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Carvedilol induces biased β_1 adrenergic receptor-nitric oxide synthase 3-cyclic guanylyl monophosphate signalling to promote cardiac contractility

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Aims β-blockers are widely used in therapy for heart failure and hypertension. β-blockers are also known to evoke additional diversified pharmacological and physiological effects in patients. We aim to characterize the underlying molecular signalling and effects on cardiac inotropy induced by β-blockers in animal hearts.

Methods and results Wild-type mice fed high-fat diet (HFD) were treated with carvedilol, metoprolol, or vehicle and echocardiogram analysis was performed. Heart tissues were used for biochemical and histological analyses. Cardiomyocytes were isolated from normal and HFD mice and rats for analysis of adrenergic signalling, calcium handling, contraction, and western blot. Biosensors were used to measure β-blocker-induced cyclic guanosine monophosphate (cGMP) signal and protein kinase A activity in myocytes. Acute stimulation of myocytes with carvedilol promotes β_1 adrenergic receptor (β_1AR)- and protein kinase G (PKG)-dependent inotropic cardiac contractility with minimal increases in calcium amplitude. Carvedilol acts as a biased ligand to promote β_1AR coupling to a G_i-PI3K-Akt-nitric oxide synthase 3 (NOS3) cascade and induces robust β_1AR-cGMP-PKG signal. Deletion of NOS3 selectively blocks carvedilol, but not isoproterenol-induced β_1AR-dependent cGMP signal and inotropic contractility. Moreover, therapy with carvedilol restores inotropic contractility and sensitizes cardiac adrenergic reserves in diabetic mice with minimal impact in calcium signal, as well as reduced cell apoptosis and hypertrophy in diabetic hearts.

Conclusion These observations present a novel β_1AR-NOS3 signalling pathway to promote cardiac inotropy in the heart, indicating that this signalling paradigm may be targeted in therapy of heart diseases with reduced ejection fraction.

Keywords Carvedilol • Cyclic guanosine monophosphate • Heart dysfunction • β adrenergic receptor • Contractility

1. Introduction

Stimulation of cardiac β adrenergic receptors (βARs) presents the key regulatory mechanism to enhance cardiac contractile function in response to sympathetic stress. In the classic paradigm, activation of the major cardiac β_1 adrenergic receptor (β_1AR) subtype by catecholamines leads to G_s-dependent activation of adenylate cyclases, which produces the secondary messenger cyclic adenosine monophosphate (cAMP) to activate protein kinase A (PKA).[1] PKA plays a key role in enhancing cardiac contractility by promoting protein phosphorylation involved in excitation–contraction (E-C) coupling.[2] In pathological conditions such as heart failure (HF), β-blockers target the β_1AR by blocking the receptor-mediated cardiotoxic cAMP signalling in hearts. Interestingly, clinical observations indicate that a class of β-blockers such as nebivolol and

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carvedilol are implicated in promoting nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) signalling in the vasculature, offering additional benefits in treatment of HF.[3,4] However, the mechanisms underlying the β-blocker-induced NO/cGMP signalling remains unclear.

In comparison, activation of β_2AR and β_3AR subtypes is known to lead to dual coupling of receptors to both G_s and G_i proteins.[5] While the G_s-coupling by these βAR subtypes promotes small and restricted cAMP signal with minimal impacts on cardiac contractility, the G_i-coupling of β_2AR and β_3AR promotes activation of Akt and nitric oxide synthase 3 (NOS3)-cGMP cascades for diverse cellular responses.[6,7] Recent studies indicate that stimulation of β_1AR can also transduce diversified βAR signalling outputs.[8,9] For example, a mutant β_1AR lacking of scaffold tethering is capable of coupling to G_i in neonatal cardiomyocytes when activated by isoproterenol, leading to reduction of cAMP signal.[9] A recent study indicates that the β-blocker carvedilol is capable of promoting β_1AR coupling to G_i to transduce MAPK pathway in fibroblasts.[8] Accumulating evidence has validated carvedilol as a βAR biased agonist and the cardioprotective effects of carvedilol may associate with its biased agonism of β_1AR signalling.[10–12] However, it is not known whether the carvedilol-induced β_1AR coupling to G_i is connected to cGMP signal observed in the clinical settings. Investigating the signalling mechanisms underlying these observations will not only help us to understand the effects of this class of β-blockers in therapy but also offer potential novel targets for more efficacious strategies to treat cardiovascular diseases.

In this study, we show that carvedilol biasedly stimulates a more robust and far-reaching β_1AR-cGMP signal relative to a minimal and restricted β_1AR-cAMP signal. This biased β_1AR-cGMP signalling is dependent on NOS3 activated by a receptor-G_i-PI3K-Akt pathway. This novel β_1AR pathway promotes PKG-dependent increases in contractility with minimal impacts on calcium transient amplitude in both healthy hearts and diabetic cardiomyopathy. Our data indicate that this novel β_1AR-cGMP signalling pathway can be targeted to improve depressed cardiac ejection and to protect against myocyte apoptosis in diabetic cardiomyopathy.

2. Methods

2.1 Reagents and materials

All reagents and materials were obtained from Millipore-Sigma (St. Louis, MO, USA) unless otherwise specified.

2.2 Animals

All experiments including mouse feeding, treatment, and tissue collection were approved by the Institutional Animal Care and Use Committees (protocol: 20234 and 20957) of the University of California at Davis and follow NIH guidelines and ARRIVE guidelines.[13–15]NOS1-KO, NOS3-KO, and wide-type (WT) C57BL/6 male mice were ordered from The Jackson Laboratory (Bar Harbor, ME, USA). WT rats were purchased from Harlan Laboratories Inc (Indianapolis, IN, USA). All experimental animals were housed at room temperature with a 12 h–12 h light cycle.

2.3 High-fat diet model establishment and *in vivo* treatment

C57BL/6 WT male mice (6- to 8-week old) were fed with low-fat diet (LFD) formula D12450J (3.85 kcal/g; containing 20% of calories from protein, 70% of calories from carbohydrate, and 10% of calories from fat) or with a matched high-fat diet (HFD) formula D12492 (5.24 kcal/g; containing 20% of calories from protein, 20% of calories from carbohydrate, and 60% of calories from fat, Research Diets Inc. New Brunswick, NJ, USA).[16] Details are described in the Supplementary material online, *Methods*.

2.4 Echocardiograph

Cardiac function was assessed with a Vevo 2100 Imaging System using a 22–55 MHz MS550D linear probe (VisualSonics, Toronto, ON, Canada). Mice were anaesthetized with isoflurane in oxygen (2% for induction and 1% for maintenance) and placed on a warm pad at the supine position. Body temperature, respiratory rate, and electrocardiograph were monitored; and heart rate was kept consistent between 450–500 beats per minute to avoid variation. After recording baseline cardiac function, mice were injected with (-)-isoproterenol hydrochloride (ISO, 100 μg/kg i.p.), carvedilol (100 μg/kg i.p), or metoprolol (100 μg/kg i.p). Cardiac function was measured 2 min after drug injection. All measurements represent the average of three cardiac cycles and were performed in a blind fashion with group identifiers removed from the imaging using Vevo 2100 software (v3.1.1). Details are described in the Supplementary material online, *Methods*.

2.5 Blood glucose and intraperitoneal glucose tolerance test

Fast blood glucose level and intraperitoneal glucose tolerance test (IPGTT) were performed after 6-h fasting from venous blood by snipping the tail. Fast glucose levels were recorded as time 0, and glucose levels were obtained at 15 min, 30 min, 60 min, and 120 min after i.p. injection of 1 mg glucose/1g body weight. Glucose time course was plotted and the area under curve (AUC) was calculated to diagnose the degree of the glucose tolerance impairment.

2.6 Histological staining

Mice were sacrificed with heart harvest after anaesthesia with 1–2% isoflurane. Hearts were collected and rinsed in 1 mM KCl to maintain an end-diastolic cardiac anatomy. The hearts were fixed and processed for histochemical analysis. Details are described in the Supplementary material online, *Methods*.

2.7 Myocyte isolation and *in vitro* treatment

Adult left ventricular myocytes (AVMs) from WT, NOS1-KO, and NOS3-KO mice and WT rats were isolated according to the protocol previously reported.[17] Animals were euthanized to harvest hearts after anaesthesia with 1–2% isoflurane. Details are described in the Supplementary material online, *Methods*.

2.8 Adult myocyte contractility and calcium transient assay

As described previously, fresh AVMs were recovered in beating buffer for 30 min and then were stained with 5 μM Fluo-4 AM (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min in the dark at room temperature and rinsed with PBS without calcium for 10 min.[16,17] Contractility and calcium transient were analysed in the same AVMs as detailed in the Supplementary material online, *Methods*. The average value from multiple cells of each animal was plotted as one data point. Data were collected from five animals per group.

2.9 Western blot

Heart tissue lysates were prepared using FastPrep-24™ classic homogenizer (MP Biomedicals, Santa Ana, CA, USA) in RIPA buffer with phosphatase and protease inhibitors. Isolated myocytes were lysed using an ultrasonic cell disruptor (Fisher Scientific, Pittsburgh, PA, USA) on ice after treatment. Cell and tissue lysates were subjected to western blot analysis detailed in the Supplementary material online, *Methods*.

2.10 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) was performed as previously described.[18] Freshly isolated adult rat and mouse myocytes were cultured and then infected with the cytosolic cGM Psensor, cGi500 (Cyto-cGMP),[4] or cGMP sensors anchored on the plasma membrane (PM) (PM-cGMP), the sarcoplasmic reticulum (SR) (SR-cGMP), or the myofilament (MF-cGMP) with targeting sequences for 36 h in serum-free FRET media as described previously.[19] Similarly, cells were infected with Cyto-AKAR3 (cytosolic PKA sensor)[13] or PM-AKAR3 (PM PKA sensor)[5] biosensors for 36 h in serum-free FRET media. Live cells on coverslip were subjected to FRET analysis as described in the Supplementary material online, *Methods*. The average value from multiple cells of each animal was plotted as one data point. Data were collected from five animals per group.

2.11 Statistical analysis

Pooled data were represented as the mean ± SEM. Group sizes were determined by an *a priori* power analysis for a two-tailed, two-sample *t*-test with an α of 0.05 and power of 0.8, in order to detect a 10% difference signal at the endpoint. Animals were grouped with no blinding but randomized during the experiments. Fully blinded analysis was performed with different persons carrying out the experiments and analysis, respectively. Male animals were used for all experiments. No samples or animals were excluded from analysis. Representative figures/images reflected the average levels of each experiment. Normality of the data was assessed using two-sided Kolmogorov–Smirnov test in GraphPad Prism 8 (GraphPad Inc., San Diego, CA, USA). All statistical tests were two-sided and evaluated at a significance of 0.05. Unpaired two-tailed Student's *t* test was utilized for comparisons of two-independent variables. One-way analysis of variance (ANOVA) and one-way nested ANOVA were used to compare ≥3 groups followed by Tukey's multiple comparison. Two-way ANOVA and two-tailed Student's *t* test were utilized for comparisons of two variables. The correlation between cell shortening and calcium transient amplitude of each mouse was plotted; and the slope of before and after treatment was evaluated using linear regression.

