study, we found that compared with HF alone, LV myocyte ANF mRNA, as a marker of cardiac wall stress and the PLB-to-SERCA2a ratio, an index of SERCA2a inhibition trended to decrease (did not reach significance, due to relatively small group size) in G1-treated HF. These LV myocyte mRNA expressions might suggest a favorable functional effect of G1 in HF males. However, the protein level and activity of SERCA2a have not been determined. Future studies show that G1 treatment reverses HF-induced cardiac downregulation of SERCA2a in males are necessary. Of importance, a large body of evidence indicates that increased oxidative stress contributes directly to cellular damage, impaired $[Ca^{2+}]_i$ regulation and remodeling during HF, whereas GPR30 activation was sufficient to protect against myocardial death, cardiac apoptosis and adverse LV remodeling [4, 7]. Although how G1 treatment alters isoproterenol-induced fibrosis and cell death in the heart in males with HF is unclear, the anti-fibrosis and anti-cell death ability of GPR30 activation to counteract pathologic cardiac remodeling certainly is another important mechanism by which G1 treatment could ameliorate HF. We speculate that in the current study,

G1 reverses HF-caused contrast changes in cardiac β_1-AR and β_3-AR expression and activity and downregulation of SERCA2a, thereby leading to restoration of normal SERCA2a activity and $[Ca^{2+}]_i$ regulation. These changes may be the key molecular mechanism for repairing LV and myocyte contractile defects, restoration of cardiac β-AR responsiveness and reversal of HFpEF and prevention of its progression. Clearly, further studies on the molecular basis for chronic GPR30 activation in HF are urgently needed.

4.3. Limitations and Future Studies

Several study limitations should be considered and need to be addressed. First, we used a mouse model of isoproterenol-induced HF. Although pathologic changes in isoproterenol-treated rats and mice resemble those of myocardial infarction [20-22, 24], and isoproterenol-induced HF mimics many structural, functional and neurohormonal changes of clinical HF [21], we cannot ascertain that these results are applicable to clinical HF or HF from other causes such as pure pressure overloaded cardiomyopathy or pure volume overload cardiomyopathy. Second, in the current investigation, G1 was initiated at the onset of HFpEF. Although the isoproterenol-induced HFpEF is consistent with previously well-characterized histological findings, LV structure and function alterations from others [20-25], we do not know if the late initiation of G1 is capable of achieving the same efficacy given that dynamic nature (time dependency) of isoproterenol-induced HF. Third, the present study focused on a phenotypic change in hemodynamic measurements as well as cardiomyocyte contraction, relaxation, calcium handling, and β-adrenergic reserve between the heart failure group and those animals receiving G1 in male mice which provides foundational information for further studies on the mechanisms underlying cardioprotection in males by GPR30. In order to get adequate numbers of high-yield and high-quality myocytes for many subgroup studies in HF mice, unfortunately, whole heart structure

changes were not determined in this study. Future studies are in progress emphasizing potential GPR30-related alterations in whole heart morphometric and histopathology. Fourth, we did not assess how G1 treatment alters isoproterenol-induced fibrosis and cell death in the heart in the current investigation. Indeed, several groups using different cardiovascular disease models suggested that GPR30 activation attenuates adverse cardiac structural remodeling by its actions of anti-inflammatory, anti-apoptosis and anti-fibrosis [7, 16, 27]. Previously, our group showed that GPR30 agonist G1 limited the OVX-induced increase in LV mass, wall thickness, and interstitial collagen deposition thereby attenuating hypertrophic remodeling in OVX-mRen2.Lewis rats. Further, Kang et al [16] reported that chronic G1 treatment reduced fibrosis and cell death in OVX female rats with ISO-induced HF. It suggested that GPR30 may improve cardiac function in female OVX mice by activating the PI3K/AKT pathway and reducing myocardial infarct size and fibrosis. Acute G-1 induces the activation of both Akt and ERK1/2. G-1 activation of GPER improves functional recovery and reduces infarct size in isolated rat hearts following I/R through a PI3K dependent, gender-independent mechanism [38]. It is possible that the G1 treatment caused beneficial actions on LV and myocyte functional performance are largely attributable to its anti-apoptosis and anti-fibrosis actions in HF. It is, therefore important that future work focuses on this point in males with HF. Fifth, the downstream signaling mechanisms of GPR30 in the heart are still unclear. It has been shown that GPR30 activates signaling mechanisms that involve ERK, Akt and PI3K [7, 14, 38-40]. It suggested that GPR30 may improve cardiac function in female OVX mice by activating the PI3K/AKT pathway and reducing myocardial infarct size and fibrosis [14]. In ISO-induced HF model of OVX rats showed that PIK-AKT pathway is the downstream pathway of GPR 30, and G1 treatment increased phosphorylation of AKT [16] we did not examine the contributions of

