β1-Blockade Prevents Post-Ischemic Myocardial Decompensation Via β3AR-Dependent Protective Sphingosine-1 Phosphate Signaling

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ABSTRACT

BACKGROUND Although β-blockers increase survival in patients with heart failure (HF), the mechanisms behind this protection are not fully understood, and not all patients with HF respond favorably to them. We recently showed that, in cardiomyocytes, a reciprocal down-regulation occurs between β1-adrenergic receptors (ARs) and the cardioprotective sphingosine-1-phosphate (S1P) receptor-1 (S1PR1).

OBJECTIVES The authors hypothesized that, in addition to salutary actions due to direct β1AR-blockade, agents such as metoprolol (Meto) may improve post-myocardial infarction (MI) structural and functional outcomes via restored S1PR1 signaling, and sought to determine mechanisms accounting for this effect.

METHODS We tested the in vitro effects of Meto in HEK293 cells and in ventricular cardiomyocytes isolated from neonatal rats. In vivo, we assessed the effects of Meto in MI wild-type and β3AR knockout mice.

RESULTS Here we report that, in vitro, Meto prevents catecholamine-induced down-regulation of S1PR1, a major cardiac protective signaling pathway. In vivo, we show that Meto arrests post-MI HF progression in mice as much as chronic S1P treatment. Importantly, human HF subjects receiving β1AR-blockers display elevated circulating S1P levels, confirming that Meto promotes S1P secretion/signaling. Mechanistically, we found that Meto-induced S1P secretion is β3AR-dependent because Meto infusion in β3AR knockout mice does not elevate circulating S1P levels, nor does it ameliorate post-MI dysfunction, as in wild-type mice.

CONCLUSIONS Our study uncovers a previously unrecognized mechanism by which β1-blockers prevent HF progression in patients with ischemia, suggesting that β3AR dysfunction may account for limited/null efficacy in β1AR-blocker-insensitive HF subjects. (J Am Coll Cardiol 2017;70:182-92)
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ignaling through myocyte G-protein-coupled receptors (GPCRs), particularly β-adrenergic receptors (β1, β2, and β3ARs), regulates the rate and force of contraction in response to increased workload [1]. However, after myocardial infarction (MI), this signaling is profoundly altered, and cardiac βAR dysregulation and desensitization largely account for chronic post-MI decompensation [2]. Accordingly, βAR-blockers attenuate the noxious effects of sympathetic catecholamines on the heart and prevent further βAR down-regulation. These compounds remain crucial to improving survival and preventing further left ventricular (LV) decompensation in patients who have experienced an ischemic event [1,3]. Clinical β-blocker use is unequivocally proven to reduce oxygen consumption, prevent cardiac adverse remodeling, blunt cardiomyocyte apoptosis, inhibit βAR down-regulation, and reduce the risk of fatal arrhythmias [4]. Yet, whether correcting βAR molecular perturbations justifies all mechanisms of β-blocker-induced cardioprotection in heart failure (HF) is a relevant question, as additional mechanisms appear to participate. Moreover, because not all patients respond to β-blockers, the overall clinical experience raises the question of how exactly β-blockers attenuate progression of HF after MI. Furthermore, delineating whether distinct, clinically useful β-blockers have different mechanisms may explain specific patient responses and lead to fine-tuning of personalized HF therapy.

β-Blockers, such as alprenolol and carvedilol, act as classical receptor antagonists. However, they can also stimulate signaling pathways in a G protein-independent fashion [5]. Moreover, selective β1AR-blockers, such as metoprolol (Meto) or nebivolol, can also promote β3AR up-regulation and/or activity in the heart, thus enhancing the cardioprotective signaling of this βAR subtype [6-8]. Thus, answering these mechanistic questions is important, and may help tailor β-blockade therapies for patients with HF. Herein, we evaluated the role of sphingosine-1-phosphate receptor 1 (S1PR1), a key GPCR expressed in the heart, which mediates the cardioprotective effect of sphingosine-1-phosphate (S1P), a natural lysophospholipid that appears tightly linked to β1AR-signaling [9,10]. In fact, β1AR down-regulation can occur after S1P stimulation, whereas S1PR1 down-regulation can be triggered by isoproterenol (ISO) treatment [9]. This direct receptor cross-talk is also physiologically relevant, as these GPCRs interact and show reciprocal down-regulation in a rat model of post-MI HF [9]. For these reasons, we hypothesize that, aside from the protective effects of β-blockade against catecholamine damage or βAR regulation in the myocardium [11], there are additional beneficial effects involving S1P and S1PR1 signaling.

METHODS

AGONISTS AND INHIBITORS.
S1P was purchased from Cayman Chemicals (26993-30-6, Ann Arbor, Michigan); ISO (I6504), BRL 37344 (BRL, B169), and Meto (M5391) were all purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri). For additional information please see the Methods section in the Online Appendix.

CELL CULTURE AND TRANSFECTION.
Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from the hearts of 1- to 2-day-old rats, as previously described [12]. HEK293 cells obtained from the American Type Culture Collection (Manassas, Virginia) were transfected as previously described [9]. For additional information please see the Methods section in the Online Appendix.

CELL FRACTIONATION AND WESTERN BLOT ANALYSIS.
Immunoblotting was performed as previously described [12]. Protein levels of S1PR1 (Y080010, ABM Inc., Richmond, Canada; 1: 1,000), sphingosine kinase-1 (SphK1; sc-48825, Santa Cruz Biotechnology, Dallas, Texas; 1: 1,000), SphK2 (ab37977, Abcam, Cambridge, Massachusetts, 1: 1,000), β3AR (PAB8502, Abnova, Walnut, California, 1: 1,000); glyceraldehyde-3-phosphate dehydrogenase (sc-32233, 6C5, Santa Cruz Biotechnology, Dallas, Texas, 1: 2,000) were assessed. Plasma membrane proteins were isolated from NRVMs, as previously described [12].

ANIMAL MODELS AND EXPERIMENTAL PROCEDURES.
All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University School of Medicine. For in vivo experiments, we used wild-type C57BL/6 mice and global β3AR knockout (KO) mice [13]. All animals (females and males, 9 weeks to 12 weeks of age) were bred and maintained on a C57BL/6 background.

