β₁-Adrenergic Receptor and Sphingosine-1-Phosphate Receptor 1 (S1PR1) Reciprocal Downregulation Influences Cardiac Hypertrophic Response and Progression to Heart Failure

Protective Role of S1PR1 Cardiac Gene Therapy

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Background—The sphingosine-1-phosphate receptor 1 (S1PR1) and β₁-adrenergic receptor (β₁AR) are G-protein–coupled receptors expressed in the heart. These 2 receptors have opposing actions on adenylyl cyclase because of differential G-protein coupling. Importantly, both of these receptors can be regulated by the actions of G-protein–coupled receptor kinase-2, which triggers desensitization and downregulation processes. Although classic signaling paradigms suggest that simultaneous activation of β₁ARs and S1PR1s in a myocyte would simply result in opposing action on cAMP production, in this report we have uncovered a direct interaction between these 2 receptors, with regulatory involvement of G-protein–coupled receptor kinase-2.

Methods and Results—In HEK (human embryonic kidney) 293 cells overexpressing both β₁AR and S1PR1, we demonstrated that β₁AR downregulation can occur after stimulation with sphingosine-1-phosphate (an S1PR1 agonist), whereas S1PR1 downregulation can be triggered by isoproterenol (a β₁-adrenergic receptor agonist) treatment. This cross talk between these 2 distinct G-protein–coupled receptors appears to have physiological significance, because they interact and show reciprocal regulation in mouse hearts undergoing chronic β₁-adrenergic receptor stimulation and in a rat model of postischemic heart failure.

Conclusions—We demonstrate that restoration of cardiac plasma membrane levels of S1PR1 produces beneficial effects that counterbalance the deleterious β₁AR overstimulation in heart failure. (Circulation. 2013;128:1612-1622.)

Key Words: genetic therapy ■ heart failure ■ hypertrophy ■ receptors, adrenergic, beta ■ signal transduction

Protective Role of S1PR1 Cardiac Gene Therapy

G-protein–coupled receptors (GPCRs) transduce cell signals via heterotrimeric G proteins from neurohormones, ions, and sensory stimuli to regulate every aspect of mammalian physiology. GPCRs are regulated by GPCR kinases (GRKs) that trigger termination of signaling, a process known as desensitization. Phosphorylation of agonist-occupied receptors by GRKs induces recruitment and binding of β-arrestins that displace bound G proteins, thereby uncoupling receptors from their downstream signaling effectors. This process continues through β-arrestin–dependent internalization of receptors, which leads either to their degradation and downregulation or to recycling (resensitization) to the membrane. Moreover, β-arrestin recruitment to GRK-phosphorylated receptors has been shown to lead to novel intracellular signaling, a process called G-protein–independent signaling. In the heart, activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway has been shown to be an important GRK-mediated β-arrestin–dependent signal.

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Given their central role in cardiac pathophysiology, GPCRs are critical therapeutic targets in cardiovascular diseases. This is especially true in heart failure (HF), in which β-adrenergic receptor (βAR) antagonists and angiotensin II receptor blockers are the standard of care for patients with HF.

Clinical Perspective on p 1622

The use of βARs and angiotensin II receptor blockade is warranted as a consequence of the sympathetic nervous system and renin-angiotensin system hyperactivity that induces their overstimulation. This phenomenon represents the molecular basis for βAR downregulation in the failing human myocardium. Because GPCRs are dynamically regulated in disease processes, a better understanding of downstream signaling is imperative. In particular, GPCR dimerization and interaction between different GPCR signaling pathways have become the cornerstone of current cardiovascular research to better clarify the molecular alterations that underlie cardiovascular diseases and to identify novel potential therapeutic targets. In cardiac physiology and pathophysiology, the β1-adrenergic receptor (β1AR) is the predominant βAR that regulates the inotropic and chronotropic responses of sympathetic nervous system catecholamines through Gs-dependent activation of adenylyl cyclase. Chronic β1AR hyperstimulation results in their downregulation, and consequently, there is a marked reduction of the inotropic reserve of the failing heart. The sphingosine-1-phosphate receptor 1 (S1PR1), which mediates the effect of the lysophospholipid sphingosine-1-phosphate (S1P, a natural agonist), is also expressed on cardiomyocytes, and its signaling opposes β1AR-mediated adenylyl cyclase activation through its coupling to the adenylyl cyclase–inhibitory G protein, Gi. Thus, in the heart, S1PR1 is able to antagonize the effects of isoproterenol and other βAR agonists. Furthermore, S1PR1 and β1AR undergo GRK-mediated regulation through phosphorylation. Although GRK2, the primary GRK isoform expressed in myocytes, can regulate both receptors, β1AR is also regulated by phosphorylation by protein kinase A (PKA), whereas S1PR1 can be regulated by protein kinase C. Importantly, with respect to novel non-canonical signaling, both receptors have been implicated in activation of extracellular signal-regulated kinase, which can lead to protective signaling.

Recently, a functional interaction between β1AR and S1PR1 signaling has been reported in vivo. In fact, isoproterenol administration in mice induces cardiac hypertrophy via engagement of the S1PR1 signaling pathway; however, there is no proof of direct cross talk at the receptor level between β1ARs and S1PR1s. In the present study, we provide biochemical and functional evidence of a direct connection between these 2 highly expressed GPCRs in the heart, demonstrating their reciprocal regulation via an important regulator, GRK2, which has potential significance for cardiac pathophysiology.

Methods

Cell Culture

Human embryonic kidney (HEK) 293 cells overexpressing the mouse wild-type β1AR carrying a FLAG epitope (WTβ1AR-FLAG) or 2 mutants lacking, respectively, the putative PKA phosphorylation sites (PKA-β1AR-FLAG) and the putative GRK phosphorylation sites (GRK β1AR-FLAG), obtained as described previously, and H9c2 cardiomyoblasts obtained from the American Type Culture Collection were cultured and transfected as described briefly in the online-only Data Supplement.