Akt and ERK pathways in the protective actions in HF by G1 in the current investigation. Further studies are needed to fully characterize the intracellular pathway modulating G1 treatment. Finally, the current study did not address the molecular mechanisms underlying the therapy/rescue actions of chronic GPR30 activation on cardiac function and cardiac reserve in mice with HFpEF (as well as the transition to HFrEF) and will require further investigations.

5. Conclusions

Chronic G1 treatment restores LV systolic and diastolic function accompanied with the preservation of normal intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$ and β-adrenergic reserve, thereby limits HFpEF progression in a male mouse model of progressive HF. These data provide new insights and strong evidence that chronic GPR30 activation is able and sufficient to rescue HFpEF in males, and support the view that GPR30 plays critical roles in cardiac health in both females and males.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

1. Tomasoni, D., et al., Heart failure in the last year: progress and perspective. ESC Heart Fail. 2020;7: p. 3505-3530.DOI: 10.1002/ehf2.13124
2. Pfeffer, M.A., A.M. Shah, and B.A. Borlaug, Heart Failure With Preserved Ejection Fraction In Perspective. Circ Res, 2019. 124(11): p. 1598-1617. DOI: 10.1161/circresaha.119.313572
3. Mishra, S. and D.A. Kass, Cellular and molecular pathobiology of heart failure with

preserved ejection fraction. 2021. 18(6): p. 400-423. DOI: 10.1038/s41569-020-00480-6

4. Groban, L., et al., Female Heart Health: Is GPER the Missing Link? Front Endocrinol (Lausanne), 2019. 10: p. 919. DOI: 10.3389/fendo.2019.00919
5. Hutson, D.D., et al., Estrogen receptor profiles across tissues from male and female Rattus norvegicus. Biol Sex Differ.2019. 10(1): p. 4. DOI: 10.1186/s13293-019-0219-9
6. Patel, V.H., et al., G-protein coupled estrogen receptor 1 expression in rat and human heart: Protective role during ischaemic stress. Int J Mol Med, 2010. 26(2): p. 193-9. DOI: 10.3892/ijmm_00000452
7. Aryan, L., et al., The Role of Estrogen Receptors in Cardiovascular Disease. Int J Mol Sci. 2020. 21(12). DOI: 10.3390/ijms21124314
8. Delbeck, M., et al., Impaired left-ventricular cardiac function in male GPR30-deficient mice. Mol Med Rep, 2011. 4(1): p. 37-40. DOI: 10.3892/mmr.2010.402
9. Pei, H., et al., G Protein-Coupled Estrogen Receptor 1 Inhibits Angiotensin II-Induced Cardiomyocyte Hypertrophy via the Regulation of PI3K-Akt-mTOR Signalling and Autophagy. Int J Biol Sci, 2019. 15(1): p. 81-92. DOI: 10.7150/ijbs.28304
10. Fredette, N.C., M.R. Meyer, and E.R. Prossnitz, Role of GPER in estrogen-dependent nitric oxide formation and vasodilation. J Steroid Biochem Mol Biol, 2018. 176: p. 65-72. DOI: 10.1016/j.jsbmb.2017.05.006
11. Machuki, J.O., et al., Estrogen regulation of cardiac cAMP-L-type Ca(2+) channel pathway modulates sex differences in basal contraction and responses to β(2)AR-mediated stress in left ventricular apical myocytes. Cell Commun Signal. 2019. 17(1): p. 34. DOI: 10.1186/s12964-019-0346-2
12. Ogola, B.O., et al., G Protein-Coupled Estrogen Receptor Protects From Angiotensin II-Induced Increases in Pulse Pressure and Oxidative Stress. Front Endocrinol (Lausanne), 2019. 10: p. 586. DOI: 10.3389/fendo.2019.00586
13. Meoli, L., et al., Sex- and age-dependent effects of Gpr30 genetic deletion on the metabolic and cardiovascular profiles of diet-induced obese mice. Gene, 2014. 540(2): p. 210-6. DOI: 10.1016/j.gene.2014.02.036
14. Wang, X., et al., GPR 30 reduces myocardial infarct area and fibrosis in female ovariectomized mice by activating the PI3K/AKT pathway. Life Sci, 2019. 226: p. 22-32. DOI: 10.1016/j.lfs.2019.03.049