3. Results

3.1 Carvedilol acute treatment enhances PKG-dependent contractility of normal adult myocytes

Beta-blockers are widely used in treatment of HF primarily through blocking the function of catecholamine; however, there are nuances in pharmacological actions among distinct β-blockers. Among them, carvedilol is unique, due to its ability in promoting cardiac contractility of HF mice.[20] Here, we observed a significant increase in contractile shortening of AVMs from normal mice after acute stimulation with non-selective β-agonist ISO and non-selective β-blocker carvedilol. The carvedilol-induced increases were about 45% of those induced by ISO (*Figure 1A–D*). By recording the calcium transient signalling simultaneously in the same AVMs, ISO was found to induce robust increases in calcium cycling with higher amplitudes of calcium transient and faster calcium decay Tau, however, carvedilol failed to affect calcium cycling (*Figure 1E* and *F*, Supplementary material online, *Figure S1A and C*). Meanwhile, a control β_1AR selective blocker metoprolol did not change contractility and calcium cycling in AVMs (*Figure 1B, D–F*, Supplementary material online, *Figure S1B*). The correlation between mean contractile function and mean calcium transient amplitude of each mouse revealed an up-regulated of calcium sensitivity for myocyte shortening in carvedilol treated AVMs with a higher slope than those treated with ISO or metoprolol (Supplementary material online, *Figure S1L*).

In the classic paradigm, stimulation of β_1AR enhances myocyte contractility *via* increasing cAMP signal and PKA activity. Carvedilol is a beta-blocker that blocks PKA activity. However, carvedilol is also implicated in mobilizing NO-cGMP signalling in clinical settings.[4] We examined signalling pathways involved in carvedilol-induced contractility. Carvedilol-induced increases in contractility of AVMs were completely abolished by PKG inhibitor DT-2, but not sensitive to inhibition of PKA with PKI (*Figure 1G–J*). Carvedilol minimally affected calcium transient and decay tau, which led to an increased slope of relationship between contractility and calcium transient amplitude (*Figure 1K and L*, Supplementary material online, *Figure S1L*). PKI or DT-2 did not affected calcium transient amplitude; but DT-2 reduced calcium decay tau under carvedilol treatment (*Figure 1K and L*, Supplementary material online, *Figure S1D–F*). Together, these data indicate that carvedilol transduces a PKG pathway to promote myocyte contractility *in vitro*.

3.2 Carvedilol promotes biased β_1AR-cGMP signalling in cardiomyocytes

Using FRET-based biosensors for PKA (AKAR3) and cGMP (cGi500), we then dissected the effects of carvedilol on cAMP-PKA and cGMP-PKG signalling in rat AVMs. A set of other β-blockers were examined in comparison. None of the β-blockers induced significant increases in PKA activity in the cytoplasm (*Figure 2A*). We suspected that some β-blockers may induce small increases in PKA activity in the local vicinity of activated receptors, which is not detectable in the cytoplasm. A PKA biosensor AKAR3 anchored on PM (PM-AKAR3) was used. Carvedilol, but not other β-blockers, induced small yet significant increases in PKA activity at the PM (*Figure 2B*). Furthermore, the carvedilol-induced PKA activity on the PM was blocked by non-selective βAR antagonist, CGP12177, but not blocked by β_2AR selective antagonist, ICI118551 (*Figure 2C*), indicating a β_1AR-dependent action. In comparison, among the compounds tested, carvedilol and nebivolol induced significant increases in cGMP signal in the cytoplasm in AVMs (*Figure 2D*). Meanwhile, β-agonist ISO-induced robust increases in PKA activity at both the PM and cytoplasm as well as cGMP signal in the cytoplasm of AVMs (*Figure 2A–D*). When comparing with the responses induced by ISO, carvedilol biasedly promoted cGMP signal in AVMs (*Figure 2E*). In myocytes, activated PKG promotes phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser239, whereas PKA enhances the phosphorylation of VASP at Ser157.[21] We assessed the downstream phosphorylation of VASP as a proxy of carvedilol-induced cGMP-PKG and cAMP-PKA signalling detected in FRET assays. Carvedilol treatment significantly promoted

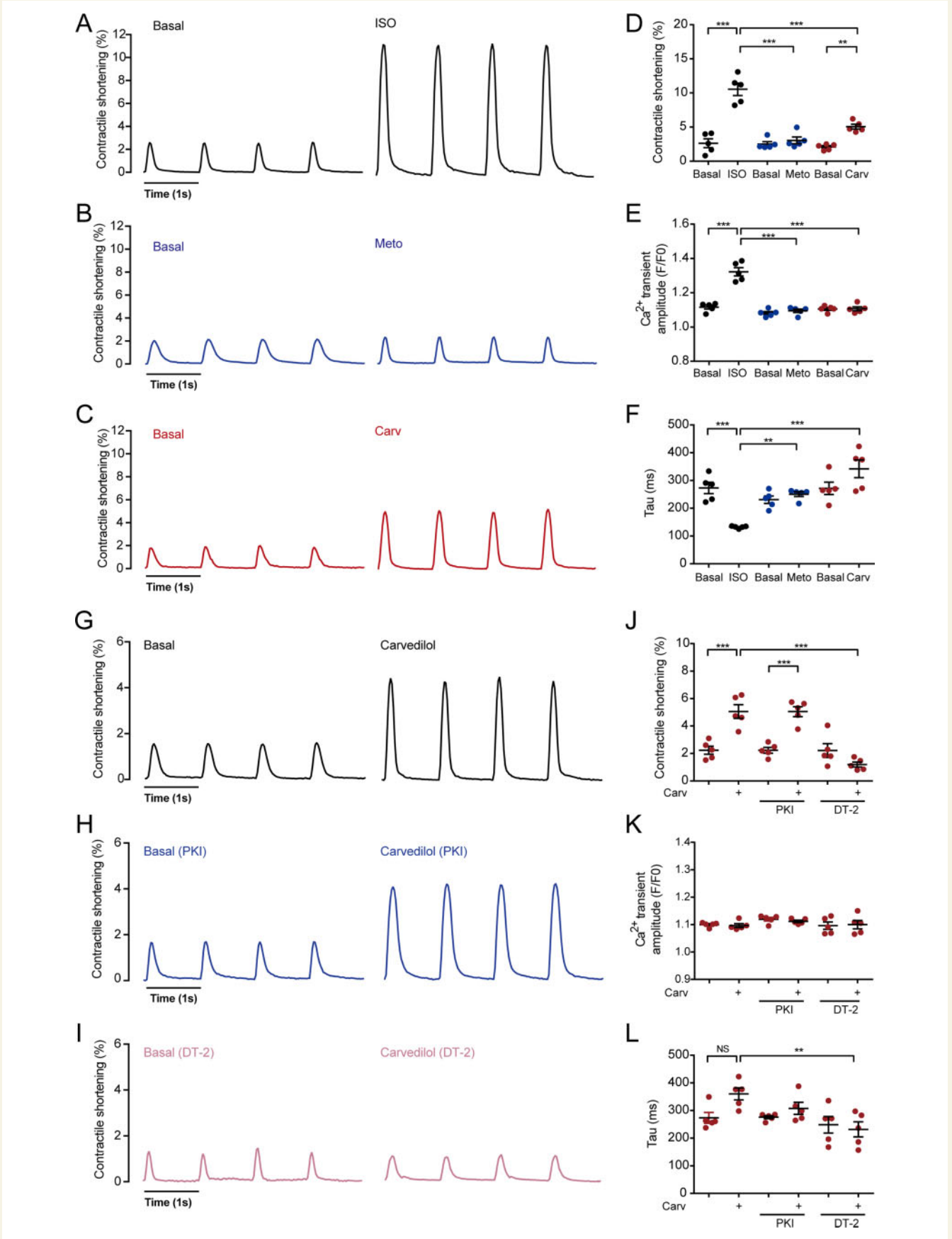

Figure 1 Acute stimulation with carvedilol enhances mouse cardiomyocyte contractility without enhancing calcium transient in a PKG-dependent manner. Mouse AVMs were treated with ISO (100 nM), carvedilol (1 μM), or metoprolol (1 μM) for 5 min, then the contractile function and calcium

the phosphorylation of Ser239 but did not enhance the phosphorylation of Ser157 of VASP in AVMs (*Figure 2F*). As control, ISO promoted phosphorylation at both PKA and PKG sites on VASP; metoprolol failed to do neither (*Figure 2F*). These data indicate that carvedilol preferentially promotes a cGMP-PKG signalling and phosphorylation of substrates in myocytes while ISO is capable of transducing both β_1AR-cGMP and cAMP-PKA signalling in AVMs.

3.3 Carvedilol-induced β_1AR-cGMP signalling preferentially targets to the myofilaments in cardiomyocytes

To understand how the β_1AR-cGMP signalling is involved in enhancing myocyte contractility, we further analysed subcellular distribution of cGMP signal after stimulation with carvedilol. We applied a series of anchored FRET-based cGMP biosensors in AVMs: SR-cGi500 on the SR, PM-cGi500 on the PM, and MF-cGi500 on the myofilament (Supplementary material online, *Figure S2A*). ISO significantly induced cGMP signal at all three cellular compartments (*Figure 3A–C*) with a smaller signal at the SR than at other subcellular compartments. Carvedilol promoted cGMP signal at the PM and myofilaments, but minimally at the SR (*Figure 3A–C*). The increases in cGMP signal induced by carvedilol were lower than those induced by ISO in individual subcellular compartment, but relative higher at the myofilament than other subcellular compartments. As a control, metoprolol failed to produce cGMP signal in AVMs (*Figure 3A–C*). The carvedilol-induced cGMP signal in AVMs was blocked by pretreatment with CGP12177, but not with ICI118551 or SR59230A, supporting a β_1AR-dependent mechanism (Supplementary material online, *Figure S2B–D*). These data indicate that carvedilol induces a biased β_1AR-cGMP signalling that preferentially targets to the myofilaments.

We further assessed the potential impacts of carvedilol-induced cGMP-PKG signalling on phosphorylation of troponin I (TnI) and myosin binding protein C (MyBP-C), two known PKG substrates on the myofilaments.[22,23] Carvedilol did not alter phosphorylation of serine23/24 of TnI; in comparison, carvedilol enhanced phosphorylation of serine282 of MyBP-C in AVMs. As a control, ISO increased phosphorylation of both serine 282 of MyBP-C and serine23/24 of TnI (*Figure 3D*). Carvedilol-induced phosphorylation of MyBP-C at serine282 was blocked by DT-2, but not by PKI or calmodulin kinase II inhibitor KN93 (*Figure 3E*). Additionally, metoprolol or CGP12177 did not promote phosphorylation of MyBP-C; and pretreatment with CGP12177 significantly abolished the phosphorylation of MyBP-C at serine282 evoked by carvedilol (*Figure 3D and F*). These data indicate that carvedilol induces β_1AR-cGMP signalling, which enhances phosphorylation of substrates at the myofilaments in myocytes.

