β1-Adrenergic Receptor and Sphingosine-1-Phosphate Receptor 1 Reciprocal Down-Regulation Influences Cardiac Hypertrophic Response and Progression Toward Heart Failure: Protective Role of S1PR1 Cardiac Gene Therapy

Running title: Cannavo et al.; Reciprocal down-regulation between S1PR1 and β1AR

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Abstract

Background—The Sphingosine-1-phosphate receptor 1 (S1PR1) and β1-adrenergic receptor (β1AR) are G protein-coupled receptors (GPCRs) expressed in the heart. These two GPCRs have opposing actions on adenylyl cyclase due to differential G protein-coupling. Importantly, both of these receptors can be regulated by the actions of GPCR kinase-2 (GRK2), which triggers desensitization and down-regulation processes. Although, classical signaling paradigms suggest that simultaneous activation of β1ARs and S1PR1s in a myocyte would simply be opposing action on cAMP production, in this report we have uncovered a direct interaction between these two receptors with a regulatory involvement of GRK2.

Methods and Results—In HEK293 cells overexpressing both β1AR and S1PR1, we demonstrate that β1AR down-regulation can occur after sphingosine 1-phosphate (S1PR1 agonist) stimulation while S1PR1 down-regulation can be triggered by isoproterenol (βAR agonist) treatment. This cross-talk between these two distinct GPCRs appears to have physiological significance since they interact and show reciprocal regulation in mouse hearts undergoing chronic βAR stimulation and also in a rat model of post-ischemic heart failure (HF).

Conclusions—We demonstrate that restoring cardiac plasma membrane levels of S1PR1 produce beneficial effects counterbalancing deleterious β1AR overstimulation in HF.

Key words: gene therapy, heart failure, hypertrophy, receptors, adrenergic, beta, signal transduction
Introduction

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, therefore uncoupling receptors from their downstream signaling effectors. This process continues through β-arrestin-dependent internalization of receptors, that lead either to their degradation and down-regulation or 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\(^1\). In the heart, an important GRK-mediated β-arrestin-dependent signal has been shown to be ERK MAP kinase activation\(^2\). Given the central role in cardiac pathophysiology, GPCRs are critical therapeutic targets in cardiac diseases. This is especially true in heart failure (HF) where β-adrenergic receptor (βAR) antagonists and angiotensin II receptor blockers are standard of care for human HF patients.

βAR and angiotensin II receptor blockade are warranted as a consequence of the sympathetic nervous system (SNS) and renin-angiotensin system (RAS) hyperactivity that induces their overstimlation. This phenomenon represents the molecular basis for βAR down-regulation in the failing human myocardium. Since GPCRs are dynamically regulated in disease processes a better understanding of downstream signaling is imperative. In particular, GPCR dimerization\(^3\) and interaction between different GPCR signaling pathways\(^4,5\) have become the cornerstone of current cardiovascular research in order to better clarify molecular alterations underlying cardiovascular diseases and to identify novel potential therapeutic targets. In cardiac
physiology and pathophysiology, the β1AR is the predominant βAR that regulates inotropic and chronotropic responses of SNS catecholamines through Gs-dependent activation of adenylyl cyclase (AC)\(^6\). Chronic β1AR hyperstimulation results in their down-regulation and consequently there is a marked reduction of the inotropic reserve of the failing heart\(^8\). 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\(^10\) and its signaling opposes β1AR-mediated AC activation through its coupling to the AC-inhibitory G protein, Gi\(^11\). Thus, in the heart, S1PR1 is able to antagonize the effects mediated by isoproterenol (ISO) and other βAR agonists\(^11,12\). Furthermore, S1PR1 and β1AR undergo GRK-mediated regulation through phosphorylation\(^13,14,15\). Although GRK2, the primary GRK isoform expressed in myocytes, can regulate both receptors, the β1AR is also regulated by phosphorylation by protein kinase A (PKA)\(^13,14\), while the S1PR1 can be regulated by protein kinase C (PKC)\(^15\). Importantly, in respect to novel non-canonical signaling both receptors have been implicated in ERK activation that can lead to protective signaling\(^2\).