Confocal Microscopy

Green fluorescent protein (GFP)-tagged S1PR1 (S1PR1-GFP) and β1AR-GFP internalization of S1PR1 tagged with green-fluorescent protein (S1PR1-GFP) and β1AR-Flag was visualized by confocal laser scanning microscopy. After stimulation with (−)-isoproterenol bitartrate (1 μmol/L; Sigma-Aldrich, St. Louis, MO) or S1P (250 nmol/L; Sigma-Aldrich), cells were fixed and visualized as described previously. After fixation, cells were incubated with an anti-FLAG Cy3-conjugated mouse IgG (Sigma-Aldrich, St. Louis, MO) using a dilution of 1:100 in PBS containing 0.5% BSA for 1 hour at room temperature. Confocal laser scanning microscopy was performed at 488 nm (GFP) or 568 nm (Cy3). The fluorescent data sets were analyzed by LSM 510 software (Carl Zeiss Microscopy, Jena, Germany). Cells treated with isoproterenol were pretreated with the selective β2AR antagonist ICI 118,551 hydrochloride (ICI, 10 μmol/L; Sigma-Aldrich, St. Louis, MO). Each experiment was repeated separately 23 times.

Immunoblotting

Immunoblotting on cells and left ventricular (LV) samples was performed as described previously and briefly reported in the online-only Data Supplement.

Treatment Protocol for Mice

As described previously, C57BL/6 mice (n=5) were injected subcutaneously, twice per day, with isoproterenol dissolved in 0.002% ascorbic acid at the total rate of 3 mg·kg⁻¹·d⁻¹ over a period of 7 days. Control mice (sham, n=5) were injected with vehicle (0.002% ascorbic acid). At the time the animals were killed, after calculation of the heart weight to body weight ratio, the hearts were removed and cardiac chambers dissected.

Measurement of Hypertrophic Growth in H9c2 Cardiomyoblasts

Hypertrophy was assessed by measurement of relative cell surface area of H9c2 cells as described previously (online-only Data Supplement).

TUNEL Staining

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) was performed on H9c2 cells (online-only Data Supplement).

Rat Myocardial Infarction Model

Myocardial infarction (MI) in rats was performed as described previously and briefly reported in the online-only Data Supplement.

Myocardial In Vivo Gene Delivery

Myocardial gene transfer in rats was achieved by direct intramyocardial injection 8 weeks after MI, as described previously (online-only Data Supplement).

Echocardiography

Echocardiography was performed as described previously and briefly reported in the online-only Data Supplement.

Catheter-Based In Vivo Hemodynamic Measurements

Cardiac function was measured 12 weeks after gene therapy, as described previously.
Myocardial Perfusion Studies

Myocardial perfusion was determined with 15-μm fluorescent microspheres (Triton Technology, Inc, San Diego, CA). Cardiac and blood samples were processed for microsphere determination. Myocardial blood flow was measured at baseline and after maximal vasodilation with dipyridamole (6 mg·kg⁻¹·min⁻¹ IV).

Measurement of Infarct Size

Infarct size was examined in all experimental rats at the end of the study period, as described previously²⁳ and briefly reported in the online-only Data Supplement.

Histology

LV paraffin-embedded specimens were immunohistochemically stained for S1PR1 (anti-S1PR1 mouse monoclonal antibody, 1:100; abm [Applied Biological Materials Inc], Richmond, British Columbia, Canada) and GFP (anti-GFP mouse monoclonal antibody, 1:200; Upstate [EMD Millipore], Billerica, MA) or stained for capillary density determination as described previously¹⁶ and briefly reported in the online-only Data Supplement.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from cardiac samples with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and was reversed transcribed to generate cDNA. To evaluate the expression of recombinant human S1PR1-GFP and GFP, we performed polymerase chain reaction using specific primers (hS1PR1-forward 5′-CAGCAAATCGGACAATTCT-3′, hS1PR1-reverse 5′-GAACCTCAGGTCCAGCTTGC-3′, GFP-forward 5′-GACCTAAAAACGCCACAAGTT-3′, GFP-reverse 5′-AAGTCTGTGCCTCTCATGTG-3′) with amplified products of 250 and 180 base pairs (bp), respectively.

βAR Radioligand Binding

Receptor binding with 20 μg of protein from plasma membrane was performed with [¹²⁵I]cyanopindolol (350 pmol/mL) as described previously¹⁹ and briefly reported in the online-only Data Supplement.

Statistical Analysis

Normally distributed continuous variables with sample size > 10 were expressed as mean±SEM and compared by 1-way or repeated-measures ANOVA test followed by Bonferroni post hoc correction, as appropriate. When the sample size was <10, exact tests (Mann-Whitney or Kruskal-Wallis, as required), followed by Bonferroni post hoc correction, were performed, and data were represented with median and dot plots. Normality was tested with the Shapiro-Wilk test (<50 values) or Kolmogorov-Smirnov test (≥50 values), as appropriate. All analyzed data showed no significant departure from normal distribution. Statistical analysis was performed with SPSS software (SPSS Inc, Chicago, IL). Level of statistical significance was set to P≤0.05.

Results

In Vitro Reciprocal Downregulation of β1AR and S1PR1

To establish a functional correlation between β1AR and S1PR1 signaling, HEK293 cells stably expressing the mouse wild-type β1AR (WTβ1AR) or 2 mutants lacking, respectively, the putative PKA phosphorylation sites (PKA−β1AR) or the putative GRK phosphorylation sites (GRK−β1AR) were transfected with S1PR1 cDNA. By confocal microscopy experiments, we evaluated β1AR and S1PR1 internalization after isoproterenol and S1P stimulation. As shown in Figure 1A, isoproterenol and S1P stimulation resulted in a marked loss of both WTβ1AR and S1PR1 from the cell surface and, surprisingly, in a colocalization of the 2 receptors in the cytosol that peaked after 30 minutes of treatment. The absence of PKA phosphorylation sites (PKA−β1AR) did not affect reciprocal internalization and cytosolic colocalization after isoproterenol or S1P stimulation (Figure 1B). In contrast, as shown in Figure 1C, in GRK−β1AR cells, isoproterenol stimulation induced β1AR but not S1PR1 internalization. On the other hand, S1P stimulation induced S1PR1 downregulation with no effect on β1AR localization. Interestingly, isoproterenol and S1P induced a similarly significant increase in levels of phosphorylated extracellular signal-regulated kinase in all cell subtypes (Figure 1A–1C). To better determine whether β1AR and S1PR1 form stable complexes, β1AR and S1PR1 were expressed at the same level in HEK293 cells, and then a coimmunoprecipitation assay was