3.4 Carvedilol promotes NOS3-dependent β_1AR-cGMP signalling in cardiac myocytes

We then analysed the carvedilol-induced cGMP signalling pathway using the cGMP biosensor in FRET assays. The carvedilol-induced cGMP signal was also abolished by CGP12117, but not by ICI118551 or SR59230A in AVMs (*Figure 4A*). Moreover, the carvedilol-induced cGMP signal was blocked by the G_i inhibitor pertussis toxin (PTX), the PI3K inhibitor LY294002, and the Akt inhibitor, MK2206 (*Figure 4B*). Under adrenergic stimulation, activated NOS produces NO, which stimulates soluble guanylyl cyclases (sGCs) to synthesize cGMP. Two NOSs, NOS1 (also known as neuronal NOS, nNOS) and NOS3 (also known as endothelial NOS, eNOS) play essential roles in heart function.[24] Genetic deletion of NOS3, but not NOS1, significantly blocked carvedilol-induced cGMP signal in AVMs, indicating that carvedilol promotes cGMP synthesis through the NOS3 pathway (*Figure 4C*).

NOS3 is known to be phosphorylated and activated by Akt.[25] Carvedilol, but not metoprolol or CGP12177, increased the phosphorylation of NOS3 on Serine1177, one of the phosphorylation events contributing to activation of the enzyme (*Figure 4D*). The specificity of NOS3 primary antibodies was validated by blotting with protein extracts from NOS3 knockout (KO) mouse (Supplementary material online, *Figure S3*). Meanwhile, CGP12177 significantly blocked carvedilol-induced phosphorylation of NOS3 at Ser1177, while neither ICI118551 nor SR59230A blocked the responses (*Figure 4E*). Moreover, inhibition of Akt with MK2206, but not inhibition of PKA with PKI, blocked carvedilol-induced phosphorylation of NOS3 at Ser1177 (*Figure 4F*). Together, these data indicate that carvedilol induces a β_1AR-cGMP-PKG signalling pathway through a G_i-PI3K-Akt-NOS3 cascade.

3.5 Carvedilol promotes NOS3-dependent increases in myocytes contractility *in vitro* and cardiac ejection fraction *in vivo*

Deletion of NOS3 also blocked carvedilol, but not ISO-induced phosphorylation of MyBP-C at serine282 (*Figure 5A*). As controls, ISO, but not carvedilol, promoted PKA phosphorylation of phospholamban (PLB) at serine16; and metoprolol did not affect the phosphorylation of either MyBP-C or PLB (*Figure 5A*). Moreover, acute injection of carvedilol in WT littermate mice induced significant increases in cardiac ejection fraction (EF) (*Figure 5B*). Deletion of NOS3 abolished the carvedilol effects on both heart rates and EF (*Figure 5B and C*). Deletion of NOS3 did not affect ISO-induced increases in heart rates and EF (*Figure 5D and E*). As negative controls, metoprolol minimally affected cardiac EF in both WT and NOS3-KO mice (*Figure 5F*), but significantly reduced heart rates (*Figure 5G*). Additionally, deletion of NOS3 also inhibited carvedilol-induced increases in contractility but did not affect ISO-induced response in isolated AVMs (*Figure 5H*). Deletion of NOS3 did not affect

Figure 1 Continued

transient were detected in the same cell before and after stimulation. (*A–C*) Representative traces of contractility both at basal line and in response to ISO, metoprolol, or carvedilol treatment, (*D*) The maximal increases in contractile function, (*E*) amplitude of calcium transient, and (*F*) Calcium decay Tau were measured and plotted. (*G–I*) AVMs were pre-treated with PKI (1 μM) or DT-2 (100 nM) for 10 min before carvedilol (1 μM) stimulation. Representative traces of contractility at basal level (after pretreatment) and at 5 min of additional carvedilol treatment. (*J*) The maximal increases in contractile function, (*K*) Amplitude of calcium transient, and (*L*) calcium decay Tau were measured and plotted. Data were presented as mean value of each mouse and from total five mice per group. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by one-way ANOVA followed by *post hoc* Tukey's test.

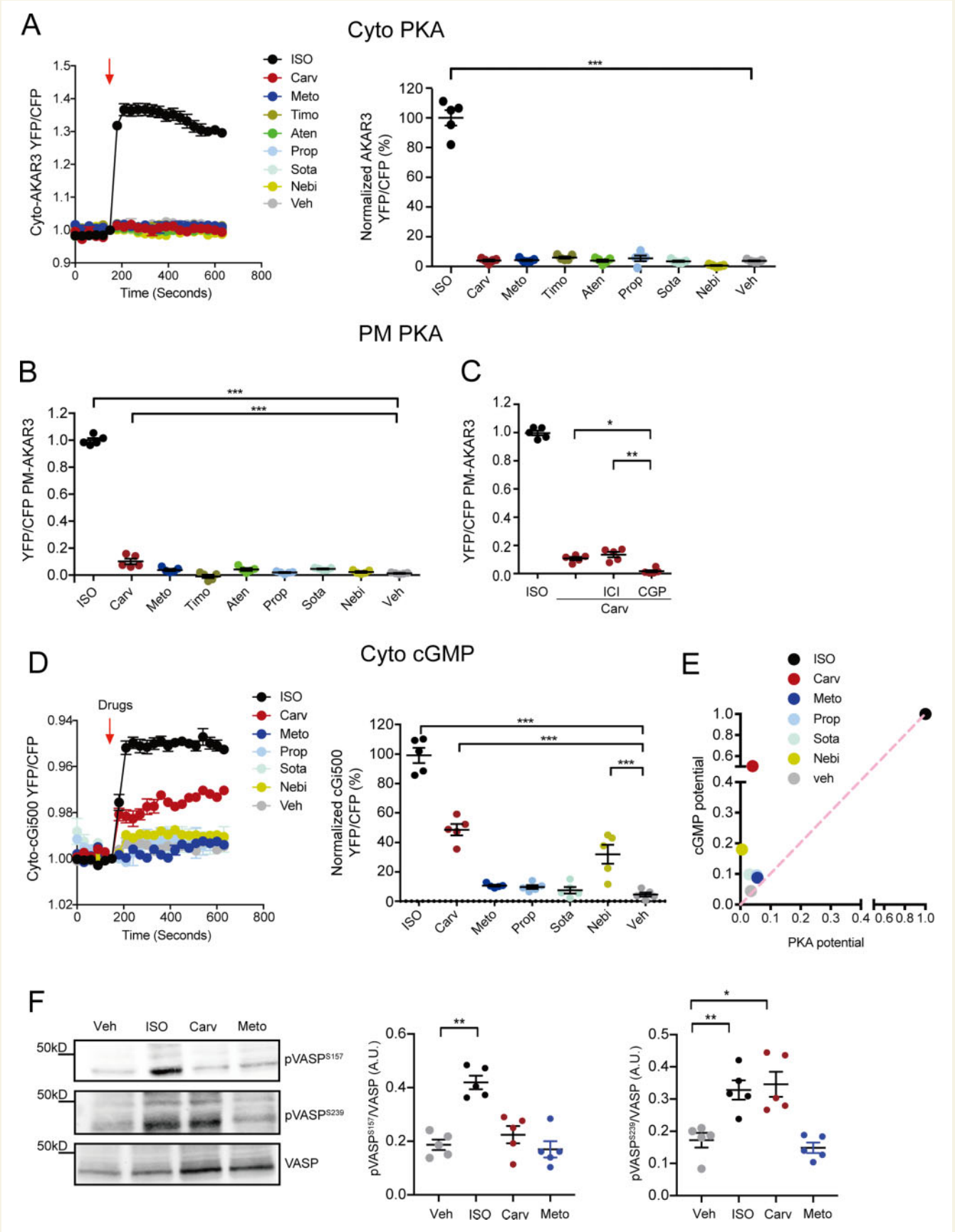

Figure 2 Carvedilol promotes biased cGMP signalling in rat cardiomyocytes. (*A*) Cytoplasmic PKA activity was measured with FRET biosensor Cyto-AKAR3 after treatment with ISO (100 nM), β-blockers (1 μM), or vehicle in rat AVMs. (*B*) PKA activity at the PM was measured with FRET biosensor PM-AKAR3 after treatment with ISO (100 nM), β-blockers (1 μM), or vehicle in rat AVMs. (*C*) Rat AVMs were pretreated with CGP12177, or ICI118551

calcium cycling in isolated AVMs after either ISO or carvedilol treatment (*Figure 5I and J*).

3.6 Carvedilol enhances cardiac contractile function in diabetic hearts

To investigate the clinical significance of this novel β_1AR-cGMP-PKG signalling pathway triggered by carvedilol, we treated HFD-induced cardiomyopathy with carvedilol or metoprolol. The metabolic and cardiac function of mice fed with HFD or LFD were monitored as outlined (Supplementary material online, *Figure S4A*). After feeding with HFD for 4 weeks, the fasting blood glucose levels of mice were further up-regulated than those of LFD fed mice; the increased levels of fasting blood glucose in HFD mice were also associated with glucose intolerance (Supplementary material online, *Figure S4B–D*). Mice fed with HFD for 14 weeks displayed an impaired systolic cardiac function with significantly reduced EF and fractional shortening (FS) when compared with mice feeding with LFD (*Figure 6A and B*), consistent with cardiomyopathy described in previous studies.[13] Subsequently, mice fed with HFD for 14 weeks were subjected to carvedilol and metoprolol therapies for 4 weeks (Supplementary material online, *Figure S4E*). Therapies with both β-blockers effectively rescued cardiac EF and FS in HFD hearts (*Figure 6C and D*). In addition, carvedilol and metoprolol therapies significantly decreased in cross sectional area of myocytes of left ventricle, left ventricular wall thickness, and myocardial fibrosis (Supplementary material online, *Figure S5A–C*). These observations were accompanied with a restoration of both systolic and diastolic cardiac function in HFD mice (Supplementary material online, *Table S1*).