Recently, a functional interaction between βAR and S1PR1 signaling has been reported in vivo. In fact, ISO administration in mice induces cardiac hypertrophy via engagement of the S1PR1 signaling pathway\(^12\). 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 two 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 (PKAβ1AR-Flag) and the putative GRK phosphorylation sites (GRKβ1AR-Flag) obtained as previously described\textsuperscript{14} and cardiomyoblasts H9c2 obtained from American Type Culture Collection (ATCC), were cultured and transfected as briefly described in online methods.

**Confocal microscopy**

S1PR1-GFP and β1AR-Flag internalization was visualized by confocal laser scanning microscopy (CLSM). Following (-)-isoproterenol bitartrate (ISO, 1 µM; Sigma-Aldrich) or sphingosine-1 phosphate (S1P, 250 nM; Sigma-Aldrich) stimulation cells were fixed and visualized as previously described (16). After fixation cells were incubated with an anti-Flag Cy3 conjugated mouse IgG (Sigma-Aldrich) using a dilution 1:100 in PBS containing 0.5% BSA for 1h at room temperature, CLSM was performed at 488 nm (GFP) or 568 nm (Cy3). The fluorescent data sets were analyzed by LSM 510 software. The cells treated with ISO were pre-treated with selective β2AR-antagonist ICI-118,551-HCl (ICI, 10 µM; Sigma-Aldrich). Each experiment was separately repeated at least three times.

**Immunoblotting**

Immunoblotting on cells and left ventricular (LV) samples were performed as previously described\textsuperscript{16} and briefly reported in the online supplemental methods.

**Treatment protocol for mice**

As previously described\textsuperscript{2,17,18}, C57BL/6 mice (n=5) were subcutaneously injected, twice a day, with ISO, dissolved in 0.002% ascorbic acid, at the total rate of 3 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.

**Measurement of hypertrophic growth in H9c2 cardiomyoblasts**

Hypertrophy was assessed by measurement of relative cell surface area of H9c2 cells as previously described\(^{19,20,21}\) (please see online supplemental methods).

**TUNEL staining**

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on H9C2 cells (please see online supplemental methods).

**Rat MI model**

Myocardial infarction (MI) in rats was performed as previously described\(^ {22}\) and briefly reported in the online supplemental methods.

**Myocardial in vivo gene delivery**

Myocardial gene transfer in rats was achieved by direct intra-myocardial injection 8 weeks post-MI as previously described\(^ {23}\) and briefly reported in the online supplemental methods.

**Echocardiography**

Echocardiography was performed as previously described\(^ {22,24}\) and briefly reported in the online supplemental methods.

**Catheter-based in vivo hemodynamic measurements**

Cardiac function was measured 12 weeks following gene therapy as previously described\(^ {23}\).

**Myocardial perfusion studies**

Myocardial perfusion was determined using 15 \(\mu\)m fluorescent microspheres (Triton Inc.). Cardiac and blood samples were processed for microspheres determination. Myocardial blood flow was measured basal and after maximal vasodilation with dipyridamole (6 mg kg\(^{-1}\) min\(^{-1}\)
i.v.).

**Measurement of infarct size**

Infarct size was examined in all experimental rats at the end of the study period, as previously described\(^{23}\) and briefly reported in the online supplemental methods.

**Histology**

Left ventricular paraffin embedded specimens were immunohistochemically stained for S1PR1 (anti-S1PR1 mouse monoclonal, 1:100; abm) and GFP (anti-GFP mouse monoclonal, 1:200; Upstate) or stained for capillary density determination as previously described\(^{16}\) and briefly reported in online methods.

**RT-PCR**

Total RNA was isolated from cardiac samples using TRIzol reagent (invitrogen) according to the manufacturer’s instructions and was reversed transcribed to generate cDNA. To evaluate the expression of recombinant human S1PR1-GFP and GFP was performed a PCR using specific primers (hS1PR1-For 5’-CAGCAAATCGGACAATTCCT-3’, hS1PR1-Rev 5’-GAACCTTCAGGGTCAGCTTG-3’, GFP-For 5’-GACGTAAACGGCCACAAGT-3’, GFP-Rev 5’-AAGTCGTGCTGCTTCATGTG-3’) with respectively amplified products of 250 bp and 180 bp.