15. Wang, H., et al., Cardiomyocyte-specific deletion of the G protein-coupled estrogen receptor (GPER) leads to left ventricular dysfunction and adverse remodeling: A sex-specific gene profiling analysis. Biochim Biophys Acta Mol Basis Dis, 2017. 1863(8): p. 1870-1882. DOI: 10.1016/j.bbadis.2016.10.003
16. Kang, S., et al., Chronic activation of the G protein-coupled receptor 30 with agonist G-1 attenuates heart failure. PLoS One, 2012. 7(10): p. e48185. DOI: 10.1371/journal.pone.0048185
17. Whitcomb, V., et al., Regulation of beta adrenoceptor-mediated myocardial contraction and calcium dynamics by the G protein-coupled estrogen receptor 1. Biochem Pharmacol, 2020. 171: p. 113727. DOI: 10.1016/j.bcp.2019.113727
18. Feng, Y., et al., Activation of G protein-coupled oestrogen receptor 1 at the onset of reperfusion protects the myocardium against ischaemia/reperfusion injury by reducing mitochondrial dysfunction and mitophagy. Br J Pharmacol. 2017. 174(23): p. 4329-4344. DOI: 10.1111/bph.14033
19. Zhao, Z., et al., Role of estrogen in diastolic dysfunction. Am J Physiol Heart Circ Physiol, 2014. 306(5): p. H628-40. DOI: 10.1152/ajpheart.00859.2013
20. Teerlink, J.R., J.M. Pfeffer, and M.A. Pfeffer, Progressive ventricular remodeling in response to diffuse isoproterenol-induced myocardial necrosis in rats. Circ Res, 1994. 75(1): p. 105-13. DOI: 10.1161/01.res.75.1.105
21. Grimm, D., et al., Effects of beta-receptor blockade and angiotensin II type I receptor antagonism in isoproterenol-induced heart failure in the rat. Cardiovasc Pathol, 1999. 8(6): p. 315-23. DOI: 10.1016/s1054-8807(99)00021-6
22. Brooks, W.W. and C.H. Conrad, Isoproterenol-induced myocardial injury and diastolic dysfunction in mice: structural and functional correlates. Comp Med, 2009. 59(4): p. 339-43.
23. Li, T., et al., Reversal of angiotensin-(1-12)-caused positive modulation on left ventricular contractile performance in heart failure: Assessment by pressure-volume analysis. Int J Cardiol, 2020. 301: p. 135-141. DOI: 10.1016/j.ijcard.2019.09.004
24. Zhang, X., et al., Cellular basis of angiotensin-(1-7)-induced augmentation of left ventricular functional performance in heart failure. Int J Cardiol, 2017. 236: p. 405-412. DOI: 10.1016/j.ijcard.2017.01.071