Figure 1. G-protein–coupled receptor kinase (GRK) phosphorylation site removal inhibits cross talk between β1-adrenergic receptor (β1AR) and sphingosine-1-phosphate receptor 1 (S1PR1). Human embryonic kidney 293 cells stably expressing wild-type β1AR (WTβ1AR) or 2 mutants lacking, respectively, the putative PKA phosphorylation sites (PKA−β1AR) or the putative GRK phosphorylation sites (GRK−β1AR) carrying a FLAG epitope (WTβ1AR-FLAG; A), protein kinase A-negative β1AR carrying a FLAG epitope (PKA−β1AR-FLAG; B), and GRK-negative β1AR carrying a FLAG epitope (GRK−β1AR-FLAG; C) and transfected with S1PR1-GFP (green fluorescent protein) were pretreated with the β2AR antagonist ICI 118,551 hydrochloride (ICI, 10 μmol/L), then stimulated with (−)-isoproterenol bitartrate (ISO; 1 μmol/L) or sphingosine 1-phosphate (S1P; 250 nmol/L) for 30 minutes and compared with unstimulated (NS) cells. Representative immunofluorescence images of S1PR1-GFP and β1AR-FLAG show cumulative data of multiple independent experiments in WTβ1AR-FLAG+S1PR1-GFP (A), PKA−β1AR-FLAG+S1PR1-GFP (B), or GRK−β1AR-FLAG+S1PR1-GFP (C). Arrowheads indicate receptor internalization. Representative immunoblots (IB) show activation of extracellular signal–regulated kinase 1 and 2 (pERK) after 5 minutes of stimulation with ISO (1 μmol/L) or S1P (250 nmol/L) in WTβ1AR-FLAG (A), PKA−β1AR-FLAG (B), and GRK−β1AR-FLAG (C). GAPDH was used as loading control.
performed. Notably, as shown in Figure I in the online-only Data Supplement, immunoprecipitation of S1PR1 resulted in communoprecipitation of β1AR in the absence or presence of isoproterenol or S1P, which confirms our hypothesis of a direct receptor-receptor interaction.

To further investigate how GRK-dependent phosphorylation was involved in the cross talk between β1AR and S1PR1, we also used a mutated form of S1PR1 (S1PR1-Δ32) that lacked GRK2 phosphorylation sites.15 As shown in Figure IIA in the online-only Data Supplement, S1PR1-Δ32 lost the ability to be cointernalized with β1AR after stimulation with agonists. Furthermore, phosphorylation of extracellular signal-regulated kinase 1 and 2 increased after isoproterenol stimulation but at a lower extent than after S1P stimulation (Figure IIA in the online-only Data Supplement).

β1AR- and S1PR1-Dependent GRK2 Upregulation

Because it is known that both β1AR and S1PR1 are substrates of GRK2 phosphorylation,13–15 and the present data suggested a crucial role of this kinase in the codependence of internalization of these 2 receptors, we further explored the potential role of GRK2. A 2-fold increase in GRK2 levels in WTβ1AR cells stimulated for 12 hours with either isoproterenol or S1P was observed (Figure 2A). In PKA-β1AR cells, isoproterenol or S1P stimulation resulted in blunted yet significant GRK2 upregulation (Figure 2B). Importantly, GRK-β1AR cells showed enhanced GRK2 expression only after S1P administration (Figure 2C).

In Vitro Protective Role of S1PR1 on Deleterious β1AR Overstimulation

It is known that both β1AR and S1P are able to induce cardiomyocyte hypertrophy.24,25 Therefore, we tested the effects of chronic isoproterenol and S1P administration in vitro in H9c2 cells stimulated for 48 hours in the absence or presence of the β1AR antagonist metoprolol or the S1PR1-selective antagonist W146. Treatment with both agonists resulted in a consistent increase of cell surface area, which indicates a hypertrophic response, which was blocked with the respective antagonist (metoprolol or W146; Figure 3A). Notably, only isoproterenol induced an increase in cardiomyocyte apoptosis (Figure 3B). This apoptotic response in isoproterenol-stimulated cells was prevented by metoprolol pretreatment (Figure 3B). Interestingly, S1P induced a robust hypertrophic response and blunted apoptotic reaction in metoprolol-pretreated cells (Figure 3A and 3B). Reciprocally, isoproterenol stimulation in the presence of S1PR1 antagonist did not induce a hypertrophic response but increased apoptosis (Figure 3A and 3B).

In Vivo Reciprocal β1AR and S1PR1 Downregulation

As widely demonstrated,2,17,18 chronic in vivo isoproterenol administration induces cardiac hypertrophy, pathology, and strong cardiac β1AR plasma membrane downregulation. Thus, to determine whether our in vitro findings could be translated in vivo, we analyzed S1PR1 density in crude myocardial membrane preparations from mice after 7 days of isoproterenol administration. As expected, after chronic isoproterenol stimulation, mice exhibited a significant increase in heart to body weight ratio (Figure 4A) and LV septum thickness (Figure 4B) and a robust cardiac GRK2 upregulation compared with untreated mice (Figure 4C). Notably, 7 days of isoproterenol administration resulted in S1PR1 downregulation at the plasma membrane level (Figure 4D).

In addition, to evaluate whether the S1PR1-selective agonist was able to induce similar β1AR downregulation in vivo, we treated mice for 7 days (via daily intraperitoneal injections) with the S1PR1 agonist SEW2871 (Figure III in the online-only Data Supplement). Interestingly, SEW2871 treatment resulted in a consistent increase in heart weight to body weight ratio (Figure IIIA in the online-only Data Supplement) and a strong increase in GRK2 protein levels (Figure IIIB in the online-only Data Supplement) compared with untreated mice. Consistent with our in vitro observations, treatment with this S1PR1-selective agonist resulted in a robust decrease in β1AR cardiac plasma membrane levels.
compared with the untreated group (Figure IIC in the online-only Data Supplement).