**β-Adrenergic Receptor Radioligand Binding**

Receptor binding with 20 µg of protein from plasma membrane was performed using \(^{[125]}I\)cyanopindolol (350 pM) as previously described\(^{24}\). Receptor density (fmol) was normalized to milligrams of membrane protein.

**Statistical analysis**

Normally distributed, continuous variables with sample size greater than 10 are expressed as

\[ \text{mean} \pm \text{standard deviation} \]
mean ± SEM and compared by one-way or repeated measures ANOVA test followed by Bonferroni post hoc correction, as appropriate. When sample size was less than 10, exact tests (Mann-Whitney or Kruskal-Wallis as required), followed by Bonferroni post hoc correction, were performed and data represented with median and dot plots. Normality was tested using Shapiro-Wilk test (<50 values) or Kolmorov-Smirnov test (≥50 values), as appropriate. All analyzed data showed no significant departure from normal distribution. Statistical Analysis was performed using SPSS software (SPSS Inc., Chicago, Illinois). Level of statistical significance was set top ≤ 0.05.

Results

In vitro reciprocal downregulation of β1AR and S1PR1

In order 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 (PKAβ1AR) or the putative GRK phosphorylation sites (GRK β1AR) were transfected with S1PR1 cDNA. By confocal microscopy experiments we evaluated β1AR and S1PR1 internalization following ISO and S1P stimulation. As shown in Figure 1A, ISO and S1P stimulations resulted in a marked loss of both WTβ1AR and S1PR1 from the cell surface and surprisingly, in a co-localization of the two receptors in the cytosol peaking after 30 min of treatment. The absence of PKA phosphorylation sites (PKAβ1AR) did not affect the reciprocal internalization and cytosolic co-localization following ISO or S1P stimulation (Figure 1B). In contrast, as shown in Figure 1C, in GRKβ1AR cells, ISO stimulation only induced β1AR but not S1PR1 internalization. On the other hand, S1P stimulation induced S1PR1 downregulation with no effect on β1AR localization. Interestingly,
ISO or S1P induced a similar significant increase in pERK levels in all cell subtypes (Figure 1A-B-C). To better determine whether β1AR and S1PR1 form stable complexes, β1AR and S1PR1 were expressed at same level in HEK293 cells and then a co-immunoprecipitation (Co-IP) assay was performed. Notably, as shown in Supplemental Figure 1, IP of S1PR1 resulted in a Co-IP of β1AR in the absence (NS) or in presence of ISO or S1P, confirming 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) lacking GRK2 phosphorylation sites15. As shown in supplementary Figure 2A, S1PR1-Δ32 lost the ability to be co-internalized with β1AR following agonists stimulation. Furthermore, ERK1/2 phosphorylation resulted increased following ISO stimulation but at a lower extent following S1P stimulation (Supplemental Figure 2A).

β1AR- and S1PR1-dependent GRK2 upregulation
Since it is known that both β1AR and S1PR1 are substrates of GRK2 phosphorylation13,14,15, and our data suggest a crucial role of this kinase in the co-dependence of internalization of these two receptors, we further explored the potential role of GRK2. A 2-fold increase in GRK2 levels in WTβ1AR cells stimulated for 12 hrs either with ISO or S1P, was observed (Figure 2A). In PKAβ1AR cells, ISO or S1P stimulation resulted in blunted, yet significant, GRK2 upregulation (Figure 2B). Importantly, GRKβ1AR cells showed enhanced GRK2 expression only following S1P administration (Fig. 2C).