25. Suzuki, M., et al., Altered inotropic response of endothelin-1 in cardiomyocytes from rats with isoproterenol-induced cardiomyopathy. Cardiovasc Res, 1998. 39(3): p. 589-99. DOI: 10.1016/s0008-6363(98)00166-7
26. Groban, L., et al., Progressive diastolic dysfunction in the female mRen(2). Lewis rat: influence of salt and ovarian hormones. J Gerontol A Biol Sci Med Sci, 2008. 63(1): p. 3-11. DOI: 10.1093/gerona/63.1.3
27. Wang, H., et al., Activation of GPR30 attenuates diastolic dysfunction and left ventricle remodelling in oophorectomized mRen2.Lewis rats. Cardiovasc Res, 2012. 94(1): p. 96-104. DOI: 10.1093/cvr/cvs090
28. Lindsey, S.H., et al., Estrogen receptor GPR30 reduces oxidative stress and proteinuria in the salt-sensitive female mRen2.Lewis rat. Hypertension, 2011. 58(4): p. 665-71. DOI: 10.1161/hypertensionaha.111.175174
29. Shao, Q., et al., Overexpression myocardial inducible nitric oxide synthase exacerbates cardiac dysfunction and beta-adrenergic desensitization in experimental hypothyroidism. Int J Cardiol, 2016. 204: p. 229-41. DOI: 10.1016/j.ijcard.2015.11.040
30. He, Q., J. Cheng, and Y. Wang, Chronic CaMKII inhibition reverses cardiac function and cardiac reserve in HF mice. Life Sci, 2019. 219: p. 122-128. DOI: 10.1016/j.lfs.2019.01.010
31. Segers, P., et al., Conductance catheter-based assessment of arterial input impedance, arterial function, and ventricular-vascular interaction in mice. Am J Physiol Heart Circ Physiol, 2005. 288(3): p. H1157-64. DOI: 10.1152/ajpheart.00414.2004
32. Cheng, H.J., et al., Upregulation of functional beta(3)-adrenergic receptor in the failing canine myocardium. Circ Res, 2001. 89(7): p. 599-606. DOI: 10.1161/hh1901.098042
33. Morimoto, A., et al., Endogenous beta3-adrenoreceptor activation contributes to left ventricular and cardiomyocyte dysfunction in heart failure. Am J Physiol Heart Circ Physiol, 2004. 286(6): p. H2425-33. DOI: 10.1152/ajpheart.01045.2003
34. Cheng, H.-J., et al., Abstract 14636: Upregulation of Cardiac β3-Adrenergic Signaling Promotes Heart Failure Progression: Evidence from Chronic β3-Adrenoceptor (AR) deficiency or β3-AR Blockade Mice. Circulation, 2014. 130(suppl_2): p. A14636-A14636. DOI: doi:10.1161/circ.130.suppl_2.14636
35. Jessup, J.A., et al., Attenuation of salt-induced cardiac remodeling and diastolic

dysfunction by the GPER agonist G-1 in female mRen2.Lewis rats. PLoS One, 2010. 5(11): p. e15433. DOI: 10.1371/journal.pone.0015433