AVMs were isolated from treated mice to assess the impacts of β-blockers on contractility and calcium handling. AVMs from HFD mice with vehicle therapy displayed impaired calcium cycling and contractile shortening relative to those from mice fed with LFD (*Figure 6E–G*). AVMs from carvedilol-treated HFD mice displayed significant increases in contractile shortening over those from vehicle-treated HFD mice (*Figure 6E*). However, these increases were associated with reduced calcium amplitude and a fast calcium decay time (*Figure 6F and G*). In comparison, metoprolol minimally affected calcium cycling and contractile shortening in HFD myocytes relative to vehicle-treated controls (*Figure 6E–G*). These observations suggest that therapies with carvedilol and metoprolol promoted distinct remodelling on E-C coupling. We then examined the activity and expression of related proteins in HFD hearts after therapies. We found therapy with neither carvedilol nor metoprolol significantly affected the expression of sarco-endoplasmic reticulum calcium ATPase 2 (SERCA2) in HFD heart (Supplementary material online, *Figure S6A*). While both drugs promoted the phosphorylation of PLB at Ser16, the increases in phosphorylation of PLB induced by carvedilol were significantly higher than those of metoprolol treated ones (Supplementary material online, *Figure S6A*). Both drugs significantly elevated the phosphorylation of ryanodine receptor 2 (RyR2) at Ser2814 relative to the LFD controls (Supplementary material online, *Figure S6B*). Metoprolol, but not carvedilol, increased the phosphorylation of RyR2 at Ser2808 and TnI at Ser23/24 in HFD hearts (*Figure 6H*; Supplementary material online, *Figure S6B*). Carvedilol therapy selectively promoted phosphorylation of MyBP-C at the Ser282, but not at serine 273 and 302 in HFD hearts (*Figure 6I*). In addition, therapy with carvedilol promoted an elevation of phosphorylation of Ser1177 of NOS3 and phosphorylation of Akt at Ser473 in HFD hearts when compared with either vehicle- or metoprolol-treated group (Supplementary material online, *Figure S6C*). Together, these data indicate that these two drugs promote remodelling of substrate phosphorylation involved in calcium handling and contraction in HFD hearts via inducing different signalling pathways.

3.7 Carvedilol therapy sensitizes adrenergic reserves in HFD hearts with attenuated calcium cycling and myocytes apoptosis

Since HFD hearts display modified contractility and calcium cycling together with modified protein phosphorylation after therapy with carvedilol, we assessed whether chronic treatment with carvedilol modulates adrenergic reserves in response to stress in HFD hearts. After 4 weeks of therapy, LFD and HFD mice were challenged with ISO. HFD mice with vehicle therapy produced smaller responses in EF and FS relative to LFD mice (*Figure 7A and B*; Supplementary material online, *Table S2*). HFD mice with carvedilol or metoprolol therapy displayed robust increases in EF and FS in response to ISO stimulation, which was equivalent to those in LFD mice (*Figure 7A and B*; Supplementary material online, *Table S2*). AVMs were isolated from HFD mice after therapy to independently analyse the sensitivity to adrenergic stimulation. In comparison to those from LFD mice, AVMs from HFD mice with vehicle therapy displayed smaller responses in E-C coupling after stimulation with ISO (*Figure 7C–E*). AVMs from carvedilol-treated HFD mice displayed robust contractile shortening in response to ISO stimulation; however, these myocytes displayed minimal to small responses in calcium transient amplitude and decay Tau when compared with myocytes from vehicle-treated HFD mice (*Figure 7C–E*). In comparison, myocytes isolated from HFD mice with metoprolol treatment restored the sensitivity to ISO stimulation, which were comparable to those of LFD groups (*Figure 7C–E*). These results demonstrate that carvedilol is capable of enhancing cardiac inotropy in HFD mice without enhancing calcium cycling, which may prevent potentially detrimental remodelling to myocardia under elevated sympathetic drive in pathological conditions. The impacts of carvedilol therapy on structural properties in HFD hearts were examined. HFD hearts with vehicle therapy displayed increased cardiac apoptosis compared to LFD hearts. Carvedilol therapy significantly reduced cell death in HFD hearts; this beneficial effect was not observed in the metoprolol-treated group (*Figure 7F and G*, Supplementary material online, *Figure S7*).

Figure 2 Continued

before carvedilol (1 μM) stimulation. ISO (100 nM) or carvedilol induced PKA activity at the PM is measured with the FRET biosensor PM-AKAR3. (*D*) Cytoplasmic cGMP signal was measured with FRET biosensor Cyto-cGi500 after treatment with ISO (100 nM), β-blockers (1 μM), or vehicle in rat AVMs. (*E*) β-blocker- or vehicle-induced responses in PKA activity and cGMP signal were normalized against the responses induced by ISO and plotted on the horizontal *X*-axis and *Y*-axis, respectively. Data were presented as mean value of each animal ($n = 5$). (*F*) AVMs were treated with ISO (100 nM), carvedilol (1 μM), metoprolol (1 μM), or vehicle for 10 min, the phosphorylation of VASP at Ser239 and Ser157, as well as the expression of total VASP was detected ($n = 5$). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ by one-way ANOVA followed by *post hoc* Tukey's test.

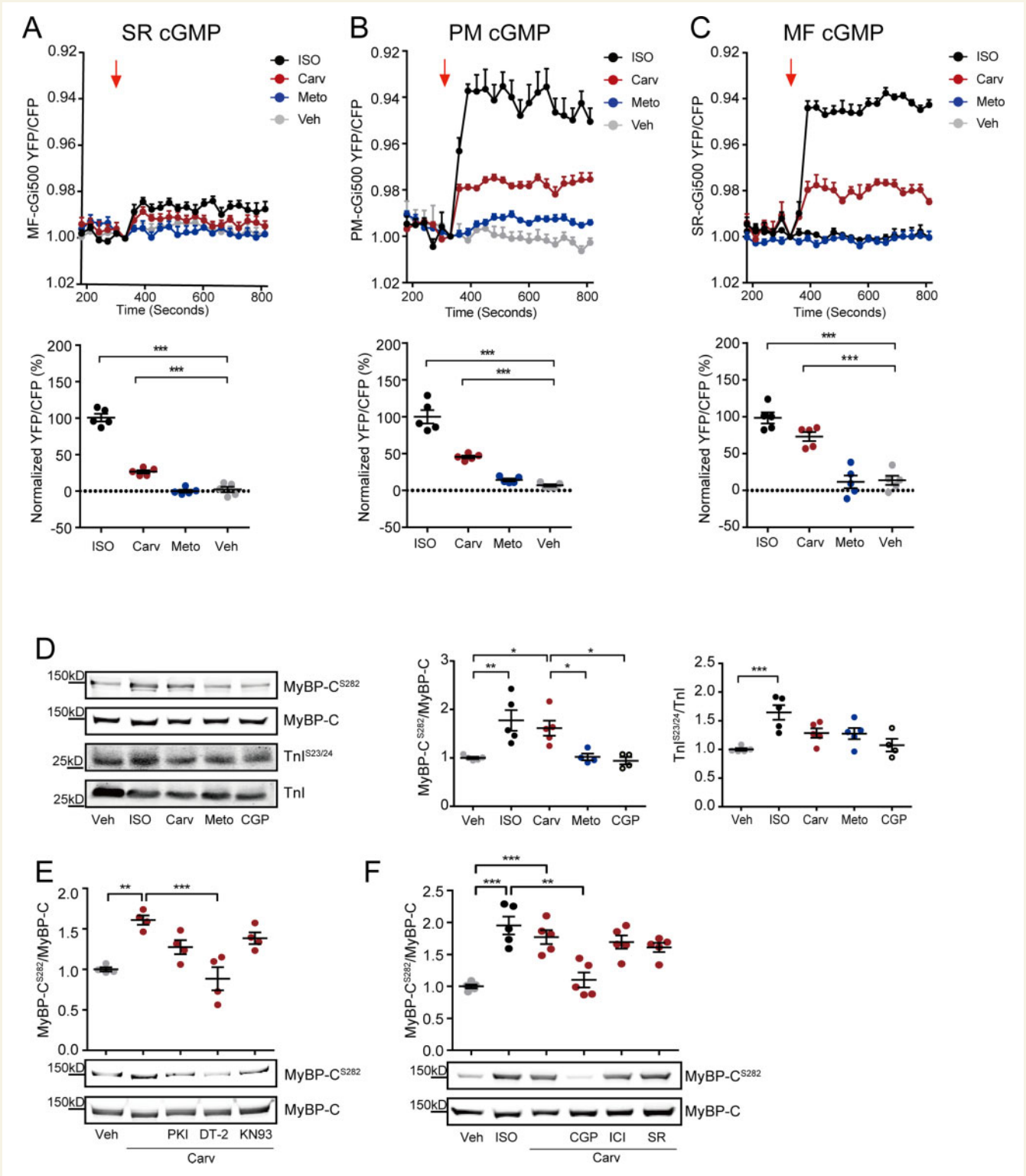

Figure 3 Carvedilol promotes cGMP signalling preferentially targets to myofilaments in cardiomyocytes. (*A–C*) Rat AVMs expressing targeted cGi500 biosensors were treated with ISO (100 nM), carvedilol (1 μM), metoprolol (1 μM), or vehicle. The time courses in FRET ratio were recorded. The maximal changes in FRET ratio normalized against ISO were plotted. Data were presented as mean value of each animal and from total five animals per group. (*D*) Rat AVMs were treated with ISO (100 nM), carvedilol (1 μM), metoprolol (1 μM), or CGP12177 (1 μM) for 10 min and cells were lysed and subjected to western blot with antibodies against phosphor-S23/24 and total TnI and phospho-S282 and total MyBP-C. The levels of phosphorylation were normalized with total protein ($n = 4–5$). (*E* and *F*) Rat AVMs were acutely stimulated with carvedilol (1 μM) for 10 min after 10-min pretreatment with PKI (1 μM), DT-2 (100 nM), KN93 (1 μM), CGP12177 (1 μM), ICI118551 (1 μM), or SR59230 A (1 μM), and phosphorylation of MyBP-C at Ser282 was determined. The western blots were quantified. ($n = 4–5$) $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by one-way ANOVA followed by *post hoc* Tukey's test.