The β3AR KO genotype was assessed using specific primers: wild-type (WT) forward 5' GTTGCGAACTGTGGACGTCAGTGG 3'; KO forward 5' CGCATCGCCTTCTATCGCCTTCTTG 3'; and KO/WT reverse (common) 5' AATGCCGTTGGCGCTTAGCCAC 3'.

Surgically induced MI was performed as previously described [14]. Seven days post-MI, mice were randomly assigned to 1 of the following groups: MI;

FIGURE 1   Meto Abolishes β_1AR-Dependent Sarcolemmal S1PR$_1$ Internalization
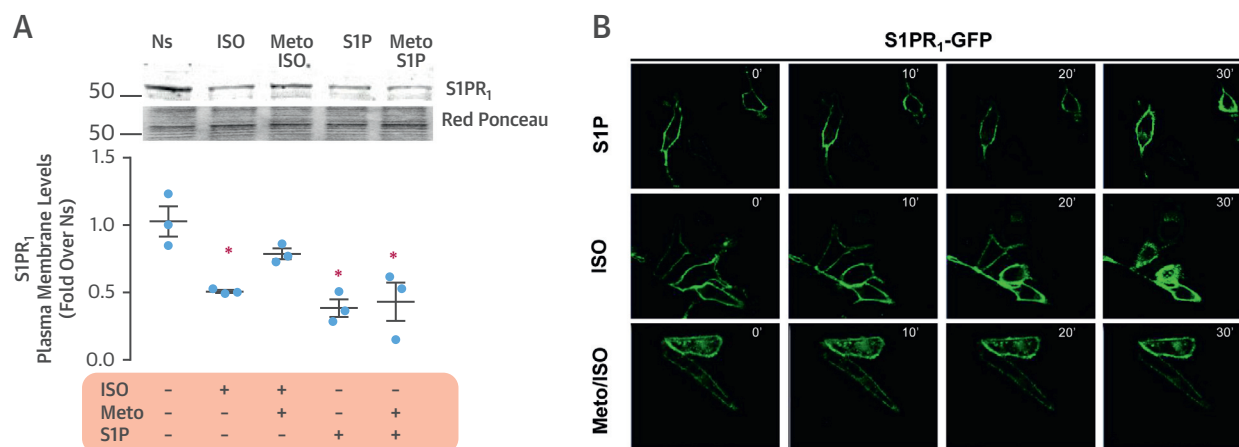
(A) Representative immunoblots **(upper panels)** and densitometric quantitative analysis **(lower panel)** of multiple (n = 3) independent experiments to evaluate S1PR$_1$ in crude plasma membrane preparations from NRVMs. Cells were nonstimulated (Ns) or stimulated with ISO (1 µmol/l) or S1P (250 nmol/l) for 30 min. Before ISO or S1P stimulation, a group of cells was pre-treated with Meto (10 µmol/l) for 30 min. Ponceau Red staining was used to assess total protein loading. *p < 0.05 versus NS **(B)** Representative immunofluorescence images (scale bar: 10 µm) of HEK293 cells overexpressing β_1AR-FLAG and S1PR$_1$-GFP **(green)**. S1PR$_1$-GFP internalization over a 30-min time course (0 min to 30 min) in cells treated with ISO (1 µmol/l) or S1P (250 nmol/l) is shown. Before ISO stimulation, a group of cells was pre-treated with Meto (10 µmol/l for 30 min). β_1AR = beta-1 adrenergic receptor; FLAG = fludarabine, ara-C, and filgrastim; GFP = green fluorescent protein; ISO = isoproterenol; Meto = metoprolol; NRVM = neonatal rat ventricular myocytes; S1P = sphingosine-1-phosphate; S1PR$_1$ = sphingosine-1-phosphate receptor-1.

MI + Meto; MI + S1P; and MI + Meto/S1P. Meto was administered in drinking water (250 mg/kg/day) (15), whereas S1P (10 µmol/l) was dissolved in phosphate-buffered saline and was continuously infused subcutaneously into mice via an osmotic mini-pump (ALZET, DURECT Co., Cupertino, California) (16). Transthoracic echocardiography was performed to assess cardiac structure and function at baseline, 7 days, and 4 weeks post-MI, using a VisualSonics VeVo 2100 system (VisualSonics, Toronto, Canada), as previously described (12).

HISTOLOGY. Cardiac-specimens were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and rehydration, 5-µm-thick sections were prepared, mounted on glass slides, and stained with 1% Sirius Red in picric acid (Sigma-Aldrich, St. Louis, Missouri) to detect interstitial fibrosis, as previously described (12). Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) was performed as previously described (12). For additional information please see the Methods section in the Online Appendix.

ANALYSIS AND STATISTICS. All procedures in mice were performed in a blinded fashion. Moreover, all LV tissue and blood samples derived from them were also coded, and the operators were blind to these codes until data quantification was complete. Data are expressed as mean ± SE. Statistical significance was determined by a Student *t* test or Mann-Whitney *U* test when sample size was <10. For multiple comparisons, 1-way analysis of variance, followed by Bonferroni post-hoc correction, was performed. All data were analyzed using GraphPad Prism software version 6 (GraphPad Software, La Jolla, California).

RESULTS

METO PREVENTS ISO-INDUCED IN VITRO S1PR1 MEMBRANE DOWN-REGULATION. The βAR agonist ISO induces robust internalization and/or desensitization of the S1PR$_1$, leading to inhibition of its protective signaling, which is dependent on down-regulation of β_1AR (9,10). Here, we tested whether selective β_1AR-blockade via Meto affects this reciprocal β_1AR-S1PR$_1$ down-regulation. To this end, we first treated neonatal rat ventricular myocytes (NRVMs) with ISO (1 µmol/l) and S1P (250 nmol/l) (each for 30 min), which resulted in a robust S1PR$_1$ plasma membrane down-regulation (**Figure 1A**). Next, we pre-treated NRVMs with Meto (10 µmol/l) for 30 min, and then stimulated them with ISO (1 µmol/l)