36. Machuki, J.O., et al., Molecular pathways of oestrogen receptors and β-adrenergic receptors in cardiac cells: Recognition of their similarities, interactions and therapeutic value. Acta Physiol (Oxf), 2018. 222(2). DOI: 10.1111/apha.12978
37. Alencar, A.K., et al., Effect of Age, Estrogen Status, and Late-Life GPER Activation on Cardiac Structure and Function in the Fischer344×Brown Norway Female Rat. J Gerontol A Biol Sci Med Sci, 2017. 72(2): p. 152-162. DOI: 10.1093/gerona/glw045
38. Deschamps, A.M. and E. Murphy, Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. Am J Physiol Heart Circ Physiol, 2009. 297(5): p. H1806-13. DOI: 10.1152/ajpheart.00283.2009
39. Tang, H., et al., GPR30 mediates estrogen rapid signaling and neuroprotection. Mol Cell Endocrinol, 2014. 387(1-2): p. 52-8. DOI: 10.1016/j.mce.2014.01.024
40. Filice, E., et al., A new membrane G protein-coupled receptor (GPR30) is involved in the cardiac effects of 17beta-estradiol in the male rat. J Physiol Pharmacol, 2009. 60(4): p. 3-10.

Figure 1. A schematization of the experimental procedures and experimental timeline.

Figure 2. Examples of the effects of chronic G1 in HF on LV systolic and diastolic functional responses. **A-D**, steady-state average LV Pressure (P)-Volume (V) loops from 4 mice: one control mouse (A), one mouse at two weeks after initial isoproterenol injection (HFpEF) (B), one mouse 4 weeks after isoproterenol injection (HF) (C), and one mouse 2 weeks after isoproterenol and then received G1 treatment for 2 weeks (HF/G1) (D). Each loop was generated by averaging the data obtained during a 10 -12-second recording period, spanning several respiratory cycles. Compared with control, 2 weeks after isoproterenol injection caused HFpEF with markedly upward shifts of the P-V loop with marked increases in LV P_{ED} and LV min, indicating impaired LV diastolic performance. After isoproterenol injection at 4 weeks, there was upward and

rightward shift of P-V loop with further elevated LV P_{ED} and LV min and decreased stroke volume. In contrast, concomitant G1 prevented isoproterenol-induced abnormal upward and rightward shifts of P-V loops and the decreased SV in HF.

E-H, LV end-systolic pressure (P_{ES})-end-systolic volume (V_{ES}) relationships produced by inferior vena cava occlusions obtained from these same animals. The slope and position of this line provide a load-insensitive measure of LV contractility. Compared with control (E), 2 weeks after isoproterenol injection the LV P_{ES}-V_{ES} relations (F) are relatively unchanged. In contrast, 4 weeks after isoproterenol injection (G), the LV P_{ES}-V_{ES} relations were shipped to the right with markedly reduced slope, indicating LV systolic dysfunction. Compared with control, concomitant G1-treated HF mouse (H) had similar the LV P_{ES}-V_{ES} relations, indicating restoration of normal LV contractility.

Figure 3. Representation of individual cases and the means of Doppler parameters obtained from the sub-group animals before (baseline) and 2 weeks after isoproterenol injection. Data are shown as mean (± SE). N=4. (A) Percent fractional shortening, (B) Early mitral annular velocity (e′), and (C) The ratio of transmitral early filling-to-early mitral annular descent (E/e′), or index of filling pressures.

Figure 4. Examples of the effects of chronic G1 in HF on myocyte contractile function and $[Ca^{2+}]_{iT}$ response at baseline and response to acute isoproterenol (ISO) (β-adrenergic reserve) in . Myocytes isolated from the LV were obtained from one mouse in control (A), HF (B) and HF/G1 (C) groups. Shown are superimposed traces of analog recordings of myocyte contractile and $[Ca^{2+}]_{iT}$ responses in electrically stimulated myocytes at baseline and after acute superfusion of isoproterenol (10^{-8} M).

Figure 5. LV myocyte functional performance at baseline of dL/dt_{max} (A), dR/dt_{max} (B), and

$[Ca^{2+}]_{iT}$ (C), and □-adrenergic reserve (D-F), calculated as % changes from baseline after acute isoproterenol stimulation. Data are shown as mean (± SD). N=12/group. * p<0.05 vs. Control group.