Cardiac S1PR1 Membrane Downregulation During HF

Because the present data showed that S1PR1 internalization occurred in vitro and in vivo after chronic isoproterenol stimulation, we studied potential S1PR1 dysregulation in a clinically relevant experimental model of HF, a pathological condition characterized by a sustained elevation of circulating catecholamines. Accordingly, plasma membranes were extracted from LV lysates of rats 8 weeks after MI. Importantly, we found a significant downregulation of S1PR1 in HF rats compared with control (Figure 5A). Accordingly, to assess whether S1PR1 downregulation during HF was induced by an increase in S1P levels, we performed an ELISA for S1P on blood serum and an immunoblot on LV lysates to assess the expression of sphingosine kinase 1 in the HF and sham groups (Figure IVA and IVB in the online-only Data Supplement). Notably, both the circulating levels of S1P and sphingosine kinase 1 expression were robustly reduced in the HF group compared with sham, which proves that S1PR1 behaves similarly to β1AR in a significant pathophysiological setting such as HF. 22 In contrast, S1PR1 gene therapy was able to restore sphingosine kinase 1 expression to the levels observed in sham animals and to increase circulating levels of S1P compared with the HF control group.

In Vivo S1PR1 Gene Therapy

Because we demonstrated that S1PR1 signaling is beneficial in cardiomyocytes in vitro and that this receptor is downregulated in our animal model of post-MI HF, we next explored the effects of long-term S1PR1 receptor overexpression during HF. Adult male Sprague-Dawley rats underwent MI (n=30) or sham operation (n=10). Eight weeks after surgery, HF rats were randomly assigned to one of the following groups: (1) HF saline (HF, n=10), (2) HF recombinant adenovirus-associated type 6–GFP (HF-rAAV6-GFP, n=10), or (3) HF recombinant adenovirus-associated type 6–S1PR1-GFP (HF-rAAV6-S1PR1, n=10). A baseline echocardiogram was performed in all groups 1 day before treatment onset to confirm the presence of similar levels of LV dysfunction before gene delivery. All groups were then studied over the course of an additional 12 weeks (20 weeks after MI; Figure 5B), and all assays in the HF groups were compared with a control sham-operated group that underwent neither MI nor gene transfer. At 12 weeks after gene delivery, both transgenes (S1PR1 and GFP) were robustly expressed in the LV, as assessed by immunohistochemistry (Figure 5C). Consistently, reverse-transcription polymerase chain reaction analysis (Figure 5D) and immunoblots (Figure 5E) confirmed the expression of human S1PR1 in the hearts of the rAAV6-S1PR1 group and of GFP in the hearts of both the rAAV6-S1PR1 and rAAV6-GFP groups. As shown in Figure 5F, 20 weeks after MI, cardiac...
rAAV6-S1PR1 gene therapy resulted in restoration of plasma membrane S1PR1 to the level observed in sham animals.

**Effects of S1PR1 Overexpression on In Vivo Cardiac Function at 12 Weeks After Gene Delivery**

Eight weeks after experimental MI, LV ejection fraction was decreased dramatically and end-diastolic diameter was increased as expected (Figure 6A and 6B). Treatment with GFP or saline had no impact on cardiac performance, with a further deterioration of cardiac function 12 weeks later. On the other hand, S1PR1 overexpression ameliorated LV contractility. In fact, ejection fraction was increased significantly in S1PR1-infected rats compared with HF controls (Figure 6B). Adverse LV remodeling as measured by ventricular dilatation progressed further in the saline and GFP groups, and this was prevented by S1PR1 gene delivery. As expected, no differences in infarct size were observed among the HF groups, because gene therapy was performed 8 weeks after MI, when the infarct scar was completely established (data not shown). Of note, S1PR1 gene delivery affected the immune response, as observed by hematoxylin-and-eosin staining of cardiac sections. In fact, S1PR1 overexpression resulted in reduced infiltration of immune cells compared with the HF control group (Figure VA in the online-only Data Supplement). Interestingly, S1PR1 overexpression in the LV induced a consistent decrease in heart rate. LV invasive hemodynamic analysis in rats performed at the end of the study period (12 weeks after gene delivery) revealed significant decreases in +dP/dt and −dP/dt in all HF groups compared with sham, which proved the HF-related reduction in LV contractility and relaxation (Table; Figure 6C). LV systolic pressure was reduced significantly and LV end-diastolic pressure was increased significantly in all HF groups compared with sham (Table). Cardiac S1PR1 overexpression significantly improved LV contractility and relaxation 12 weeks after treatment (Table; Figure 6C).

Furthermore, S1PR1 gene delivery increased LV systolic pressure and decreased end-diastolic pressure compared with the HF control groups (Table). Notably, total plasma membrane βAR density was completely restored in rAAV6-S1PR1 hearts compared with HF controls (Figure 6D). Accordingly, S1PR1-infected rats showed significantly improved LV +dP/dt and LV −dP/dt after maximal βAR stimulation by isoproterenol (Table; Figure 6C).

**Effect of S1PR1 on Cardiac Remodeling and Angiogenesis**

The beneficial effect of S1PR1 overexpression on cardiac function was accompanied by a hypertrophic response of the failing heart. This observation was evident at echocardiographic evaluation, where we found increased anterior wall diastolic thickness and posterior wall diastolic thickness in S1PR1 rats compared with HF controls (Table). Consistently, the heart weight to body weight ratio was significantly higher in hearts treated with S1PR1 than in GFP- and saline-treated groups (Table). Importantly, the S1PR1-dependent increase in LV mass was accompanied by a significant decrease in LV systolic and diastolic internal diameter compared with rAAV6-GFP HF hearts, thus indicating that S1PR1 gene therapy induced a compensatory hypertrophic response able to counteract LV dilation. In accordance with previous observations reported by us and others, 25-26 this adaptive LV remodeling was associated with a significant growth of the cardiac capillary network. In fact, S1PR1 gene delivery resulted in a significant increase in capillary density
compared with the HF groups and a complete recovery of myocardial blood flow that was indistinguishable from sham (Figure 7A and 7B). At the molecular level, we investigated the effects on Akt activation because this kinase is one of the major proangiogenic molecules involved in S1PR1 signaling.27,28 Notably, S1PR1 downregulation was associated with a robust reduction in Akt activation compared with sham (Figure VIA in the online-only Data Supplement). S1PR1 gene therapy was able to enhance Akt activation (Figure VIA in the online-only Data Supplement).