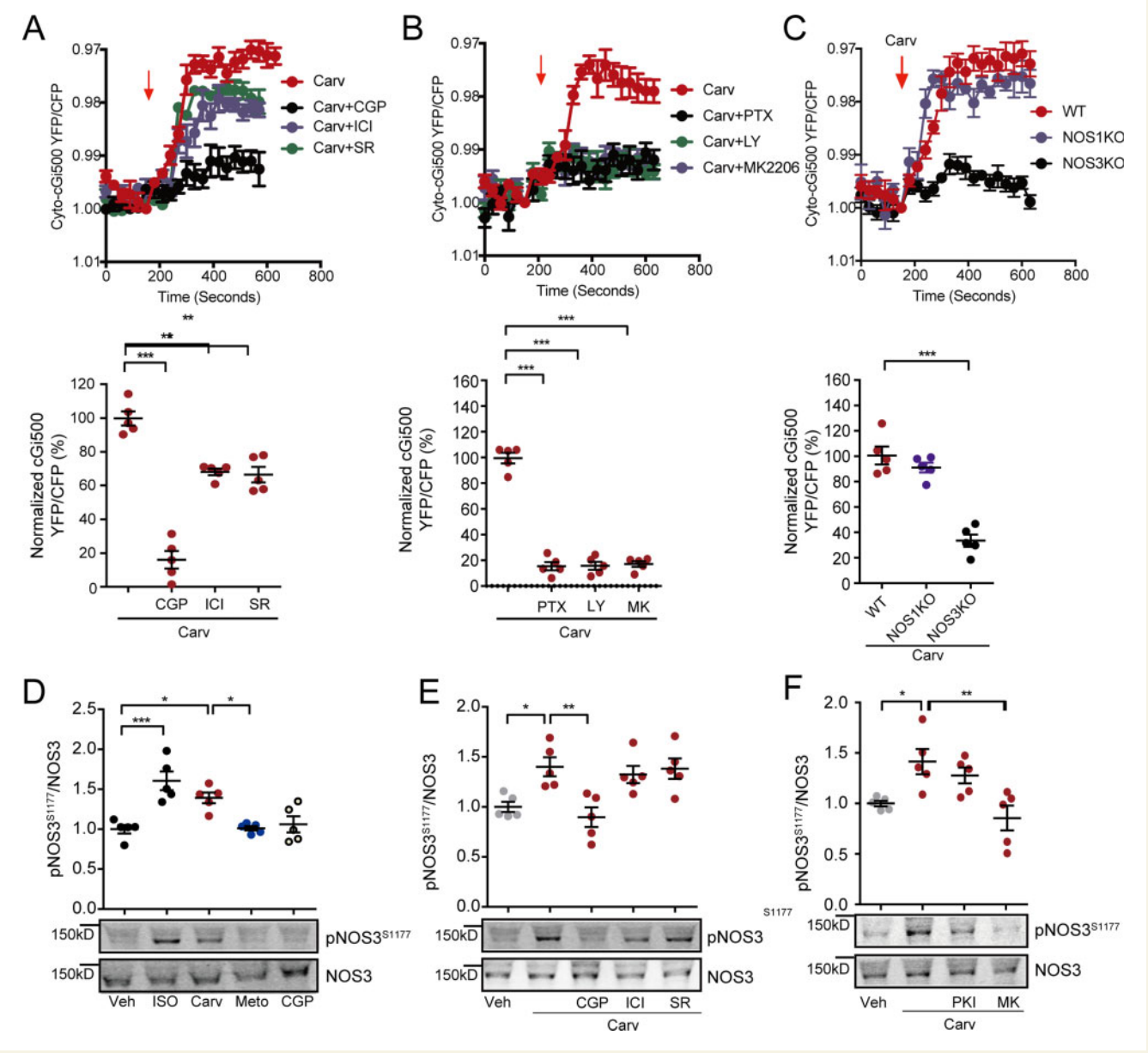

Figure 4 Carvedilol-induced β_1AR-cGMP signalling is dependent on a G_i-Akt-NOS3 pathway in cardiomyocytes. (*A*) Rat AVMs expressing Cyto-cGi500 biosensor were pretreated with CGP12177 (1 μM), ICI118551 (1 μM), or SR59230 A (1 μM) for 10 min before stimulation with carvedilol (1 μM). The time courses in FRET ratio were recorded; and the maximal changes in FRET ratio were normalized against carvedilol. (*B*) Rat AVMs expressing Cyto-Gi500 were treated with G_i inhibitor (PTX, 1 μg/mL, 3 h), PI3K inhibitor (LY294002, 1 μM, 10 min), or Akt inhibitor (MK2206, 1 μM, 10 min) before stimulation with carvedilol (1 μM). The time courses in FRET ratio were recorded; and the maximal changes in FRET ratio were normalized against carvedilol. (*C*) AVMs from WT, NOS1KO, and NOS3KO mice expressing cGMP biosensor Cyto-Gi500 were stimulated with carvedilol (1 μM). The time courses in FRET ratio were recorded. The maximal changes were normalized against carvedilol response in WT myocytes. For panel *A–C*, data were presented as mean value of each animal and from total five animals per group. (*D–F*) Rat AVMs were treated with vehicle, ISO (100 nM), carvedilol (1 μM), metoprolol (1 μM), or CGP12177 (1 μM) for 10 min. Alternatively, rat AVMs pretreated with CGP12177 (1 μM), ICI118551 (1 μM), SR59230A (1 μM), PKI (1 μM), or MK2206 (1 μM) were stimulated with carvedilol as indicated. The phosphorylation of serine1177 and total protein of NOS3 were detected and the ratio of phosphorylated NOS3 against total NOS3 was normalized. ($n = 5$) *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ by one-way ANOVA followed by *post hoc* Tukey's test.

4. Discussion

A major finding reported here is that carvedilol is capable of inducing β_1AR-cGMP signalling to promote an inotropic contractile response in healthy and HFD-induced diabetic hearts without increasing calcium signalling. The observed carvedilol-induced positive inotropy is consistent with previous observations showing that acute carvedilol injection promotes cardiac contractility in dogs and humans.[26,27] The effects of carvedilol are uncoupled from the elevation of PKA phosphorylation of substrates such as TnI and PLB, two proteins typically involved in

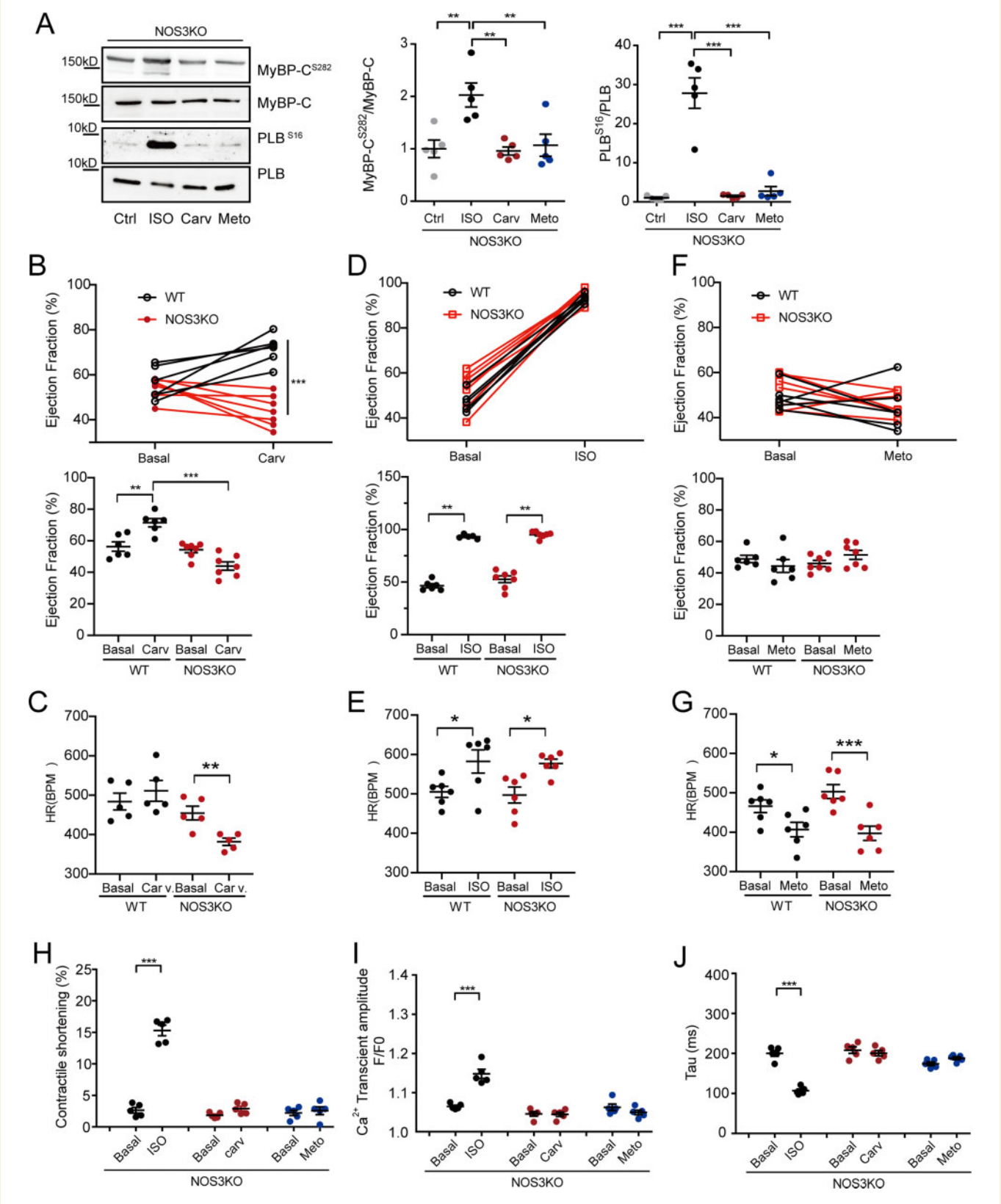

Figure 5 Carvedilol promotes NOS3-dependent increases in contractile function *in vitro* and *in vivo*. (*A*) AVMs from NOS3-KO mice were stimulated with vehicle, ISO (100 nM), carvedilol (1 μM), or metoprolol (1 μM). The phosphorylation of MyBP-C at serine282 and PLB at serine16 were examined and quantified ($n = 5$). (*B–G*) WT and NOS3-KO mice were subjected to an intraperitoneal injection of 100 μg/kg of ISO, 100 μg/kg of carvedilol, or 100 μg/kg of metoprolol. EF was measured with echocardiograph before and after drug treatment ($n = 5–7$). Heart rate was recorded before and after drug injection ($n = 5–6$). (*H–J*) AVMs from NOS3-KO mouse loaded with calcium dye were paced at 1 hz and treated with ISO, carvedilol, or metoprolol, then the contractile shortening, amplitude of calcium transient, calcium decay Tau were measured before and after 5-min treatment in the same cell, and the

adrenergic stimulation of E-C coupling. Stimulation with carvedilol induces a biased β_1AR coupling to G_i-PI3K-Akt-NOS3-dependent activation of cGMP-PKG signalling for cardiac inotropy (*Figure* 7*H*). Deletion of NOS3 blocks carvedilol but not ISO-induced cGMP signal and inotropic responses in myocytes and hearts. Furthermore, therapy with carvedilol modifies cardiac E-C coupling in the diabetic hearts without elevation of calcium signalling and sensitizes the diabetic hearts to respond to adrenergic stimulation. These alterations are accompanied by reduced cardiac cell apoptosis and hypertrophy. Meanwhile, carvedilol has been shown to increase the contractility and NO synthesis of rat AVMs.[28] Our data show that the carvedilol-induced β_1AR-NOS3 pathway is conserved in both rat and mouse. Together, these data reveal a novel cardiac β_1AR signalling paradigm to promote cardiac inotropy, with potential benefits in preventing cell apoptosis and hypertrophy in diabetic cardiomyopathy.