FIGURE 2 Meto Ameliorates MI-Induced Cardiac Dysfunction and S1PR₁ Down-Regulation
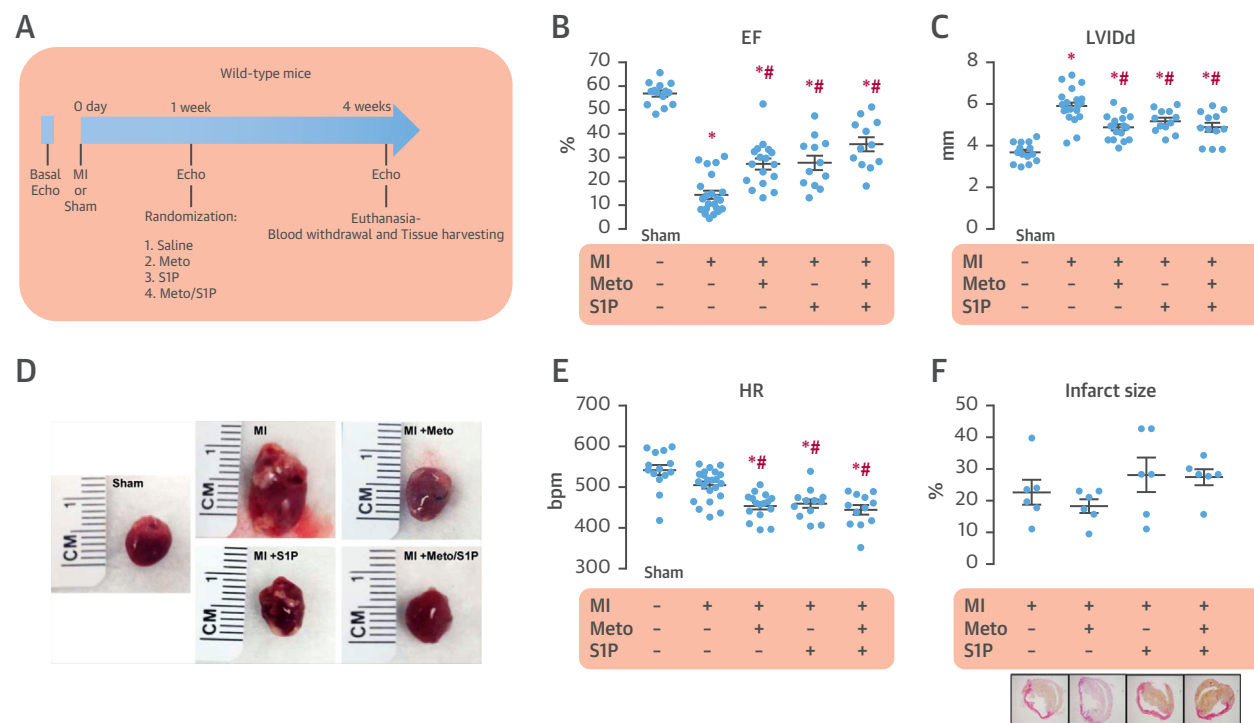
(A) Overall design of 4-week study of MI in mice. Echocardiography was performed at day 0 and then MIs were surgically induced. Sham-operated mice were used as controls. After 1 week, echocardiography was performed to assess that all mice had similar cardiac dysfunction. Mice (total n = 12 to 22 per group) were randomized for treatments: MI (controls), MI + Meto (250 mg/kg/day), MI + S1P (10 μmol/l) and MI + Meto/S1P. Four weeks post-MI (3 weeks post-treatment), echocardiography was performed and animals euthanized for tissue harvesting. **(B to C)** Dot plots showing the echocardiographic analysis performed at the end of the study period (4 weeks post-MI) of **(B)** left ventricular ejection fraction (EF, %) and **(C)** left ventricular internal diameter at diastole (LVIDd) of individual mice from each of the groups. **(D)** Representative images of whole hearts from mice. **(E)** Dot plots showing echocardiographic analysis of heart rate of individual mice from the each of the groups. **(F)** Dot plots **(upper panel)** and representative panels of Picrosirius red staining (lower panels; scale bar: 500 μm) showing the percentage of infarct size in the MI, MI + Meto, MI + S1P, and MI + Meto/S1P mice (n = 6). For **B, C, and E,** *p <0.05 versus sham; #p <0.05 versus MI. Echo = echocardiogram; MI = myocardial infarction; other abbreviations as in **Figure 1**.

or S1P (250 nmol/l) for an additional 30 min. Pre-treatment of cells with Meto prevented ISO-, but not S1P-induced S1PR₁ down-regulation (**Figure 1A**).

To confirm these observations, we used HEK293 cells over-expressing both (FLAG)-tagged β₁AR (β₁AR-FLAG) and green fluorescent protein tagged S1PR₁ (S1PR₁-GFP) (Online Figure 1) and checked for S1PR₁ internalization. Using confocal microscopy, we performed a time course evaluation (0 min to 30 min), assessing the impact of Meto on ISO- and S1P-induced S1PR₁ internalization. In line with the NRVM data, we observed that ISO and S1P induced massive receptor internalization, and that Meto prevented ISO-induced effects (**Figure 1B**). Together, these data indicate that β₁AR-blockade

prevents β₁-dependent S1PR₁ down-regulation in vitro.

BOTH METO AND S1P IMPROVE CONTRACTILITY AND PREVENT LV REMODELING AFTER MI. For in vivo support of the findings described in the preceding section, we used a mouse MI model because both β₁AR and S1PR₁ are down-regulated in infarcted myocardium (9). Five groups of randomized mice were studied: 1) sham-operated; 2) untreated MI; 3) MI treated with Meto (250 mg/kg/day); 4) MI treated with S1P (10 μmol/l); and 5) MI treated with both Meto and S1P (Meto + S1P). LV function and dimensions were evaluated by echocardiography before MI, and then 1 (Online Figures 2A and 2B) and 4 weeks after MI (study termination) (**Figure 2A**). Four weeks after