Discussion

HF is a major and growing public health problem that affects 1% to 6% of the US population.29 It is a disease characterized by LV dysfunction associated with a complex of symptoms that relate to inadequate perfusion of tissues and pulmonary congestion. One of the consequences is the activation of the sympathetic nervous system, which plays a crucial role in adapting circulatory homeostasis to changes in environment. Furthermore, circulating levels of catecholamines are increased in HF in proportion to the severity of the disease.30 However, sympathetic hyperactivity can also initiate or accelerate cardiac dysfunction and provoke major cardiovascular events, thus justifying why HF patients with higher plasma levels of norepinephrine have the most unfavorable prognosis.31 These observations have led to the hypotheses that sympathetic activation may play an important role in HF progression8,32,33 and that pharmacological interference with this system can produce hemodynamic and clinical benefits.34

Currently, some of the most effective treatments for HF target β1AR, β2AR, and angiotensin II type IA receptor, which are GPCRs.4 Several studies have demonstrated that GPCRs can actually interact and can be regulated reciprocally. For instance, it has been shown that β1AR is able to transactivate the epidermal growth factor receptor, conferring a β-arrestin–dependent cardioprotective effect.2 These data have led others to hypothesize about the development of new therapies for HF that would be able not only to antagonize harmful cardiac signaling but also to potentiate the
Reciprocal Downregulation Between S1PR1 and β1AR

In that regard, a deeper understanding of the signaling mechanisms underlying the development and progression of HF is needed.

The present data show, for the first time, a direct interaction between β1AR and S1PR1, representing a dynamic regulation present between 2 important and predominant GPCRs in the heart that appears to have significant physiological effects. Using HEK293 cells overexpressing the WT β1AR and transiently transfected with S1PR1, we demonstrated in vitro that either isoproterenol or S1P stimulation induced dual internalization, with a cytosolic colocalization of both β1AR and S1PR1. As a functional consequence, activation of extracellular signal-regulated kinase occurred. Importantly, we also demonstrated that both β1AR and S1PR1 were able to form a stable complex and that reciprocal downregulation of the 2 receptors occurred only in the presence of agonist-dependent GRK2 activation. In fact, we examined the molecular mechanisms of such interaction and found that the absence of GRK phosphorylation sites but not the absence of PKA phosphorylation sites on the β1AR abolished the cross talk between the 2 receptors. Similarly, a lack of GRK2 phosphorylation sites on S1PR1 inhibited the reciprocal downregulation between β1AR and S1PR1 after stimulation with agonists. Therefore, GRK2 appears to be a nodal regulator of the cellular response to both catecholamines and S1P, and it is able to modulate the phosphorylation and the reciprocal internalization of both β1AR and S1PR1.

It is known that β1AR is a very important regulator of cardiac function in both physiological and pathophysiological settings, and its action can become deleterious over time. An important role has also been shown recently for S1PR1. In fact, this receptor can mediate a hypertrophic response in neonatal rat cardiomyocytes and is cardioprotective for adult cardiomyocytes under hypoxia and in the intact heart exposed to MI. The present data confirm the ability of S1PR1 to mediate hypertrophy in cultured cells as well as the β1AR; however, although β1AR can also induce significant apoptosis, this is not the case with S1PR1. More interestingly, the apoptotic response to isoproterenol stimulation increased when the S1PR1 receptor was blocked. The present data demonstrate that S1PR1 signaling is cardioprotective, particularly during sustained catecholamine stimulation that reproduces pathological conditions such as HF. In fact, our demonstration that S1PR1 is downregulated in the pathological cardiac hypertrophy induced by isoproterenol injection in mice, which is known to predispose to cardiac dysfunction, confirms that S1PR1 inactivation could have a role in the progression toward HF. More interestingly, we showed that in a rat model of postischemic HF, S1PR1 plasma membrane levels were significantly downregulated. Furthermore, the
The observed S1PR1 downregulation was not correlated with an S1P increase. In fact, this molecule was strongly reduced in blood serum of post-MI HF mice compared with sham, which strengthens our hypothesis of an active role of catecholamine overstimulation in vivo on reciprocal downregulation between β1AR and S1PR1.

**Table. Echocardiographic, Hemodynamic, and Physical Parameters at 12 Weeks After rAAV6-Mediated Cardiac Gene Delivery**

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<td>LV dP/dt, mmHg/s</td>
<td>6494.8±244</td>
<td>4461±179.8*</td>
<td>5260±245.7†</td>
</tr>
<tr>
<td>LV −dP/dt, mmHg/s</td>
<td>6968±327.8</td>
<td>3626±106.1*</td>
<td>4251.4±128.8†</td>
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<tr>
<td>LVEDP, mmHg</td>
<td>2.2±0.3</td>
<td>13.5±1.1*</td>
<td>7.7±0.9†</td>
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<td>LVESP, mmHg</td>
<td>132.2±3.6</td>
<td>104.1±4.9*</td>
<td>107.8±3.3*</td>
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</tbody>
</table>

Data are mean±SEM. Effect of S1PR1 gene therapy on LV function evaluated at 12 weeks after gene delivery is shown. ANOVA and Bonferroni test were used between all groups. In vivo characteristics were assessed in sham (n=10), HF+saline (n=10), HF+rAAV6-S1PR1 (n=10), and HF+rAAV6-GFP (n=10) rats. Ratio of heart weight to body weight and fractional shortening were also measured in all groups. Awd indicates anterior wall diastolic thickness; Aws, anterior wall systolic thickness; EF, ejection fraction; HF, heart failure; HF+rAAV6-GFP, rats with heart failure given recombinant adeno- virus type 6 and green fluorescent protein; HF+rAAV6-S1PR1, rats with heart failure given recombinant adeno- virus type 6 and sphingosine-1-phosphate receptor 1; HF+Saline, rats with heart failure given saline; HR, heart rate; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; LVIDd, left ventricular internal diameter at diastole; LVIDs, LV internal diameter at systole; PWd, posterior wall thickness at diastole; PWs, posterior wall thickness at systole; rAAV6, recombinant adeno- virus type 6; and Sham, control mice injected with vehicle.