Canonically, activation of cardiac β_1AR selectively couples to G_s to promote cAMP-PKA activity and cardiac contractility; in this paradigm, PKA is the most critical and essential enzyme for enhancing contractile responses. In comparison, activation of β_2AR and β_3AR leads to a dual coupling of receptors to both G_s and G_i proteins.[6,7,29] The coupling of β_2AR to G_s leads to limited and restricted distribution of cAMP signal at the PM, whereas the coupling of β_2AR and β_3AR to G_i not only restricts and limits G_s-dependent cAMP-PKA activity but also promotes beneficial cardiac Akt activity and NO-cGMP signal.[7,8,30] Similarly, the current data shows that carvedilol promotes β_1AR coupling to both G_s and G_i in the heart: the G_s-dependent cAMP signal is restricted at the PM whereas the carvedilol-induced β_1AR-G_i-cGMP signal is far-reaching in myocytes. The carvedilol-induced β_1AR dual coupling to different G proteins appears to be dependent on concentration: carvedilol at high concentrations promotes β_1AR coupling to G_i in fibroblasts while acting as a classic beta-blockade at low concentrations.[8] Additionally, mutation of the C-terminal PDZ motif on β_1AR releases the receptor from its scaffolding proteins and permits the receptor coupling to G_i when stimulated with ISO.[9] Meanwhile, carvedilol activates β_2AR in hippocampal neurons and induces a local cAMP signal sufficient to promote activation of L-type calcium channel in the local vicinity.[31] While carvedilol promotes β_1AR-G_i signalling to arrestin-ERK pathway,[8] it does not do so with β_2AR.[31] Moreover, our data show that nebivolol: a highly selective β_1AR blocker also promotes G_i-NO-cGMP pathway in cardiomyocytes.[32] The mechanisms underlying the divergent βAR subtype specific signalling are currently unclear and remain to be explored. These data depict the diversified βAR signalling outputs in myocardium that are dictated not only by the ligand binding-induced receptor conformation changes, but also by the receptor-associated signalling partners within subcellular environments in both healthy and disease states.

Considering the dominant expression of β_1AR in hearts, the β_1AR-cGMP signalling may have much greater impacts on cardiac function relative to those induced by β_2AR and β_3AR. While cardiac cGMP-PKG signalling is generally linked to lusitropic effects, the inotropic effects of PKG have been previously reported.[33] Our data show that the positive effect of carvedilol on inotropy is mediated by the β_1AR-PKG signalling but is independent of increases in phosphorylation of PLB and calcium transient. Thus, the carvedilol effects are different from those induced by β-arrestin-biased signalling induced by angiotensin II type 1 receptor (AT1R) and β_2AR, which are associated with phosphorylation of PLB and increases in calcium signalling.[34,35] Mechanistically, carvedilol enhances a global cGMP-PKG transduction while restricting cAMP-PKA signalling at the PM. These biased signalling induced by carvedilol are further validated by the increase of phospho-VASP at PKG site of Ser239 but not at the PKA site of Ser157. An early study shows that the β_3AR-induced cGMP-PKG signalling has a negative impact on E-C coupling in myocytes, which is linked to the phosphorylation of TnI.[36] While the carvedilol-induced β_1AR-cGMP signal is also preferentially targeted to the myofilaments, our data show that carvedilol selectively promotes PKG-dependent phosphorylation of MyBP-C but not TnI. MyBP-C has three sites (Ser273, Ser282 and Ser302) in cardiac-specific M-domain that are the main targets of different enzymes that regulate the force of contraction.[37,38] The increased phosphorylation of MyBP-C at Ser282 likely plays a role in cooperating with other phosphorylation events to regulate the function of myofilaments.[37,38] These data however don't rule out the involvement of other substrates in the carvedilol-induced contractile shortening. Future studies are merited to pursue the precise mechanisms on how carvedilol-induced cGMP-PKG signalling promotes cardiac inotropy.

This and previous studies indicate that both β_1AR and β_3AR are capable of coupling to NOS3 but induce distinct subcellular pools of cGMP to facilitate the phosphorylation of specific substrates locally.[33] Similarly, distinct pools of cAMP are involved in differential phosphorylation of TnI and MyBP-C in cardiomyocytes after adrenergic stimulation.[39] Along the same vein, distinct subcellular distribution of cGMP signal has been reported after activation of natriuretic peptide receptor A and B in cardiomyocytes.[40] Additionally, alteration of phosphodiesterase distribution facilitates cross talk between ANP-induced cGMP signal and β_1AR-induced cAMP signal in myocytes from hypertrophic hearts induced by pressure overload,[41] through which ANP enhances ISO-induced cardiac contractility in hypertrophic hearts. Thus, the subcellular pools of cGMP may be further modified by expression and distribution of βAR subtypes and phosphodiesterase isoforms to modulate cardiac contractility in diseased conditions. For example, the carvedilol activated β_2AR-cAMP signalling may also influence β_1AR-cGMP signal through crosstalk between cAMP/PKA and cGMP/PKG pathways.[31] Further dissection of subcellular distribution of βAR subtypes and their associated phosphodiesterases will help us understand the dynamics of cyclic nucleotide signal and their impacts on E-C coupling in healthy and diseased hearts. Indeed, we have shown that chronic therapy with carvedilol can reduce expression of PDE4D in HFD hearts.[16] Potential crosstalks between cAMP/PKA and cGMP/PKG pathway may further affect the observed outcomes in HFD hearts in the current study.[40,42] In all, our data support a potential beneficial role of β_1AR-cGMP signalling in enhancing cardiac inotropy in cardiac diseases with reduced EF, consistent with evidence supporting that both β_2AR- and β_3AR-cGMP signalling are beneficial in HF therapy in animal models.[7,43,44]

In healthy mice and myocytes, carvedilol induces NOS3-dependent positive inotropy in the absence of increases in calcium amplitude and calcium decay Tau. These observations are different from the effects of NOS1 in adrenergic stimulation of intracellular calcium signalling and

Figure 5 Continued

maximal increases were plotted. Data were presented as mean value of each animal and from total five animals per group. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by one-way ANOVA followed by *post hoc* Tukey's test. Differences of EF between WT and NOS3-KO mice after carvedilol stimulation were assessed by two-tailed unpaired *t*-test.

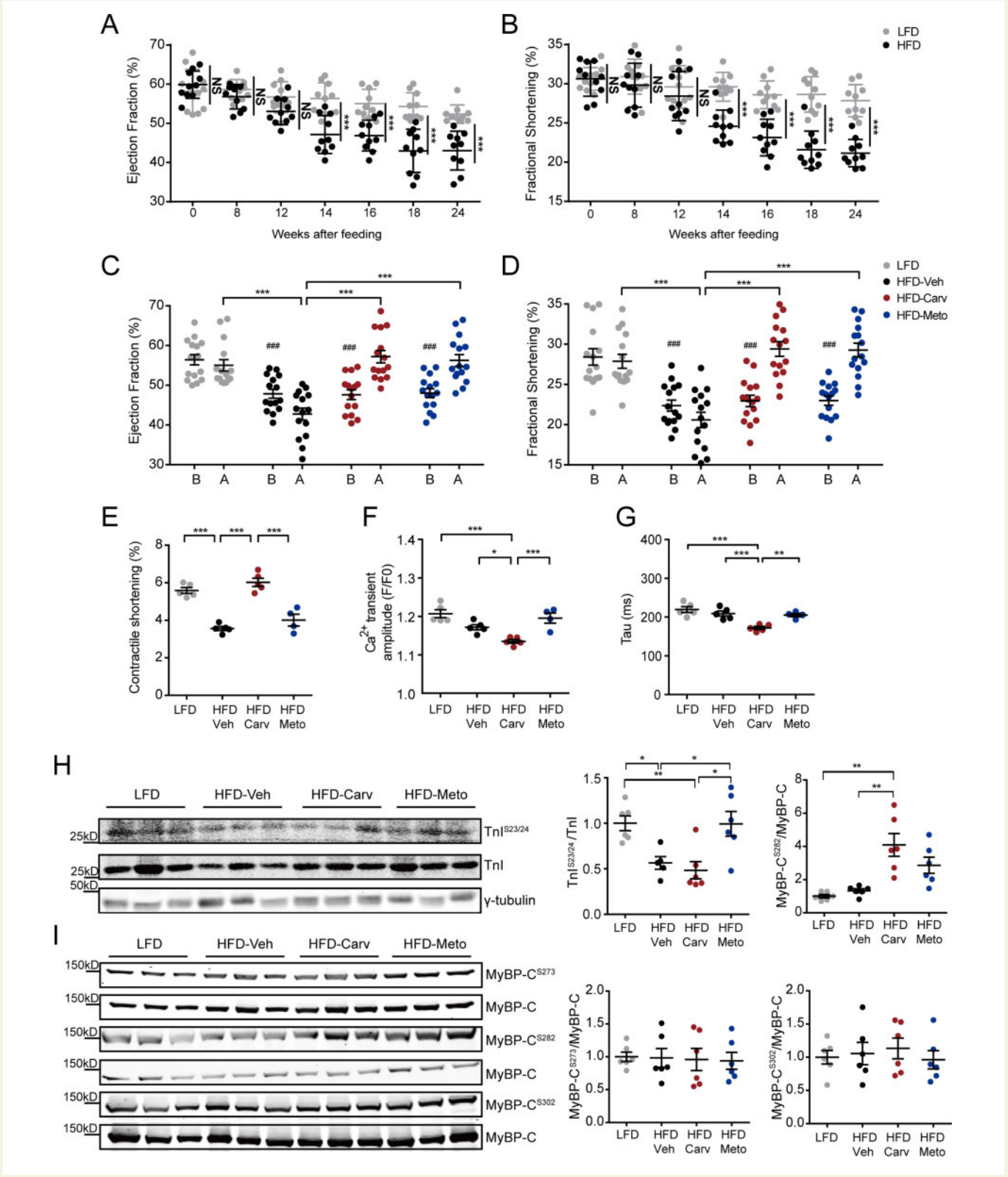

Figure 6 Chronic therapy with carvedilol promotes cardiac contractile function in diabetic hearts. (*A* and *B*) Mice were fed with LFD or HFD for 24 weeks, and systolic cardiac function EF and FS was assessed at 0, 8, 12, 14, 16, 18, 24 weeks after feeding ($n = 10$). (*C* and *D*) Mice were fed with LFD or HFD for 14 weeks; and the HFD fed mice were then subjected additionally to 4-week therapy (5% DMSO in water vehicle, 2.5 mg/kg/day carvedilol, or 10 mg/kg/day metoprolol). Cardiac function EF and FS was determined before and after therapy ($n = 15$). (*E*–*G*) AVMs were isolated from LFD and HFD mice after therapy with vehicle, carvedilol, or metoprolol. The baseline contractile shortening, amplitude of calcium transient, and calcium decay Tau were measured in the same cell. Data were presented as mean value of each mouse and from total four to five mice per group. (*H* and *I*) Cardiac tissue lysates from LFD, HFD-Veh, HFD-Carv, HFD-Meto mice were used to examine the phosphorylation of Ser23/24 on TnI, Ser273, Ser282, and Ser302 of MyBP-C and total proteins. The western blots were quantified ($n = 5$–6). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by one-way or two-way ANOVA followed by *post hoc* Tukey's test. $^{\#\#\#}P \leq 0.001$ vs. LFD mouse before treatment by two-way ANOVA.