In vitro protective role of S1PR1 on deleterious β1AR overstimulation
It is known that both β1AR and S1P are able to induce cardiomyocyte hypertrophy24,25. Therefore, we tested the effects of chronic ISO and S1P administration in vitro in H9c2 cells.
stimulated for 48 hrs in 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 indicating a hypertrophic response that was blocked with the respective antagonist (metoprolol or W146) (Figure 3A). Notably, only ISO induced an increase in cardiomyocytes apoptosis (Figure 3B). This apoptotic response in ISO stimulated cells was prevented by metoprolol pre-treatment (Figure 3B). Interestingly, S1P induced a robust hypertrophic response and blunted apoptotic reaction in metoprolol pre-treated cells (Figure 3A-B). Reciprocally, ISO stimulation in the presence of S1PR1 antagonist did not induce an hypertrophic response but increased apoptosis (Figure 3A-B).

In vivo reciprocal β1AR and S1PR1 downregulation

As widely demonstrated, chronic in vivo ISO 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 analysed S1PR1 density in crude myocardial membrane preparations from mice after 7 days of ISO administration. As expected, after chronic ISO stimulation mice exhibited a significant increase in Heart to Body Weight (HW/BW) ratio (Figure 4A), LV-septum thickness (Figure 4B) and a robust cardiac GRK2 upregulation compared to untreated mice (Figure 4C). Notably, 7 days of ISO administration resulted in a S1PR1 downregulation at the plasma membrane level (Figure 4D).

In addition, in order to evaluate whether S1PR1-selective agonist was able to induce a similar β1AR downregulation in vivo, we treated mice for 7 days (via daily intraperitoneal injections) with the S1PR1 agonist, SEW2871 (Supplemental Figure 3). Interestingly, SEW-treatment resulted in a consistent increase in HW/BW ratio (Supplemental Figure 3A) and in a strong increase in GRK2 protein levels (Supplemental Figure 3B) compared to untreated mice.
Consistently, with our in vitro observations, this S1PR1 selective agonist resulted in a robust decrease in β1AR cardiac plasma membrane levels compared to untreated group (Supplemental Figure 3C).

**Cardiac S1PR1 membrane downregulation during heart failure**

Since our data show that S1PR1 internalization occurs in vitro as well as in vivo following chronic ISO 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 8 week post-myocardial infarction (post-MI) rats. Importantly, we found a significant down-regulation of S1PR1 in HF rats compared to control (Figure 5A). Accordingly, in order to assess whether S1PR1 down-regulation during HF was induced by an increase in S1P levels we performed an ELISA assay for S1P on blood serum and an immunoblots on LV lysates to assess the expression of Sphingosine kinase 1 (SphK1) in our HF and SHAM groups (Supplemental Figure 4A-B). Notably, both S1P circulating levels and SphK1 expression were robustly reduced in HF group compared to SHAM, proving that S1PR1 behaves similarly to β1AR in a significant pathophysiological setting such as HF. In contrast, S1PR1 gene therapy was able to restore SphK1 expression at the levels observed in sham and to increase S1P circulating levels compared to HF control group.