FIGURE 3 Effects of Meto and S1P on Post-Ischemic Cardiac Fibrosis and Apoptosis
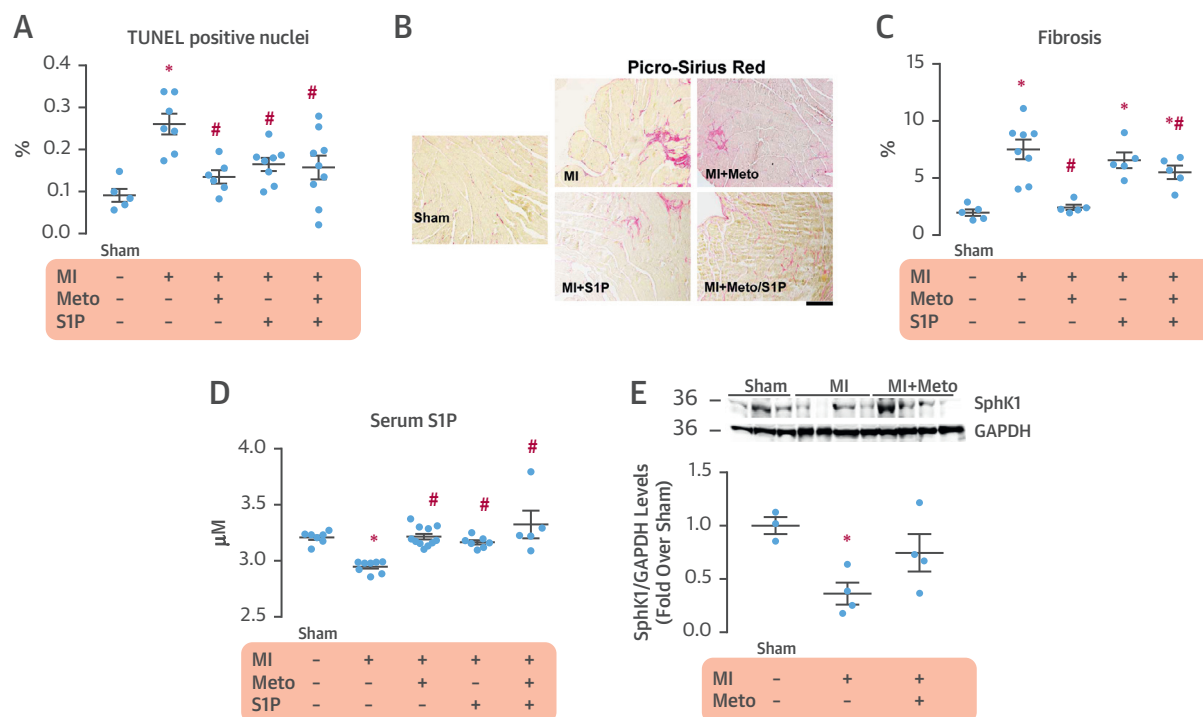
(A) Dot plots showing myocyte cell death via TUNEL staining from the following groups (n = 5 to 9): sham; MI; MI + Meto (250 mg/kg/day); MI + S1P (10 μmol/l); and MI + Meto/S1P. **(B)** Representative images (scale bar: 200 μm) and **(C)** quantitative data showing the percentage of cardiac fibrosis via Picrosirius red staining from each of the groups (n = 5 to 8). **(D)** S1P serum levels measured by ELISA assay in each of the groups (n = 7 to 11). **(E)** Representative immunoblots **(upper panels)** and densitometric quantitative analysis **(lower panel)** of multiple independent (n = 3 to 4) experiments to evaluate SphK1 protein levels in total cardiac lysates from each of the groups. GAPDH levels were used for prot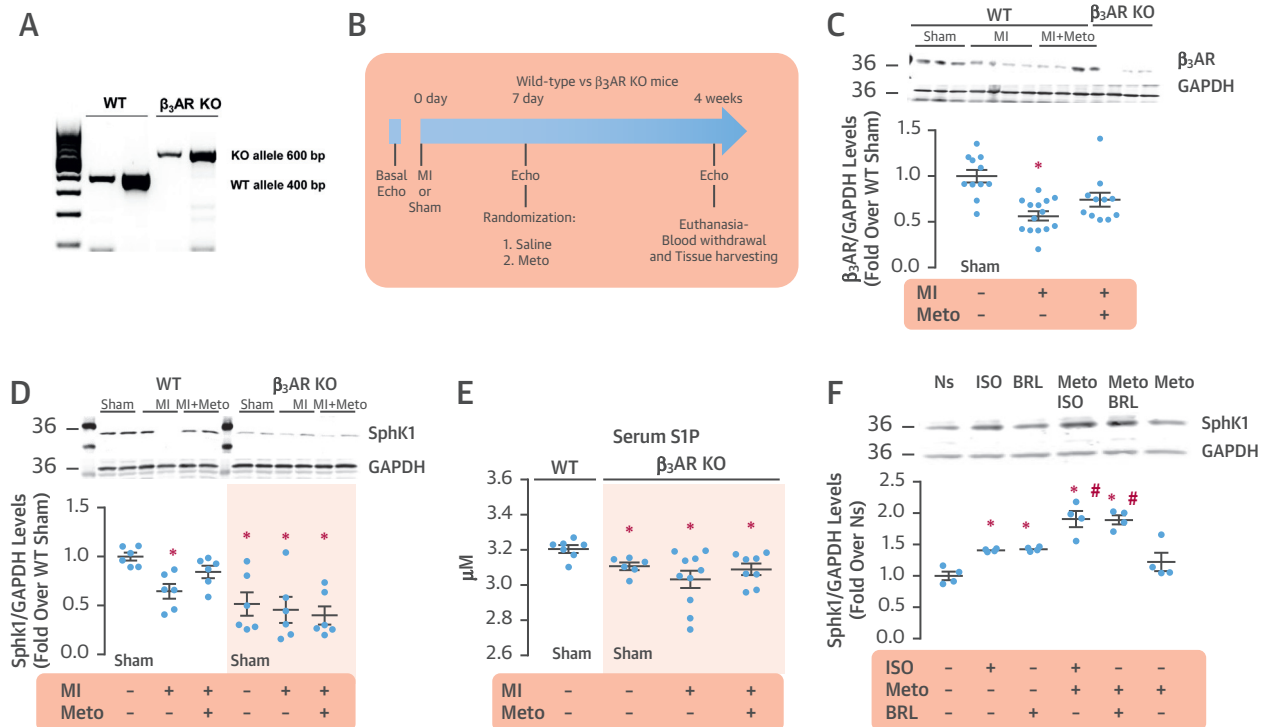ein loading controls. **A, C to E**, *p <0.05 versus sham; #p <0.05 versus MI. ELISA = enzyme-linked immunosorbent assay; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; SphK1 = sphingosine kinase-1; TUNEL = terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling; other abbreviations as in **Figures 1 and 2**.