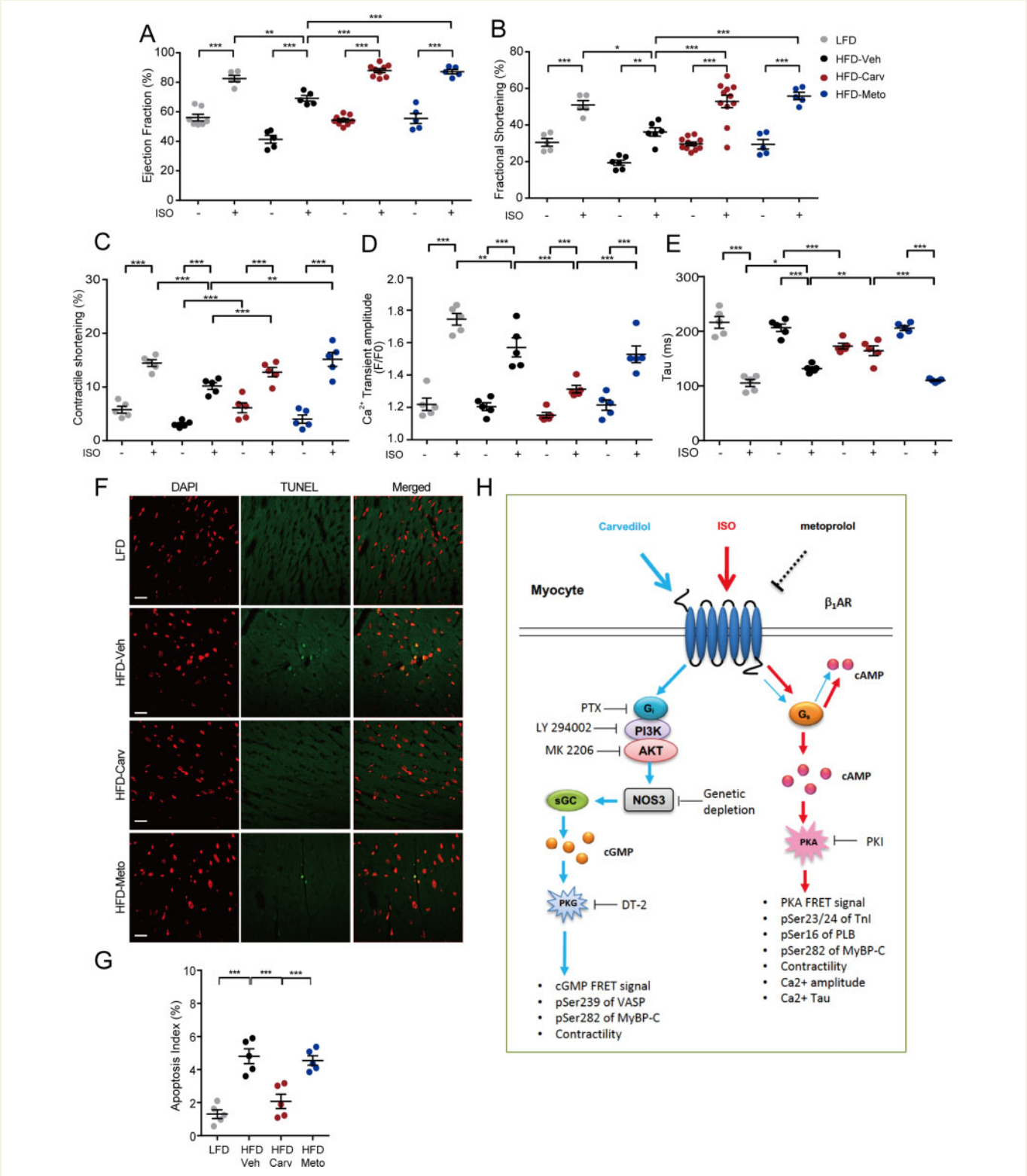

Figure 7 Carvedilol therapy sensitizes adrenergic reserves in HFD hearts with attenuated calcium cycling and myocytes apoptosis. (*A* and *B*) At least 24 h after 4-week therapy, LFD, HFD-Veh, HFD-Carv, HFD-Meto mice were intraperitoneally injected with ISO (100 μg/kg). Cardiac function was measured with echocardiogram. The maximal EF and FS after ISO treatment were plotted ($n = 5$–11). (*C*–*E*) AVMs isolated from LFD, HFD-Veh, HFD-Carv, HFD-Meto mice were treated with ISO (100 nM). The changes in contractile function, amplitude of calcium transient, and calcium decay Tau were measured in the same cell. Data were presented as mean value of each mouse and from total five mice per group. (*F* and *G*) TUNEL staining was performed on heart sections from LFD-, HFD-Veh, HFD-Carv, and HFD-Meto mice. The average percentage of TUNEL-positive myocytes from each heart was quantified and presented. Data were from five mice (scale bar, 20 μM). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ by one-way ANOVA followed by *post hoc* Tukey's test. (*H*) Working model of carvedilol induced β_1AR-NOS3-cGMP signalling pathway. Carvedilol transduces biased β_1AR signalling towards the G_i-PI3K-AKT pathway, which promotes the NOS3-NO-cGMP signal, facilitating PKG-dependent substrate phosphorylation and cardiac contractility, which is compared to isoproterenol induced cAMP-PKA-dependent substrate phosphorylation and cardiac contractility.

myocyte contractility.[34,45] Genetic deletion of NOS3 completely abolishes the effects of carvedilol in animal hearts *in vivo*. Future studies with cardiac specific deletion of NOS3 will help to clarify the direct contribution of cardiac NOS3 in promoting the inotropic effects of carvedilol in hearts. The observed β_1AR-NOS3 signalling is also distinct from a recent report that ISO induces β_1AR-NOS1-dependent increases in CaMKII activity in hearts.[46] Mechanistically, our data show carvedilol induces limited cAMP-PKA activity within the PM, which is spatially segregated from NOS1 enriched in the SR. In comparison, NOS3 is enriched at the PM and can be readily activated by G_i-PI3K-Akt signalling.[25] Therefore, carvedilol can block the detrimental NOS1-CaMKII activity induced by sympathetic drive while promoting beneficial NOS3-NO-cGMP activity. Moreover, we observed a decreased calcium transient amplitude and faster decay Tau in myocytes from HFD mice with carvedilol therapy. These observations are associated with elevation of phosphorylation of Akt and NOS3 as well as proteins involved in calcium handling such as PLB and RyR2. These changes could also prime myocytes to respond to sympathetic drive to increase contractility without elevation of calcium transient amplitude and decay Tau in diseased hearts. The absence of elevation in calcium signalling could further protect cardiomyocytes from calcium overload and cell apoptosis, which could have significant ramifications in diseased hearts during long-term therapy. In comparison, metoprolol exerts similar cardiac protective effects to HFD-induced heart dysfunction *in vivo*. However, the improved function of myocytes treated by metoprolol is accompanied with elevated calcium transient amplitude and a faster declining time to the calcium transient. The effect of metoprolol on calcium handling accounts for the negligible anti-apoptotic effect comparing with carvedilol. Moreover, the profiles of myofilament protein modifications in HFD hearts after carvedilol and metoprolol treatment are distinct, in which carvedilol promotes phosphorylation of MyBP-C whereas metoprolol primarily increases the activation of TnI. The mechanical difference between both β-blockers in treating HF may be relevant to the difference in calcium handling and cGMP production.

5. Conclusion

While β-blockers generally attenuate detrimental β_1AR-cAMP-CaMKII signalling under sympathetic drive, carvedilol also induces additional cardioprotective β_1AR-cGMP-PKG signalling *in vivo*. These findings may lend understanding to the controversial findings of the COMET trial, where carvedilol had a significant survival advantage over metoprolol in the treatment of HF.[47,48] Together, our data indicate that the biased β_1AR-cGMP-PKG signalling may be targeted to improve cardiac ejection depression and protect cardiac myocytes against apoptosis in cardiac diseases.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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References

1. Xiang Y, Kobilka BK. Myocyte adrenoceptor signaling pathways. *Science* 2003;**300**:1530–1532.
2. Catterall WA. Regulation of cardiac calcium channels in the fight-or-flight response. *CMP* 2015;**8**:12–21.
3. Mason RP, Jacob RF, Corbalan JJ, Szczesny D, Matysiak K, Malinski T. The favorable kinetics and balance of nebivolol-stimulated nitric oxide and peroxynitrite release in human endothelial cells. *BMC Pharmacol Toxicol* 2013;**14**:48.
4. Refaie MMM, El-Hussieny M, Bayoumi AMA, Shehata S. Mechanisms mediating the cardioprotective effect of carvedilol in cadmium induced cardiotoxicity. Role of eNOS and HO1/Nrf2 pathway. *Environ Toxicol Pharmacol* 2019;**70**:103198.
5. Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 1997;**390**:88–91.
6. Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci USA* 2001;**98**:1607–1612.
7. 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.
8. Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, Ahles A, Engelhardt S, Rockman HA. Galphai is required for carvedilol-induced beta1 adrenergic receptor beta-arrestin biased signaling. *Nat Commun* 2017;**8**:1706.
9. Xiang Y, Devic E, Kobilka B. The PDZ binding motif of the beta 1 adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. *J Biol Chem* 2002;**277**:33783–33790.
10. Teoh JP, Bayoumi AS, Aonuma T, Xu Y, Johnson JA, Su H, Weintraub NL, Tang Y, Kim IM. β-arrestin-biased agonism of β-adrenergic receptor regulates Dicer-mediated microRNA maturation to promote cardioprotective signaling. *J Mol Cell Cardiol* 2018;**118**:225–236.
11. Bayoumi AS, Park KM, Wang Y, Teoh JP, Aonuma T, Tang Y, Su H, Weintraub NL, Kim IM. A carvedilol-responsive microRNA, miR-125b-5p protects the heart from acute myocardial infarction by repressing pro-apoptotic bak1 and klf13 in cardiomyocytes. *J Mol Cell Cardiol* 2018;**114**:72–82.
12. Peitzman ER, Zaidman NA, Maniak PJ, O'Grady SM. Agonist binding to β-adrenergic receptors on human airway epithelial cells inhibits migration and wound repair. *Am J Physiol Cell Physiol* 2015;**309**:C847–C855.
13. Barbagallo F, Xu B, Reddy GR, West T, Wang Q, Fu Q, Li M, Shi Q, Ginsburg KS, Ferrier W, Isidori AM, Naro F, Patel HH, Bossuyt J, Bers D, Xiang YK. Genetically encoded biosensors reveal PKA hyperphosphorylation on the myofilaments in rabbit heart failure. *Circ Res* 2016;**119**:931–943.
14. Shi Q, Li M, Mika D, Fu Q, Kim S, Phan J, Shen A, Vandecasteele G, Xiang YK. Heterologous desensitization of cardiac beta-adrenergic signal via hormone-induced betaAR/arrestin/PDE4 complexes. *Cardiovasc Res* 2017;**113**:656–670.
15. Liu S, Li Y, Kim S, Fu Q, Parikh D, Sridhar B, Shi Q, Zhang X, Guan Y, Chen X, Xiang YK. Phosphodiesterases coordinate cAMP propagation induced by two stimulatory G protein-coupled receptors in hearts. *Proc Natl Acad Sci USA* 2012;**109**:6578–6583.
16. Wang Q, Liu Y, Fu Q, Xu B, Zhang Y, Kim S, Tan R, Barbagallo F, West TM, Anderson EJ, Wei W, Abel ED, Xiang YK. Inhibiting insulin-mediated β2AR activation prevents diabetes-associated cardiac dysfunction. *Circulation* 2017;**135**:73–88.
17. West TM, Wang Q, Deng B, Zhang Y, Barbagallo F, Reddy GR, Chen D, Phan KS, Xu B, Isidori A, Xiang YK. Phosphodiesterase 5 associates with β2 adrenergic receptor to modulate cardiac function in type 2 diabetic hearts. *J Am Heart Assoc* 2019;**8**:e012273.