**In vivo S1PR1 gene therapy**

Since we have demonstrated that S1PR1 signaling is beneficial in cardiomyocytes in vitro, and that this receptor is down-regulated 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). 8 weeks post-surgery HF rats were
randomly assigned to one of the following group: 1) HF Saline (HF, n=10); 2) HF Adeno-associated type 6-GreenFluorescent Protein (HF-rAAV6-GFP, n=10); 3) HF rAAV6-S1PR1-GFP (HF-rAVV6-S1PR1, n=10). A baseline echocardiogram was performed in all groups one 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 12 additional weeks (20 weeks after MI) (Figure 5B), and all assays in the HF groups were compared with a control SHAM-operated group that received 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, RT-PCR analysis (Figure 5D) and immunoblots (Figure 5E) confirmed the expression of the human S1PR1 in the heart of rAAV6-S1PR1 group and of GFP in both hearts of rAAV6-S1PR1 and –GFP groups. As shown in Figure 5F, 20 weeks following MI, cardiac rAAV6-S1PR1 gene therapy resulted in restoration of plasma membrane S1PR1 at 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 (EF) was dramatically decreased and end-diastolic diameter was increased as expected (Figure 6A-B). 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, EF was significantly increased in S1PR1 infected rats compared to HF controls (Figure 6B). Adverse LV remodeling as measured by ventricular dilatation also progressed further in saline and GFP groups, and this was prevented by S1PR1 gene delivery. As expected no differences in infarct size were observed among all HF groups since gene therapy was performed 8 weeks post-MI when the infarct scar was completely established (data not shown). Of note, S1PR1 gene delivery...
delivery affected the immune response as observed by Hematoxylin/Eosin staining of cardiac sections. In fact, S1PR1 overexpression resulted in a reduced infiltration of immune cells compared to HF control group (Supplemental Figure 5A). 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) showed significant decreases in +dP/dt and –dP/dt in all HF groups compared to SHAM, proving HF-related reduction in LV contractility and relaxation (Table 1 and Figure 6C). LV systolic pressure was significantly reduced, whereas LV end-diastolic pressure was significantly increased in all HF groups compared to SHAM (Table 1). Cardiac S1PR1 overexpression significantly improved LV contractility and relaxation 12 weeks after treatment (Table 1 and Figure 6C). Furthermore, S1PR1 gene delivery increased LV systolic pressure and decreased end-diastolic pressure compared to HF control groups (Table 1). 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 ISO (Table 1 and 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 (AWd) and posterior wall diastolic thickness (PWd) in S1PR1 rats compared to HF controls (Table 1). Consistently, HW/BW ratio was significantly higher in hearts treated with S1PR1 compared to GFP and saline treated groups (Table 1). Importantly, the S1PR1-dependent increase of LV mass was accompanied by significant decrease of LV systolic and diastolic internal diameter compared to
rAAV6-GFP HF hearts, thus indicating that S1PR1 gene therapy induced a compensatory hypertrophic response able to contrast LV dilatation. In accordance with previous observations reported by us and others\textsuperscript{25,26}, this adaptive LV remodeling was associated with a significant growth of cardiac capillary network. In fact, S1PR1 gene delivery resulted in a significant increase in capillary density compared to HF groups and a complete recovery of myocardial blood flow that was indistinguishable from SHAM (Figure 7A-B). At the molecular level, we investigated the effects on Akt activation since this kinase is one of the major pro-angiogenetic molecules involved in S1PR1 signaling\textsuperscript{27,28}. Notably, S1PR1 down-regulation was associated with a robust reduction in Akt activation compared to SHAM (Supplemental Figure 6A). Of note, S1PR1 gene therapy was able to enhance Akt activation (Supplemental Figure 6A).

Discussion

Heart failure is a major and growing public health problem affecting 1–6% of the US population\textsuperscript{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 SNS, which plays a crucial role in adapting circulatory homeostasis to changes in environment. Further, circulating levels of catecholamines are increased in HF in proportion to the severity of the disease\textsuperscript{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\textsuperscript{31}. These observations have led to the hypotheses that sympathetic activation may play an important role in HF progression\textsuperscript{8,32,33} and that pharmacological interference with this system can produce hemodynamic and clinical
benefits. Currently, some of the most effective treatments for HF target β1AR and β2AR and angiotensin II type IA receptor, which are both GPCRs. Several experimental evidences have demonstrated that GPCRs can actually interact and can be reciprocally regulated. For instance, it has been shown that the β1AR is able to transactivate EGFR, conferring a β-arrestin dependent cardioprotective effect. These data have led to hypothesize the development of new therapies for HF that would be able not only to antagonize harmful cardiac signaling, but also potentiate the beneficial pathways. In that regard, a deeper understanding of the signaling mechanisms underlying the development and progression of heart failure is absolutely needed.