MI, echocardiographic analysis revealed that LV ejection fraction (EF) was markedly decreased, and LV dimensions and dilation were substantially enlarged in untreated post-MI mice, as opposed to sham-operated animals (**Figures 2B to 2D**). However, Meto administration resulted in improved LV contractility (**Figure 2B**) and reduced LV dilation (**Figure 2C to 2D**), as compared with untreated post-MI mice. S1P treatment also significantly increased EF and reduced the LV diameter versus control MI mice (**Figure 2B to 2D**). Although Meto and S1P were compatible for coadministration, we did not observe any synergistic effects of these therapies (**Figures 2B to 2D**). Interestingly, Meto and S1P, either alone or in combination, decreased heart rate (**Figure 2E**). Of note, the percentage of infarct size (measured at the end of the study) was similar among all study groups (**Figure 2F**).

BOTH METO AND S1P REDUCE POST-MI CARDIAC APOPTOSIS AND FIBROSIS. Cardiac apoptosis and fibrosis are 2 major hallmarks of post-MI cardiac remodeling (12,17). Therefore, we compared the impact of Meto and/or S1P on these endpoints. As expected, cardiomyocyte cell death and collagen deposition were significantly increased in post-MI untreated mice versus sham animals (**Figures 3A and 3B**). These changes were accompanied by the up-regulation of profibrotic genes encoding connective tissue growth factor, and type 1 (Col1) and type 3 collagen (Col3) (Online Figures 3A to 3C). Importantly, Meto treatment induced a marked reduction of both cardiac fibrosis and apoptosis (**Figures 3A and 3B,** Online Figures 3A to 3C). Conversely, S1P treatment significantly prevented MI-dependent apoptosis (**Figure 3A**), but had no effect on cardiac fibrosis (**Figure 3B,** Online Figures 3A to 3C).

FIGURE 4 β₃AR-Dependent Expression/Activation of Cardiac SphK1 and S1P Secretion in MI Mice After Meto Treatment

(A) Representative genotype analyses of mice (in duplicate) with WT (400 bp), or deleted (600 bp) β₃AR alleles. **(B)** Overall design of 4-week study in WT and β₃AR KO mice. Echocardiography was performed at day 0, followed by surgically induced MI. Sham-operated mice were used as controls. After 1 week, echocardiography was performed to assess that all mice had similar cardiac dysfunction, and they were then randomized for treatments: MI (controls) and MI + Meto. **(C)** Representative immunoblots **(upper panels)** and densitometric quantitative analysis **(lower panel)** of multiple independent experiments (n = 10 to 13) to evaluate β₃AR protein levels in total cardiac lysates from the following groups of WT mice: sham; MI; and MI + Meto. Total cardiac lysates from β₃AR KO mice were used as negative controls. GAPDH levels were used for protein loading. *p <0.05 versus sham. **(D)** Representative immunoblots **(upper panels)** and densitometric quantitative analysis **(lower panel)** of multiple independent experiments (n = 6) to evaluate SphK1 protein levels in total cardiac lysates from the following groups: WT (sham, MI, and MI + Meto) and β₃AR KO (sham, MI, and MI + Meto). GAPDH levels were used for protein loading controls. *p <0.05 versus WT sham. **(E)** S1P blood serum levels measured by ELISA in the following groups (n = 7 to 10): WT (sham) and β₃AR KO (sham, MI, and MI + Meto). *p <0.05 versus WT sham. **(F)** Representative immunoblots **(upper panels)** and densitometric quantitative analysis **(lower panel)** of multiple (n = 4) independent experiments to evaluate SphK1 protein levels in NRVMs Ns or stimulated with ISO (1 μmol/l) or BRL (1 μmol/l) for 12. Before ISO or BRL stimulation a group of cells was pre-treated with Meto (10 μmol/l for 30 min). *p <0.05 versus Ns; #p <0.05 versus ISO. BRL = BRL 37344; KO = knockout; WT = wild-type; other abbreviations as in **Figures 1 to 3**.
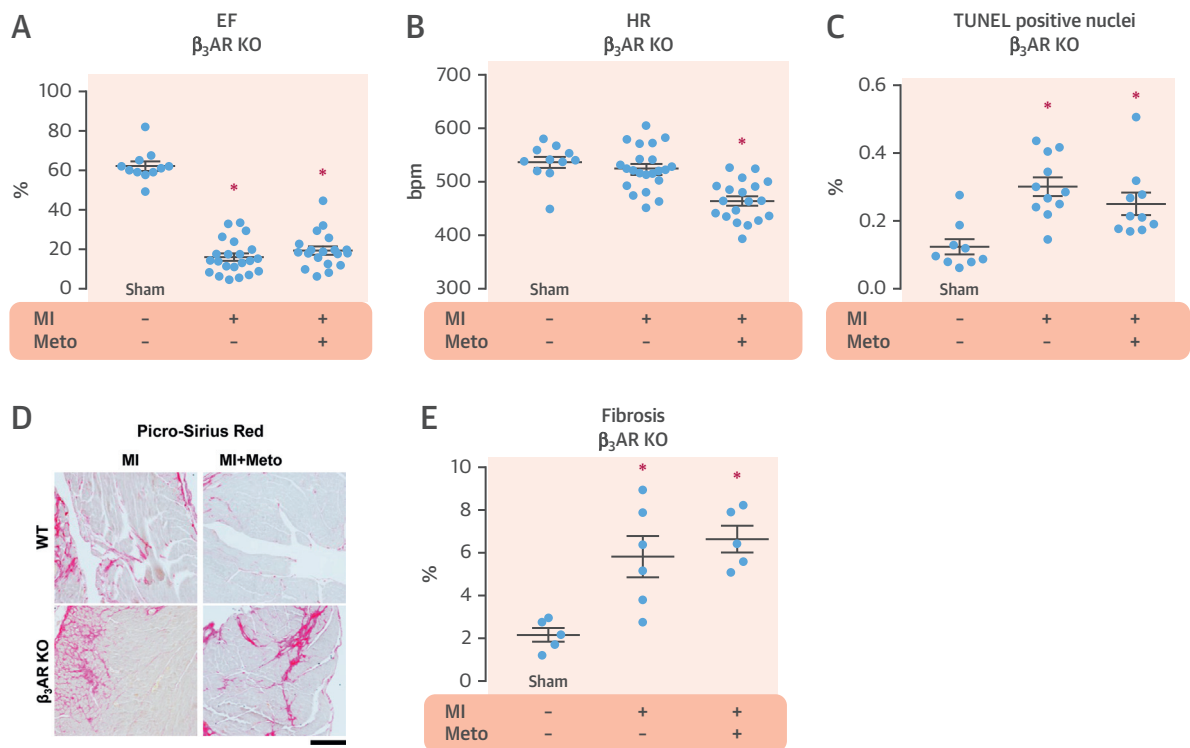
Moreover, combined Meto + S1P treatment did not improve upon Meto alone in preventing the MI-induced increase in apoptotic rate and fibrosis (**Figures 3A and 3B,** Online Figures 3A to 3C).