*P<0.05 vs sham; †P<0.05 vs HF control groups.

**Figure 7.** Beneficial effects of sphingosine-1-phosphate receptor 1 (S1PR1) gene delivery on cardiac angiogenesis. A. Total myocardial blood flow measured at basal condition and after maximal (Max) coronary dilation. B. Bar graph shows capillary density evaluated by staining of capillaries with lectin from Bandeiraea simplicifolia I (BS-I) in cardiac section obtained from heart failure (HF) control groups and HF+AAV6-S1PR1. Statistical significance between groups was determined by repeated-measures ANOVA followed by Bonferroni post hoc correction. n=10 for each group. *P<0.05 vs sham; †P<0.05 vs HF.
Of note, rAVV6 gene therapy, as described previously, enabled us to obtain long-term and stable myocardial gene expression of rAAV6-S1PR1 cardiac gene transfer. For the first time, we directly investigated the therapeutic effects of 12-week expression of S1PR1 in the failing heart. We were able to demonstrate that rAAV6-S1PR1 gene delivery restored S1PR1 plasma membrane levels and its signaling, observed by a consistent increase in sphingosine kinase 1 expression in the heart and active secretion of S1P in blood serum, exerting an important functional and structural cardiac recovery, improving cardiac function, and blocking the negative remodeling in our post-MI rat HF model. Three months after gene delivery, we found significantly increased LV ejection fraction, dP/dt, and systolic blood pressure, whereas LV end-diastolic diameter and pressure were decreased compared with the control HF animals.

The beneficial effects of S1PR1 gene therapy were also evident on post-MI βAR dysfunction. In fact, maximal inotropic responses during βAR stimulation were almost completely restored after S1PR1 gene delivery. It is reasonable to hypothesize that the favorable action of S1PR1 overexpression in the post-MI heart is probably attributable to either a direct cardioprotective effect or an increase of the angiogenic response that promotes an adaptive, angiogenesis-dependent LV hypertrophy instead of transitioning to a maladaptive state. Consistent with this, S1PR1 gene delivery induced activation of Akt signaling, which prevented capillary rarefaction and completely restored myocardial blood flow in our experimental groups. Taken together, the present data show that S1PR1 downregulation, probably induced by excessive catecholamine stimulation during HF, could be responsible for progression toward a further decrease in cardiac performance. Importantly, in the present study, we focused on S1PR1, which represents the S1PR subtype with the highest expression in the heart, and further studies will be needed to uncover potential relevant roles of S1PR2 and S1PR3 in HF. In conclusion, the ability of S1PR1 gene delivery to prevent LV failure in a setting of established myocardial damage suggests that this molecule has the potential to be a candidate for HF treatment.

Sources of Funding
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Disclosures
None.

References


CLINICAL PERSPECTIVE

In chronic heart failure (HF), sympathetic nervous system overdrive induces the upregulation of G-protein–coupled receptor kinase 2 (GRK2) with a consequent β-adrenergic receptor downregulation/desensitization. Importantly, in failing myocardium, β-adrenergic receptor dysregulation is clinically seen by loss of inotropic reserve. Currently, β-blockers represent a solid “pillar” of HF therapy that at the molecular level efficiently counteracts both β-adrenergic receptor downregulation and GRK2 upregulation. GRK2 inhibition represents a promising new strategy to rescue the failing heart, and we have recently demonstrated that in some animal models, it could be used as a substitute for or in conjunction with β-blockers. GRK2 inhibition may be effective by interfering with other intracellular processes. The present study provides the first evidence of a direct and GRK2-dependent interaction between the β1-adrenergic receptor and the sphingosine-1-phosphate receptor 1 (S1PR1). We show that the reciprocal downregulation between these 2 predominant G-protein–coupled receptors in the heart appears to have significant physiological effects on cardiac hypertrophy, apoptosis, and remodeling. Clinically, HF-related S1PR1 downregulation worsens left ventricular dysfunction in a setting of established myocardial damage. We show that the increase of S1PR1 density, through gene therapy, in failing cardiomyocytes may represent a novel therapeutic strategy for HF. This study clearly opens a new chapter in the understanding of the molecular mechanisms involved in HF, demonstrating that besides the well-documented therapeutic effects of β-adrenergic receptor signaling “resensitization” in HF via GRK2 inhibition or β-blockade, a new field of research could be the reconstitution of S1PR1 cardioprotective signaling as a new therapeutic target along with other unexplored signaling pathways.
Failure: Protective Role of S1PR1 Cardiac Gene Therapy
Alessandro Cannavo, Giuseppe Rengo, Daniela Liccardo, Gennaro Pagano, Carmela Zincarelli, Maria Carmen De Angelis, Roberto Puglia, Elisa Di Pietro, Joseph E. Rabinowitz, Maria Vittoria Barone, Plinio Cirillo, Bruno Trimarco, Timothy M. Palmer, Nicola Ferrara, Walter J. Koch, Dario Leosco and Antonio Rapacciuolo

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell culture
HEK293 (WTβ1AR-Flag,PKAβ1AR-Flag and GRKβ1AR-Flag) and H9c2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 200 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells were then transfected with 1 μg of cDNA encoding for S1PR1 conjugated with GFP (S1PR1-GFP) while H9c2 cells were also transfected with 1 μg of cDNA encoding for WTβ1AR-Flag. All transfections were performed using FuGENE6 reagent according to the manufacturer’s instructions (Roche Applied Science). Following transfection cells were incubated overnight in serum-free medium supplemented with 0.1% BSA, 10 mM HEPES (pH 7.4), and 1% penicillin prior to stimulation. Under serum starvation conditions, cells were stimulated as described in the figure legends.