Our data show, for the first time, a direct interaction between β1AR and S1PR1, representing dynamic regulation present between two 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 ISO or S1P stimulation induce a dual-internalization with a cytosolic co-localization of both β1AR and S1PR1. As a functional consequence, ERK activation occurs. Importantly, we also demonstrated that both β1AR and S1PR1 are able to form a stable complex and that the reciprocal downregulation of the two receptors occurs only in presence of agonist-dependent GRK2 activation. In fact, we examined the molecular mechanisms of such interaction, and interestingly 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 two receptors. Similarly, the lack of GRK2-phosphorylation sites on S1PR1 inhibited the reciprocal down-regulation between β1AR and S1PR1 following agonists stimulation. Therefore, GRK2 seems to be a nodal regulator of the cellular response to both cathecolamines 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 both in physiological and pathophysiological settings\(^\text{36,37}\) and its action can become deleterious over time. An important role has also been recently shown for the S1PR1. In fact, this receptor can mediate an hypertrophic response in neonatal rat cardiomyocytes\(^\text{38}\) and it is cardioprotective for adult cardiomyocytes under hypoxia\(^\text{10}\) and in the intact heart exposed to myocardial infarction\(^\text{39,40,41,42}\). Our data confirm S1PR1 ability to mediate hypertrophy in cultured cells as well as the β1AR. However, while β1AR is also able to induce significant apoptosis, this is not the case of S1PR1. More interestingly, the apoptotic response to ISO stimulation increases if the S1PR1 receptor is blocked. Our data demonstrate that S1PR1 signaling results to be cardioprotective, in particular during a sustained catecholamine stimulation which reproduces pathological condition such as HF. In fact, our demonstration that S1PR1 is downregulated in the pathological cardiac hypertrophy induced by ISO 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 show that in a rat model of post-ischemic HF, S1PR1 plasma membrane levels are significantly downregulated. Further, our results showed that the observed S1PR1 down-regulation was not correlated to an S1P increase. In fact, this molecule was even strongly reduced in blood serum of post-MI HF mice compared to SHAM, strengthening our hypothesis of an active role of catecholamine overstimulation \textit{in vivo} on reciprocal down-regulation between β1AR and S1PR1.

Of note, rAVV6 gene therapy, as previously described\(^\text{43}\), allowed us to obtain the 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 weeks 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 SphK1 expression in the heart and S1P active secretion in blood serum, and exerts an important functional and structural cardiac recovery, improving cardiac function and blocking the negative remodelling in our post-MI rat HF model. Three months after gene delivery we found significantly increased LV EF, dP/dt and systolic blood pressure whereas LV-end diastolic diameter and pressure were decreased in comparison to 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 due to either a direct cardioprotective effect and to 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 the activation of Akt signaling, preventing capillary rarefaction and completely restoring myocardial blood flow in our experimental groups. Taken together, our data show that S1PR1 downregulation, probably induced by an excessive catecholamine stimulation during HF, can be responsible for the progression toward a further decrease of cardiac performance. Importantly, in the present study we focused on S1PR1 that represents the S1PR subtype with 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, confers to this molecule the potential to be a candidate for HF treatment.
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**Conflict of Interest Disclosures:** None.

**References:**


Table 1. Echocardiographic, hemodynamic and physical parameters at 12 weeks after rAAV6-Mediated Cardiac Gene Delivery.