The lack of any additional effect of Meto + S1P compared to Meto alone prompted us to further investigate the effects of Meto and S1P. In particular, we measured circulating S1P serum levels in all of our study groups. We found that although S1P in serum was significantly reduced in MI untreated mice compared with sham, Meto treatment was able to restore circulating S1P to sham levels (**Figure 3D**). As expected, S1P administration normalized circulating S1P levels, both when administered alone or

with Meto (**Figure 3D**). Importantly, the impact of Meto on circulating S1P levels was paralleled by the rescued cardiac SphK1 expression (**Figure 3E**) and activity (Online Figure 4A). SphK1 is the enzyme involved in S1P production and consequent S1PR₁ activation (18). Of relevance, Meto obviated the S1P secretion deficiency found in post-MI WT mice (Online Figure 4B).

META RESCUES β₃AR-DEPENDENT SPHK1 EXPRESSION IN INFARCTED HEARTS. To date, there is no molecular mechanism explaining how β-blockers lead to S1P production. Normally, β₃ARs, which are expressed at low levels in the myocardium, can be up-regulated

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Metoprolol Requires β₃AR/S1P Signals

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FIGURE 5 Lack of Meto-Dependent Post-MI Therapeutic Response in β₃AR KO Mice

(A, B) Dot plots showing echocardiographic analysis of **(A)** left ventricular ejection fraction (EF, %) and **(B)** heart rates of individual mice from the following groups (n = 11 to 22): β₃AR KO (sham, MI, and MI + Meto). **(C)** Dot plots showing myocyte cell death via TUNEL staining from the following groups of mice (n = 5): β₃AR KO (sham, MI, and MI + Meto); **(D, E).** Representative images (scale bar: 200 μm) and quantitative data showing percentage of cardiac fibrosis via Picrosirius red staining from the β₃AR KO (sham, MI, and MI + Meto) mouse groups (n = 5). **A to C, E,** *p <0.05 versus sham. Abbreviations as in **Figures 1–4**.

and activated by Meto (6,19). Moreover, due to metabolic effects (1), the β₃AR is implicated in regulation of SphK1 expression and S1P release, without affecting SphK2 (20). Thus, we tested whether β₃AR signaling accounts for Meto-induced post-MI cardioprotection using β₃AR KO and WT mice. At 1 week after MI, all animal groups had similar LV dysfunction (Online Figures 5A and 5B). We then randomized mice to placebo or Meto treatment (**Figures 4A and 4B**). At the end of the study period, we found significant down-regulation of cardiac β₃AR protein levels in post-MI WT mice versus sham-operated animals (**Figure 4C**). Notably, Meto obviated this change (**Figure 4C**). As expected, β₃AR protein levels were not detectable in β₃AR KO mice (**Figure 4C**). Consistent with our previous studies (9), cardiac SphK1, SphK2 protein levels, and circulating S1P were all reduced after MI in WT mice with respect to sham (**Figures 4D and 4E,** Online Figure 6). Treatment with Meto restored SphK1 expression (**Figures 4D and 4E,** Online

Figure 6). Intriguingly, SphK1 expression and S1P secretion were very low in β₃AR KO mice, regardless of MI and/or Meto status. Of note, SphK2 expression was similar in WT and in β₃AR KO mice. In aggregate, this evidence strongly supports the contention that β₃AR per se plays a crucial role in basal expression levels of SphK1 in the heart (**Figures 4D and 4E**).

To confirm the role of β₃AR on SphK1 expression, we treated NRVMs with ISO (10 μmol/l) or with the β₃AR agonist BRL 37344 (BRL, 1 μmol/l) for 12 h. Before ISO or BRL stimulation, some groups of cells were also pre-treated with Meto (10 μmol/l) for 30 min. Both ISO and BRL were able to induce SphK1 up-regulation, as compared with control, nonstimulated (Ns) cells (**Figure 4F**). More importantly, Meto pre-treatment robustly enhanced the ability of ISO and BRL to increase SphK1 expression. Meto alone enhanced SphK1 at levels comparable to those found in Ns cells (**Figure 4F**). Thus, Meto rescues β₃AR-dependent SphK1 expression through

CENTRAL ILLUSTRATION β1AR-Blocker–Dependent Activation of β3AR/S1P Protective Signaling in Cardiomyocytes
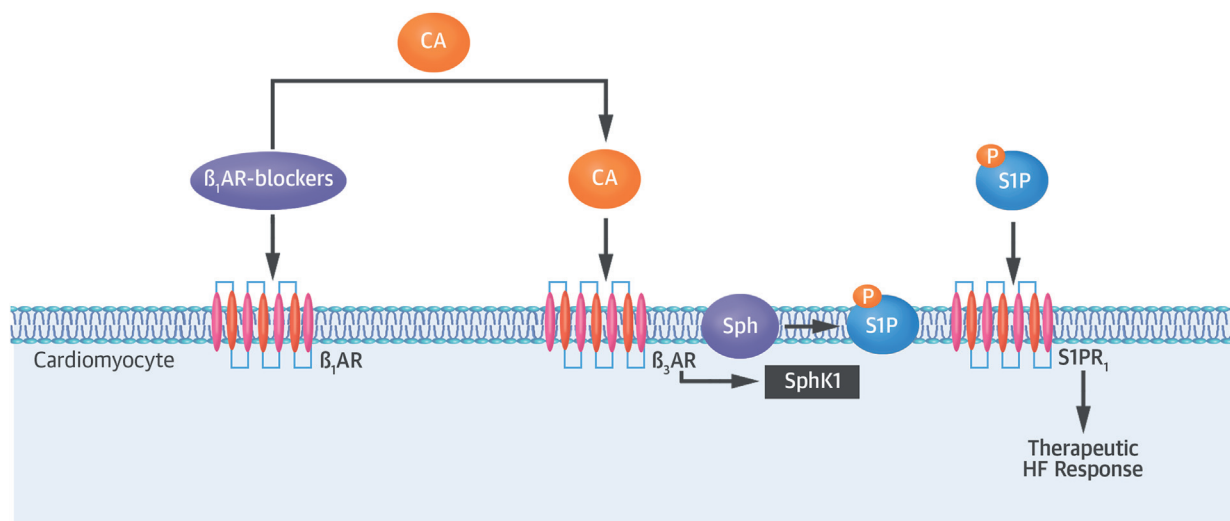
Cannavo, A. et al. J Am Coll Cardiol. 2017;70(2):182–92.