Immunoblotting
Cells and left ventricular (LV) samples were lysed in a RIPA buffer with protease and phosphatase inhibitors cocktail (Roche). Protein concentrations in all lysates were measured using a dye-binding protein assay kit (Bio-Rad) and a spectrophotometer reader (Biorad) at a wavelength of 750 nm. Phosphorylation of ERK 1/2 was detected by protein immunoblotting using a 1:1000 anti-mouse IgG (Cell Signaling). β1AR was detected by using 1:1000 anti-rabbit IgG (Thermo Scientific). GRK2 was detected by using 1:1000 anti-mouse IgG (Santa Cruz). S1PR1 was detected by using 1:1000 anti-mouse IgG (abm). Secondary antibodies were
purchased from Amersham Life Sciences Inc. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Life Sciences Inc.) according to the manufacturer’s instructions, and were quantified using densitometry (Chemidoc, Biorad, USA). Each experiment and densitometric quantification was separately repeated at least three times.

*Plasma membrane proteins fractionation and S1PR1 levels analysis*

Membrane protein purification was performed using a membrane protein extraction kit (Biovision) according to manufacturer instructions. Briefly, 100 mg of LV section were lysed in 1 mL of Homogenization Buffer. Then centrifuged at 700 g for 10 minutes at 4°C. The supernatant (cytosol fraction) was stored at -80°C while pellet (total cellular membrane protein) was further processed in order to separate organelles membrane from plasma membranes. Plasma membrane proteins were dissolved in RIPA buffer and used to evaluate the levels of S1PR1 by immunoblot. Na⁺/K⁺ ATPase (Upstate) antibody (1:1000 anti-mouse IgG) and an anti-Actin antibody (Santa Cruz) were respectively used as plasma membrane purification and loading controls.

*Measurement of hypertrophic growth in H9c2 cardiomyoblasts*

H9C2 cells were grown on glass coverslip. Following co-transfection, the cells were serum starved and treated with ISO or S1P and pre-treated also with selective β1AR blocker metoprolol tartrate (MET, 1 μM; MP biomedical) or S1PR1 antagonist W146 (10 μM; Sigma-Aldrich) as described in figure legends.

*TUNEL staining*
Tunel staining was performed with the use of an ApopTag Fluorescein Direct in Situ Apoptosis Detection kit (Chemicon, UK) according to the manufacturer’s instructions. Images were visualized by specific green fluorescence and nuclei by 4′-6-diamidino-2-phenylindole (DAPI) (nuclear counterstain). The TUNEL positives cells were examined with a microscope (Nikon TE-2000 U) and images were acquired with a digital camera (Nikon).

*Rat MI model*

30 Wistar Kyoto male rats (300 gr.) entered the study and underwent surgically induced myocardial infarction (MI) by permanent ligation of the left anterior descending coronary artery (LAD). Mortality rate was ~25% at 8 weeks post-MI and raised to ~30% the end of the study period.

*Myocardial in vivo gene delivery*

8-weeks post-MI (HF) a total of $4 \times 10^{11}$ total particles of rAAV6-S1PR1 or rAAV6-GFP in a total volume of 500 μL were injected using a 32½ G needle in five different area of the LV free wall (two injections into the anterior wall, two into the posterior and one into the lateral wall immediately above the infarct scar).

8 weeks following rAAV6-GFP (n=5 for each delivery technique) in vivo gene delivery to HF rats using an Olympus IX 71 microscope, a mercury arc light and suitable filters. Moreover, transfection efficiency of *in vivo* gene transfer was assessed by GFP fluorescence (510 nm) in sectioned hearts (10 μm) using an Olympus IX81 confocal microscope.

*In vivo Gene Therapy Efficiency*

The percentage (%) of GFP-stained isolated myocytes was assessed as previously described (23) and briefly reported in the online supplemental methods.

*Immunohistochemistry*
LV specimens were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and re-hydratation, 4 μm-thick sections were prepared and mounted on glass slides. Sections were deparaffinized, rinsed in xylene, and rehydrated. Subsequently they were quenched with 0.3% hydrogen peroxide, washed in water, treated with 2% bovine albumin in PBS and incubated with the primary antibodies at 4°C overnight. After being washed in PBS the primary antibody was detected with biotinylated anti-mouse IgG for 1 hour at room temperature. Sections were washed in PBS, reacted with horseradish peroxidase–conjugated streptavidin (1:5000; Dako), and developed with 3,3-diaminobenzidine. Negative controls (Blank) were prepared by substitution of the primary antibody with an irrelevant antibody. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

**Capillary density**

To determine capillary density, LV sections were incubated with a biotinilated lectin from Bandeiraea simplicifolia (Sigma) and amplified by a Tyramide Signal Amplification (TSA) Biotin System kit (Perkin Elmer Life Sciences, MA). Sections were washed in PBS, reacted with horseradish peroxidase–conjugated streptavidin (1:5000; Dako), and developed with 3,3-diaminobenzidine. Negative controls (Blank) were prepared by substitution of the primary antibody with an irrelevant antibody. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

**Echocardiography**

Echocardiography was performed on conscious mice (following 7 days of ISO treatment) and on rats (8-weeks post-MI and 12-weeks following gene therapy) respectively, with a Vevo770 (VisualSonics) or an HDI 5000 (Philips) echocardiograph.
**Catheter-based in vivo hemodynamic measurements**

Cardiac function was measured 12 weeks following gene therapy (20 weeks after MI) in anesthetized rats (2% isofluorane; v/v) using 2 F pressure catheter (SPR-320; Millar instruments; Houston, TX). The pressure transducer was placed into the LV cavity through the right carotid artery and the right external jugular vein was cannulated with a P-10 catheter (Becton-Dickinson, Sparks, MD) that was used for ISO administration (333 ng/Kg BW).

**Measurement of infarct size**

Briefly, hearts were frozen in liquid nitrogen and sectioned from apex to base into 2-mm slices. To delineate the infarct size, sections were incubated in 1% (wt/vol) triphenyltetrazolium chloride (TTC, Sigma) in PBS (pH 7.4) at room temperature for 15 min. For each section, the infarct size of the LV was calculated from enlarged digital photos using SigmaScan 5.0 software.

**Co-Immunoprecipitation (Co-IP) assay**

WTβ1AR-Flag cells were transfected with cDNA encoding for S1PR1-GFP. Following stimulation with ISO (1µM) or S1P (250 nM) for 30 minutes the cells were lysed and subjected to Co-IP assay, using a commercial kit (Pierce), according to manufacturer instructions. IP of S1PR1 was performed using an Anti-GFP antibody (Upstate). Total lysates and Co-IP elutions were then subjected to immunoblot. For β1AR immunoblot an anti-Flag antibody (Sigma) was used.