Figure 6. The effects of chronic G1 in HF on LV myocyte functional responses to β_1-AR or β_3-agonists. Group means of the changes on dL/dt_{max} (A), dR/dt_{max} (B), and $[Ca^{2+}]_{iT}$ (C) in response to norepinephrine stimulation, a selective β_1-AR agonist (10^{-7}M), or BRL-37,344, a selective β_3-agonist (10^{-8} M). Values shown mean (± SD). * p<0.05, norepinephrine or BRL-37,344-induced changes vs corresponding baselines; † p<0.05, norepinephrine or BRL-37,344-induced changes among groups.

Figure 7. LV myocyte mRNA expressions of atrial natriuretic factor (ANF) (A), sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) (B), and phospholamban (PLB) (C) in each group, determined by real-time PCR. Quantification of the PLB-to-SERCA2a ratio with densitometry (D). Values are shown as mean (± SE). N=4~6 mice/group.

Table 1. Effects of Chronic G1 on Body Weights (BW) and Heart Weights (HW) after Isoproterenol-Induced HF

	BW (g)	HW (mg)	HW/BW (mg/100g)
Control (N=12)	32.2±1.8	180±20	5.6±0.5
HFpEF (N=6)	32.5±2.1	193±26	5.9±0.9
HF (N=12)	32.5±1.4	260±20*†	8.0±0.6*†
HF/G1 (N=12)	32.4±1.3	190±20	5.8±0.6

Data shown as mean ± SD; N=number of mice.

HF, heart failure; HFpEF, heart failure with preserved ejection fraction

*p <0.05 vs Control;

† p <0.05, HF vs. HFpEF

Table 2. Effects of Chronic G1 on LV Function and General Hemodynamic Variables in HF

	Control (N=6)	**HFpEF** (N=6)	**HF** (N=6)	**HF HF/G1** (N=6)
Heart rate (beats/min)	633±19	647±23	636±16	628±17
LV P_{ED} (mmHg)	5.0±1.1	8.8± 1.9*	10.6 ± 1.5*	5.2±1.0
LV P_{ES} (mmHg)	114.4± 3.2	115.9±6.5	116.9±2.6	113.2 ± 4.7
LV V_{ED} (µl)	104.0 ± 5.7	104.7± 5.3	11[illegible] ± 4.8*†	105.7 ± 5.0
LV V_{ES} (µl)	45.0 ± 4.2	46.4± 5.4	76.7 ± 2.7*†	47.2 ± 5.5
SV (µl)	59.0 ± 1.8	58.3 ± 2.7	41.1 ± 2.2*†	58.5± 1.5
Maximum dP/dt (mmHg/s)	11,106± 692	10,975 ± [illegible]	7,726± 478*†	11,046 ± 545
Minimum dP/dt (mmHg/s)	-8,333 ± 432	-8,451 ± 430*	-5,514 ± 489*†	-7,663 ± 544
dV/dt_{max} (µl/s)	3,692± 245	2,746 ± 227*	2,571 ± 282*	3,620 ± 356
□□(msec)	7.7 ± 0.3	10.8 ± 0.9*	11.3 ± 0.6*	7.8± 0.6
E_A (mmHg/µl)	1.95 ± 0.08	1.99 ± 0.23	2.85± 0.10*†	1.97 ± 0.08
EF (%)	57±2	56±2	35±2*†	55 ± 3
E_{ES} (mmHg/µl)	1.85± 0.06	1.81 ± 0.36	1.28 ± 0.12*†	1.79 ± 0.05
M_{SW} (mmHg)	103.0 ± 1.6	101.9± 3.7	74.7 ± 2.6*†	101.5 ± 1.6
E_{ES}/E_A	0.95± 0.06	0.91± 0.11	0.45 ± 0.04*†	0.91 ± 0.03

Values are mean ± SD; N=number of mice.