18. Soto D, De Arcangelis V, Zhang J, Xiang Y. Dynamic protein kinase A activities induced by beta-adrenoceptors dictate signaling propagation for substrate phosphorylation and myocyte contraction. *Circ Res* 2009;**104**:770–779.
19. Russwurm M, Mullershausen F, Friebe A, Jager R, Russwurm C, Koesling D. Design of fluorescence resonance energy transfer (FRET)-based cGMP indicators: a systematic approach. *Biochem J* 2007;**407**:69–77.
20. Vanzelli AS, Medeiros A, Rolim N, Bartholomeu JB, Cunha TF, Bechara LR, Gomes ER, Mattos KC, Sirvente R, Salemi VM, Mady C, Negrao CE, Guatimosim S, Brum PC. Integrative effect of carvedilol and aerobic exercise training therapies on improving cardiac contractility and remodeling in heart failure mice. *PLoS One* 2013;**8**: e62452.
21. Pitari GM, Cotzia P, Ali M, Birbe R, Rizzo W, Bombonati A, Palazzo J, Solomides C, Shuber AP, Sinicrope FA, Zuzga DS. Vasodilator-stimulated phosphoprotein biomarkers are associated with invasion and metastasis in colorectal cancer. *Biomark Cancer* 2018;**10**:1179299X1877455.
22. Thoonen R, Giovanni S, Govindan S, Lee DI, Wang GR, Calamaras TD, Takimoto E, Kass DA, Sadayappan S, Blanton RM. Molecular screen identifies cardiac myosin-binding protein-C as a protein kinase G-lalpha substrate. *Circ Heart Fail* 2015;**8**: 1115–1122.
23. Zhang YH, Jin CZ, Jang JH, Wang Y. Molecular mechanisms of neuronal nitric oxide synthase in cardiac function and pathophysiology. *J Physiol* 2014;**592**:3189–3200.
24. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* 2002;**416**:337–339.
25. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;**399**:601–605.
26. Sawangkoon S, Miyamoto M, Nakayama T, Hamlin RL. Acute cardiovascular effects and pharmacokinetics of carvedilol in healthy dogs. *Am J Vet Res* 2000;**61**:57–60.
27. DasGupta P, Broadhurst P, Lahiri A. The effects of intravenous carvedilol, a new multiple action vasodilatory beta-blocker, in congestive heart failure. *J Cardiovasc Pharmacol* 1991;**18**:S12–S16.
28. Kurosaki K, Ikeda U, Maeda Y, Shimada K. Carvedilol stimulates nitric oxide synthesis in rat cardiac myocytes. *J Mol Cell Cardiol* 2000;**32**:333–339.
29. Hadi T, Barrichon M, Mourtialon P, Wendremaire M, Garrido C, Sagot P, Bardou M, Lirussi F. Biphasic Erk1/2 activation sequentially involving Gs and Gi signaling is required in beta3-adrenergic receptor-induced primary smooth muscle cell proliferation. *Biochim Biophys Acta* 2013;**1833**:1041–1051.
30. Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ, Lakatta EG. Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes. *Circ Res* 1999;**84**:43–52.
31. Shen A, Chen D, Kaur M, Bartels P, Xu B, Shi Q, Martinez JM, Man K-N. M, Nieves-Cintron M, Hell JW, Navedo MF, Yu X-Y, Xiang YK. Xiang YK. β-blockers augment L-type Ca2+ channel activity by targeting spatially restricted β2AR signaling in neurons. *Elife* 2019;**8**:e49464.
32. Gupta S, Wright HM. Nebivolol: a highly selective beta1-adrenergic receptor blocker that causes vasodilation by increasing nitric oxide. *Cardiovasc Ther* 2008;**26**:189–202.
33. 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.
34. Ryba DM, Li J, Cowan CL, Russell B, Wolska BM, Solaro RJ. Long-term biased beta-arrestin signaling improves cardiac structure and function in dilated cardiomyopathy. *Circulation* 2017;**135**:1056–1070.
35. Carr R 3rd, Schilling J, Song J, Carter RL, Du Y, Yoo SM, Traynham CJ, Koch WJ, Cheung JY, Tilley DG, Benovic JL. beta-arrestin-biased signaling through the beta2-adrenergic receptor promotes cardiomyocyte contraction. *Proc Natl Acad Sci USA* 2016;**113**:E4107–E4116.
36. Lee DI, Vahebi S, Tocchetti CG, Barouch LA, Solaro RJ, Takimoto E, Kass DA. PDE5A suppression of acute beta-adrenergic activation requires modulation of myocyte beta-3 signaling coupled to PKG-mediated troponin I phosphorylation. *Basic Res Cardiol* 2010;**105**:337–347.
37. Ponnam S, Sevrieva I, Sun YB, Irving M, Kampourakis T. Site-specific phosphorylation of myosin binding protein-C coordinates thin and thick filament activation in cardiac muscle. *Proc Natl Acad Sci USA* 2019;**116**:15485–15494.
38. Sadayappan S, Gulick J, Osinska H, Barefield D, Cuello F, Avkiran M, Lasko VM, Lorenz JN, Maillet M, Martin JL, Brown JH, Bers DM, Molkentin JD, James J, Robbins J. A critical function for Ser-282 in cardiac myosin binding protein-C phosphorylation and cardiac function. *Circ Res* 2011;**109**:141–150.
39. Surdo NC, Berrera M, Koschinski A, Brescia M, Machado MR, Carr C, Wright P, Gorelik J, Morotti S, Grandi E, Bers DM, Pantano S, Zaccolo M. FRET biosensor uncovers cAMP nano-domains at β-adrenergic targets that dictate precise tuning of cardiac contractility. *Nat Commun* 2017;**8**:15031.
40. Subramanian H, Froese A, Jonsson P, Schmidt H, Gorelik J, Nikolaev VO. Distinct submembrane localisation compartmentalises cardiac NPR1 and NPR2 signalling to cGMP. *Nat Commun* 2018;**9**:2446.
41. Perera RK, Sprenger JU, Steinbrecher JH, Hubscher D, Lehnart SE, Abesser M, Schuh K, El-Armouche A, Nikolaev VO. Microdomain switch of cGMP-regulated phosphodiesterases leads to ANP-induced augmentation of beta-adrenoceptor-stimulated contractility in early cardiac hypertrophy. *Circ Res* 2015;**116**:1304–1311.
42. Zaccolo M. cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. *Br J Pharmacol* 2009;**158**:50–60.
43. Woo AY, Song Y, Zhu W, Xiao RP. Advances in receptor conformation research: the quest for functionally selective conformations focusing on the beta2-adrenoceptor. *Br J Pharmacol* 2015;**172**:5477–5488.
44. Yang XQ, Xiong T, Wang DX. The potential multiple effects of beta3-adrenergic receptor agonists on the heart protection for patients with heart failure with preserved ejection fraction. *Eur Heart J* 2018;**39**:4287.
45. Jin CZ, Jang JH, Kim HJ, Wang Y, Hwang IC, Sadayappan S, Park BM, Kim SH, Jin ZH, Seo EY, Kim KH, Kim YJ, Kim SJ, Zhang YH. Myofilament Ca2+ desensitization mediates positive lusitropic effect of neuronal nitric oxide synthase in left ventricular myocytes from murine hypertensive heart. *J Mol Cell Cardiol* 2013;**60**:107–115.
46. Dries E, Santiago DJ, Johnson DM, Gilbert G, Holemans P, Korte SM, Roderick HL, Sipido KR. Calcium/calmodulin-dependent kinase II and nitric oxide synthase 1-dependent modulation of ryanodine receptors during β-adrenergic stimulation is restricted to the dyadic cleft. *J Physiol* 2016;**594**:5923–5939.
47. Poole-Wilson PA, Swedberg K, Cleland JG, Di Lenarda A, Hanrath P, Komajda M, Lubsen J, Lutiger B, Metra M, Remme WJ, Torp-Pedersen C, Scherhag A, Skene A; Carvedilol or metoprolol European trial. Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the carvedilol or metoprolol European trial (COMET): randomised controlled trial. *Lancet* 2003; **362**:7–13.
48. Ajam T, Ajam S, Devaraj S, Mohammed K, Sawada S, Kamalesh M. Effect of carvedilol vs metoprolol succinate on mortality in heart failure with reduced ejection fraction. *Am Heart J* 2018;**199**:1–6.

Translational perspective

Carvedilol transduces β_1 adrenergic receptor (β_1AR) coupling to G_i-PI3K-Akt pathway to promote NOS3-cGMP-PKG signal and evokes cardiac inotropy with minimal elevation of calcium signalling. Chronic therapy with carvedilol not only restores cardiac contractility and adrenergic reserves but also leads to reduction of cardiac remodelling with less cell death than metoprolol. These results may explain at least in part that carvedilol had a significant survival advantage over metoprolol in the treatment of heart failure in clinical studies. The carvedilol-induced β_1AR-NOS3 axis represents a novel regulatory paradigm to increase cardiac inotropy, which can be a promising therapeutic target for treating cardiac diseases with depressed cardiac ejection fraction.