**Treatment protocol for mice**

As previously described (1), C57BL/6 mice (n=5) were intra-peritoneal injected, with SEW2871, dissolved in DMSO Tween 20, at the total rate of 1 mg/kg/d over a period of 7 days. Control mice (SHAM, n=5) were injected with vehicle (0.002% ascorbic acid). At sacrifice, after heart
weight (HW) and body weight (BW) ratio calculation, the hearts were removed and cardiac chambers dissected.

*SphK1 and Akt immunoblots*

SphK1 was detected by using 1:1000 anti-mouse IgG (Santacruz). Phosphorylation of Akt was detected by protein immunoblotting using a 1:1000 anti-mouse IgG (Cell Signaling). Total Akt was detected by protein immunoblotting using a 1:1000 anti-rabbit IgG (Cell Signaling).

*ELISA assay*

Total blood serum S1P levels were measured using a commercial kit (Echelon), according to manufacturer instructions. 1 mL of blood was collected from rat groups (n=10 of each group) at the end of the study period prior to heart explantation. Then the blood was centrifuged to 2000 rpm a 15°C for 15 minutes and 25 μL of serum were used for the ELISA assay.

*Hematoxylin and Eosin staining*

Left ventricular specimens were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and re-hydratation, 4 μm-thick sections were prepared, mounted on glass slides and were stained with hematoxylin for 5 min and with eosin for 2 min. Then the slides were washed for 30 sec with tap water and then rapidly dehydrated with 100% ethanol and mounted. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

**References**

Supplementary Figure legends

Supplementary Figure 1

β1AR and S1PR1 reciprocal interaction

A. Representative panels of Co-IP assay in total lysates from HEK293 cells stably expressing WTβ1AR-Flag-S1PR1-GFP. Immunoprecipitated proteins (IP) for GFP (S1PR1) were blotted with an antibody anti-Flag (β1AR).

Supplementary Figure 2

The lack of GRKs-phosphorylation sites on S1PR1 inhibits the cross-talk between β1AR and S1PR1

A. HEK293 cells stably expressing WTβ1AR-Flag transfected with cDNA encoding for S1PR1-GFP or S1PR1-Δ32-GFP were pre-treated with β2AR antagonist ICI-118,551-HCl (ICI, 10 μM), then were stimulated with (-)-isoproterenol bitartrate (ISO) (1 μM) or sphingosine 1-phosphate (S1P) (250 nM) for 30 min and compared with unstimulated (NS). Representative panels of S1PR1-GFP, S1PR1-Δ32-GFP and β1AR-Flag immunofluorescence images, showing cumulative data of multiple independent experiments. Arrows indicate receptor internalization; Representative immunoblots showing ERK1/2 activation following 5 min of stimulation with ISO (1 μM) or S1P (250 nM) in WTβ1AR-Flag. GAPDH was used as loading control.

Supplementary Figure 3
In vivo chronic S1PR1 agonism (7 days) resulted in a GRK2 upregulation and a β1AR plasma membrane downregulation.

A. Bar graphs showing the heart weight/body weight (HW/BW) ratio in SHAM and SEW2871-7d;

B. Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple independent experiments to evaluate GRK2 levels in SEW2871-7d groups compared to SHAM (GRK2 levels Fold over SHAM);

C. Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple independent experiments to evaluate β1AR plasma membrane levels in crude LV membrane preparations from SHAM and SEW2871-7d mice (βAR Membrane levels Fold over SHAM). ACTIN was used as loading control.

Data are expressed as means ± SEM. Statistical significance between groups was determined by Mann-Whitney exact test. N= 5 for each group. *p<0.05 vs SHAM.

Supplementary Figure 4

S1PR1 natural agonist levels are decreased during HF

A. Bar graphs showing S1P levels (µM) in blood serum collection from SHAM, HF+ rAVV6-GFP and HF+rAVV6-S1PR1 groups.

B. Representative immunoblots (upper panels) and densitometric analysis (lower panel) evaluating Sphingosine kinase 1 (SphK1) levels in HF+rAVV6-GFP and HF+rAVV6-S1PR1 groups compared to SHAM (SphK1 levels Fold over SHAM). GAPDH was used as loading control.
Data are presented as mean ± SEM. Statistical significance between groups was determined by one-way ANOVA with Bonferroni post-hoc correction. N= 10 for each group. *p<0.01 vs SHAM; †p<0.05 vs HF.

**Supplementary Figure 5**

**Reduced immune cells infiltration following S1PR1 gene therapy**

A. Representative Hematoxylin/Eosin panels of remote and infarcted region from cardiac sections of HF, HF+rAVV6-S1PR1 and HF+rAAV6-GFP rats, performed at the end of the study period;

**Supplementary Figure 6**

**S1PR1 gene-delivery preserve Akt activation**

A. Representative immunoblots (upper panels) and densitometric analysis (lower panel) evaluating pAkt levels in HF+rAVV6-GFP and HF+rAVV6-S1PR1 groups compared to SHAM (pAkt levels Fold over SHAM). tAkt was used as loading control.

Data are presented as mean ± SEM. Statistical significance between groups was determined by one-way ANOVA with Bonferroni post-hoc correction. N= 10 for each group. *p<0.01 vs SHAM; †p<0.05 vs HF.
Supplementary Figure 4

A

S1P Blood serum levels (μM)

<table>
<thead>
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<th>SHAM</th>
<th>HF</th>
<th>HF+AAV6-S1PR1</th>
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<tr>
<td>S1P</td>
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<td>5</td>
<td>30</td>
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B

IB: SphK1

IB: GAPDH

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<th>HF+AAV6-S1PR1</th>
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<tbody>
<tr>
<td>S1P</td>
<td>20</td>
<td>5</td>
<td>30</td>
</tr>
</tbody>
</table>

† Significant difference compared to SHAM.
Supplementary Figure 6

A

IB: pAkt

IB: total Akt

Fold over SHAM

SHAM  HF  HF+AAV6-S1PR1

Statistical significance:
* p < 0.05 compared to SHAM
† p < 0.05 compared to HF