After binding with the β1AR, β1AR-blockers prevent direct catecholamine toxicity on the cardiac myocytes. Circulating catecholamines then bind to β3ARs, leading to up-regulation and activation of SphK1, which phosphorylates sphingosine (Sph). The S1P generated is then secreted outside the myocytes, and directly activates S1PR1 in an autocrine and paracrine fashion, which, in turn, induces a therapeutic response in heart failure. β1AR = beta-1 adrenergic receptor; β3AR = beta-3 adrenergic receptor; CA = catecholamine; HF = heart failure; S1P = sphingosine-1-phosphate; S1PR = sphingosine-1-phosphate receptor; SphK1 = sphingosine kinase-1.

direct β1AR blockade, making more catecholamines available for β3AR activation and inhibitory G protein (G_i) coupling. In fact, pre-treatment of NRVMs with the G_i inhibitor pertussis toxin (PTX, 100 ng/ml for 18 h) prevented SphK1 up-regulation due to ISO or BRL (Online Figure 7).

META-INDUCED IMPROVEMENT IN POST-MI HF DEPENDS ON BOTH β3AR AND S1PR1. The data presented so far suggests that S1PR1 resensitization and β3AR activation are important components of Meto-induced beneficial effects post-MI. To solidify this view, we compared the in vivo cardiac effects of Meto in post-MI WT and β3AR KO mice. Meto improved cardiac function in post-MI WT mice, but not β3AR KO mice (Figures 2B and 5A), despite a similar heart rate reduction in both groups (Figures 2E and 5B). Infarct sizes in β3AR KO mice were similar under any experimental conditions (Online Figure 8), and no differences in cardiac function were observed between WT and β3AR KO mice after MI (Figure 5A). Consistently, apoptosis and fibrosis were also similar in WT and β3AR KO mice after MI (Figures 3A to 3E). In contrast, whereas Meto reduced both of these endpoints in post-MI WT mice, it did not in β3AR KO mice (Figures 3A to 3C and 5C to 5E). Finally, to confirm the role of S1PR1 in

Meto-mediated cardioprotection, infarcted WT mice were treated with the S1PR1 antagonist W146. MI mice were randomized to 1 of the following groups: MI + vehicle (phosphate-buffered saline + ethanol); MI + Meto (250 mg/kg/day); MI + W146 (8 μg/ml) (21); and MI + Meto/W146. W146 was administered via mini-osmotic pumps, as done with S1P. As expected, Meto prevented LV dysfunction and adverse remodeling (Online Figure 9), and these beneficial actions were prevented by W146 (Online Figures 9A and 9B). Either vehicle or W146, taken alone had no sizable impact on post-MI decompensation. Thus, activation of the β3AR/S1PR1 axis accounts for the protection against MI afforded by Meto.

CHRONIC TREATMENT WITH META INCREASES CIRCULATING S1P LEVELS IN HUMAN HF PATIENTS. To add clinical relevance to our findings, we investigated whether β1-blocker treatment can affect circulating S1P levels in human patients with HF. Patients treated with β1AR antagonists and a similar HF group not treated with β-blockers (see Online Table 1 for specific patient characteristics) were used to asses S1P levels via an enzyme-linked immunosorbent assay (ELISA). Interestingly, clinical β1-blockade was characterized by significantly higher blood S1P levels

compared with the β-blocker-naïve control population (Online Figure 10).

DISCUSSION

Our study shows that the selective β_1-blocker Meto rescues $S1PR_1$-dependent signaling by preventing β_1AR-induced $S1PR_1$ down-regulation, which attenuates HF progression after MI in mice. This effect requires up-regulation of β_3AR signaling, which leads to increased circulating S1P levels and cardiac $S1PR_1$ stimulation, ultimately inducing pro-survival signals in the ischemic myocardium. Accordingly, the salutary actions of Meto are completely lost in β_3AR KO mice. Hence, the present study documents a previously unrecognized mechanism by which β_1-blockers, such as Meto, effectively improve survival and mitigate LV decompensation in mice with ischemic HF.

β_1-Blockers remain a forefront approach for treating acute or chronic HF of ischemic and nonischemic origin. They prevent direct cardiac catecholamine toxicity and promote βAR down-regulation in the failing heart, resulting in markedly reduced HF-related mortality [1]. However, β_1-blockers are a heterogeneous class of drugs, and some appear to attenuate HF progression through mechanisms additive to βAR resensitization and prevention of catecholamine-induced damage. Uncovering these unknown modalities of protection may help tailor βAR-blocker therapies for each HF patient. In the present study, we focused on β_1ARs and $S1PR_1$s, 2 distinct, highly-expressed cardiac GPCRs, whose cross-talk appears to have important pathophysiological repercussions [9]. In a rat model of post-MI HF, where S1P is significantly reduced and catecholamine secretion is increased, we previously found that β_1AR-hyperstimulation leads to marked $S1PR_1$ signaling dysregulation [9]. Therefore, in this study, we tested whether, among other salutary actions, β_1AR-blockers are also able to tip the balance to favor $S1PR_1$ protective signaling. Accordingly, we show that Meto pre-treatment of cardiomyocytes in vitro abolishes ISO-induced $S1PR_1$ down-regulation, whereas no effect is detectable in presence of S1P stimulation, confirming that β_1AR activation could lead to $S1PR_1$ transactivation. We validated these in vitro findings in a relevant in vivo HF model in mice after MI. We found that Meto treatment rescued LV function and prevented further adverse LV remodeling, restoring, at least in part, $S1PR_1$ cardiac plasma membrane levels. $S1PR_1$ mediates the effect of S1P, which is a natural cardioprotective sphingolipid present at high