HF, heart failure; HFpEF, heart failure with preserved ejection fraction; LV, left ventricular; P_{ED}, end- diastolic pressure; P_{ES}, end-systolic pressure; V_{ED}, end-diastolic volume; V_{ES}, end-systolic volume; SV, stroke volume; dV/dtmax, the peak rate of mitral flow; □□□LV time constant of

relaxation; E_A, arterial elastance; EF, Ejection Fraction; E_{ES}, the slope of linear P_{ES}-V_{ES} relation; M_{SW}, the slope of SW-V_{ED} relation; E_{ES}/E_A, ratio of LV-arterial coupling.

* p <0.05, vs. Control group.

† p <0.05, HF vs. HFpEF.

Table 3. Effects of Chronic G1 on Myocyte Contractile Function, $[Ca^{2+}]_i$ Transient, and β-Adrenergic Reserve in HF

	Control (N=12)		**HFpEF (N=6)**		**HF (N=12)**		**HF/G1 (N=12)**	
	Baseline	**Isoproterenol**	**Baseline**	**Isoproterenol**	**Baseline**	**Isoproterenol**	**Baseline**	**Isoproterenol**
Resting length (□m)	113.4 ± 4.6	112.5 ± 4.9	110.0 ± 8.3	109.5 ± 8.0	124.9 ± 9.1*	124.4 ± 8.9	114.3 ± 7.5	113.8 ± 7.5
SA (%)	9.3 ± 1.0	14.1 ± 1.4†	9.7 ± 0.7	14.6 ± 1.3†	6.2 ± 0.5*§	7.4 ± 0.7†‡	9.1 ± 0.4	13.8 ± 1.0†
dL/dt_{max} (□m/sec)	145.2 ± 5.5	233.1 ± 9.6†	141.8 ± 11.4	226.8 ± 14.1†	99.5 ± 3.2*§	122.8 ± 4.4†‡	140.6 ± 3.4	226.0 ± 5.0†
dR/dtmax (□m/sec)	108.1 ± 5.6	169.8 ± 9.8†	71.6 ± 7.9*	99.2 ± 7.8†‡	70.2 ± 3.6*	88.5 ± 4.0†‡	104.7 ± 4.9	163.7 ± 8.9†
$[Ca^{2+}]_{iT}$	0.20 ± 0.01	0.27 ± 0.01†	0.21 ± 0.01	0.27 ± 0.01†	0.16 ± 0.01*§	0.18 ± 0.01†‡	0.20 ± 0.01	0.27 ± 0.01†

Values are mean ± SD; N=number of mice. HF: heart failure; HFpEF, heart failure with preserved ejection fraction; SA, Percent of shortening; dL/dt_{max}, peak velocity of shortening; dR/dt_{max}, peak velocity of re-lengthening; $[Ca^{2+}]_{iT}$, the peak systolic $[Ca^{2+}]_i$ transient.

* p<0.05, vs. Control baseline.

† p<0.05, Isoproterenol response vs. corresponding baseline value.

‡ p<0.05, Isoproterenol-induced percent changes between HF vs. Control.

§ p<0.05, HF baseline value vs. HFpEF baseline value.

Credit Author Statement

C.P.C., L.G., X.W.Z. and H.J.C. Contributed conception and design of research as well as provided direction and supervision of the project; X.W.Z., T.K.L., H.J.C., L.G., and H.W. performed experiments; X.W.Z., T.K.L., H.J.C., L.G., H.W. and C.P.C. analyzed data and the statistical analysis; X.W.Z., H.J.C., C.M.F., L.G. and C.P.C. interpreted results of experiments; X.W.Z., H.W. and C.P.C. prepared figures; X.W.Z., H.J.C. and C.P.C. drafted manuscript; C.M.F., L.G., H.J., X.W.Z., T.K.L., H.W. and C.P.C. edited and revised it critically for important intellectual content; X.W.Z., T.K.L., H.J.C, H.W., C.M.F., L.G. and C.P.C. approved the final version of the manuscript.