concentrations in blood [9]. Data showing that increasing cardiac $S1PR_1$ levels contributed to significantly reduce myocyte apoptosis and fibrosis in post-MI hearts treated with Meto are confirmed by the fact that combining S1P and Meto had beneficial effects similar to those exerted by Meto alone. In keeping with this, we found that chronic administration of this selective β_1-blocker was sufficient to restore circulating S1P levels, which were markedly reduced after MI. In turn, this increased bioavailability activated cardiac SphK1, which was ultimately responsible for the rise in S1P levels in the serum of Meto-treated infarcted mice. Of note, this rise was superimposable to that achieved after direct infusion of S1P in post-MI mice. Taken together, this evidence explains why combining S1P and Meto had no additional or synergistic effects. This conclusion not only points toward new and important mechanistic insights into the beneficial effects of β_1-blockade in ischemic HF, but also stresses the functional repercussions of a loss of cardiac $S1P$-$S1PR_1$ signals due to β_1AR-hyperstimulation after ischemic injury. In essence, our study represents a significant departure from previous in vitro and in vivo data showing that only direct $S1PR_1$ activation confers cardioprotection after an ischemic insult [22–26].

Another conceptual advancement offered by the present findings is the involvement of β_3ARs in Meto-induced protection involving the $S1P$-$S1PR_1$ axis. β_3ARs were recently proposed to be cardioprotective receptors, with nodal roles in cardiac metabolism involving regulation of lipolysis via enhanced Sphk1 activity [20]. Our present findings significantly expand this view by showing that after MI, β_3AR expression is substantially down-regulated, along with markedly reduced SphK1 expression, and consequent impaired S1P secretion and worse LV remodeling. The dependence of SphK1/S1P signaling downstream of β_3AR activation is supported by the severe down-regulation of both SphK1 and S1P levels in β_3AR KO mice, and that these levels do not differ significantly from those found in WT mice after MI. Congruent with this, we observed similar LV dysfunction in post-MI WT and β_3AR KO mice. Nevertheless, the most striking finding is that Meto not only restores cardiac $S1PR_1$, but also prevents β_3AR down-regulation after MI, resulting in enhanced SphK1 activity and consequent S1P secretion. These biological effects were not seen in β_3AR KO mice. Meto did not consistently prevent HF progression in infarcted β_3AR KO mice. Thus, our data reveal that activating the β_3AR signal is a crucial and required step for β_1AR blockers (and likely other

pharmacological interventions impinging on the β_1AR signal) because Meto does not improve the HF phenotype in post-MI β_3AR KO mice.

Although appearing somewhat modest, Meto or S1P treatment of mice with severe post-MI cardiac dysfunction resulted in a significant protective effect in terms of post-MI viable myocardium or LV function. Importantly, the treatments were started at 1 week post-MI and drugs were administered for just 3 weeks. Therefore, instituting Meto or S1P within the first week post-MI and/or prolonging the treatment up to 8 weeks or more might result in more pronounced effects, as previously shown for S1P (27). These pending questions, along with possible dosing issues, should be addressed in large animals to more firmly establish the therapeutic potential of the present findings obtained in mice. Likewise, future studies to more finely dissect changes in S1PR₁ downstream signaling, particularly in β_3AR KO mice, are warranted.

All of the evidence reported here appears to be relevant to human HF. In fact, we report for the first time that patients with ischemic cardiomyopathy receiving treated with β_1-blockers (Online Table 1, Online Figure 10) had significantly higher S1P serum levels. At this time, possible β_3AR up-regulation in these patients with ischemia receiving β_1-blockers can only be inferred from evidence obtained in post-MI WT mice. However, a previous report showed up-regulation of β_3AR protein levels in heart specimens from human subjects with either ischemic or dilated cardiomyopathy who received chronic treatment with β_1AR-blockers, digitalis, angiotensin-converting enzyme inhibitors, and diuretic agents (28). Overall, these data corroborate our view that it is not the HF condition per se or its etiology, but rather the pharmacological treatment used (i.e., β_1-blockers [or others]) that effectively increases cardiac β_3AR in these human HF subjects. Additional in-depth studies must be designed to directly address this important point. However, our study suggests another important translational consideration that may help in personalizing β_1AR-blocker therapy: the cohort of patients that do not respond or responds minimally to β_1-blockers may harbor abnormalities in β_3AR signaling.

STUDY LIMITATIONS. Critical appraisal is needed for some aspects of our study. We have evaluated the impact of Meto in total β_3AR KO mice and not in cardiac-selective mouse model. Although here we have convincingly shown that S1P is secreted from the myocardium, we cannot exclude the possible contribution of other tissues, such as the adipose one in enriching the serum with S1P. Finally, future studies should be designed to finely dissect changes in S1PR1 downstream signaling, both in vitro and in vivo, and in light of the fact that this signaling pathway interrelate with S1PR1 and β_1AR.

CONCLUSIONS

Our study documents a novel mechanism by which β_1AR-blockers induce a therapeutic response after MI: β_1AR blockade by Meto leads to up-regulation of β_3ARs, followed by S1P/S1PR₁-triggered protective and beneficial signaling. The latter event is followed by β_3AR-dependent S1P release outside the cardiomyocytes, which, in turn, activates the rescued S1PR₁ aligned on the sarcolemmal membrane of neighboring myocytes to ultimately trigger intracellular protective signaling (**Central Illustration**). Therefore, Meto acting on myocardial β_1ARs actually influences the signaling of 3 distinct receptors, both directly (S1PR1 down-regulation) and indirectly (up-regulation of β_3ARs and increased S1P secretion) (**Central Illustration**). The discovery of this new protective loop triggered by β_1AR blockers such as Meto advances our understanding of ischemic HF pathophysiology.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: βAR-blockers are a key component of therapy for patients with HF, but some patients do not respond to this line of treatment. β_3-dependent S1P activation is crucial for cardiac protection, and β_3 signaling may underlie the differential responsiveness to this class of drugs.

TRANSLATIONAL OUTLOOK: Patients with HF who are resistant to β-blockade may harbor structural or functional β_3 variants, and therapies that stimulate these selectively might extend the benefit of to a broader segment of the patient population.

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APPENDIX For a supplemental Methods section as well as figures and table, please see the online version of this article.