<table>
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<tr>
<th>Echocardiography</th>
<th>SHAM</th>
<th>HF</th>
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<tr>
<td>HR, bpm</td>
<td>330.5±11.6</td>
<td>322±9.6</td>
<td>290±8.1*†</td>
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<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>6494.8±244</td>
<td>4461±179.8*</td>
<td>5260±245.7*†</td>
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<td>LV −dP/dt, mm Hg/s</td>
<td>6968±327.8</td>
<td>3626±106.1*</td>
<td>4251.4±128.8*†</td>
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<td>LVEDP, mm Hg</td>
<td>2.2±0.3</td>
<td>13.5±1.1*</td>
<td>/.<em>±0.9</em>†</td>
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<td>LVESP, mm Hg</td>
<td>132.2±3.6</td>
<td>104.1±4.9*</td>
<td>107.8±3.3*</td>
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<td><strong>Isoproterenol (333 ng/kg BW)</strong></td>
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<tr>
<td>HR, bpm</td>
<td>400±20.7</td>
<td>381±12.7</td>
<td>341.1±4.3*†</td>
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<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>15316±416.8</td>
<td>7476.5±140*</td>
<td>1003.6±272.4*†</td>
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<tr>
<td>LV −dP/dt, mm Hg/s</td>
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<td>5144.9±166.3*</td>
<td>7297.1±244.1*†</td>
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<tr>
<td>LVEDP, mm Hg</td>
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<td>11.4±0.9*</td>
<td>6.6±0.7*†</td>
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<tr>
<td>LVESP, mm Hg</td>
<td>126.7±2</td>
<td>99.7±2.7*</td>
<td>110.8±3.2*†</td>
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<td>BW, Kg</td>
<td>0.46±0.01</td>
<td>0.48±0.01</td>
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<tr>
<td>HW/BW, g/Kg</td>
<td>1.12±0.02</td>
<td>3.03±0.08*</td>
<td>3.55±0.13*†</td>
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Data are presented as mean ± SEM. Effect of S1PR1 gene therapy on LV function evaluated at 12 weeks after gene delivery is shown. In vivo Ejection fraction (EF), Anterior wall diastolic thickness (Awd), Anterior wall systolic thickness (Aws), LV internal diameter at systole (LVIDs), PWd, Posterior wall systolic thickness (PWs), Heart rate (HR), LV dP/dt, LV −dP/dt, LV end-diastolic pressure (LVEDP), LV end-systolic pressure (LVESP), 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 (FS%) was also measured in all groups ANOVA analysis and Bonferroni test were used between all groups. *p<0.05 vs SHAM; †p<0.05 vs HF control groups.

Figure Legends:

Figure 1. GRKs-phosphorylation sites removal inhibits the cross-talk between β1AR and S1PR1. HEK293 cells stably expressing WTβ1AR-Flag (A), PKAβ1AR-Flag (B), GRKβ1AR-
Flag (C) and transfected with S1PR1-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 and β1AR-Flag immunofluorescence images, showing cumulative data of multiple independent experiments in WTβ1AR-Flag+S1PR1-GFP (A), the PKA-β1AR-Flag+S1PR1-GFP (B) or the GRK-β1AR-Flag+S1PR1-GFP (C). 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 (A), PKA-β1AR-Flag (B), GRK-β1AR-Flag (C). GAPDH was used as loading control.

**Figure 2.** Reduced effect on GRK2 upregulation following GRKs-phosphorylation sites removal in β1AR. Representative immunoblots (upper panels) and dot plots (lower panels) showing GRK2 levels following 12 hours of stimulation with ISO (1 μM) or S1P (250 nM) in WTβ1AR-Flag (A), PKA-β1AR-Flag (B), GRK-β1AR-Flag (C). GAPDH was used as loading control (GRK2 levels Fold over NS mean); Statistical significance between groups was determined by exact tests (Mann-Whitney and Kruskal-Wallis) followed by Bonferroni post-hoc correction. Barred diamond represents the median. N= 9 for each group. *p<0.05 vs. NS; †p<0.05 vs. ICI/ISO.

**Figure 3.** Physiological role for β1AR and S1PR1 interaction. Representative images (upper panels) and dot plots (lower panels) showing (A) hypertrophic (relative cell surface area) and (B) apoptotic (Tunel positive nuclei) response in H9c2 cells transfected with cDNA encoding for S1PR1-GFP and the mouse WTβ1AR-Flag. Cells were pre-treated with ICI (10 μM) and
stimulated with ISO (10 μM) or S1P (250 nM); cells pre-treated with β1AR antagonist
Metoprolol tartrate (MET, 1μM) or S1PR1 selective antagonist W146 (10 μM) were then treated
with ISO (10 μM) or S1P (250 nM) for 48 h. Each experiment was independently repeated 3
(panel A) and 4 (panel B) times. Statistical significance between groups was determined by one-
way ANOVA followed by Bonferroni post-hoc correction. Barred diamond represents the mean.
*p<0.05 vs NS; † p<0.05 vs all.

**Figure 4.** *In vivo* chronic ISO treatment (7 days) resulted in a GRK2 upregulation and an S1PR1
plasma membrane downregulation. **A.** Representative images (upper panel) of sirius red staining
of mouse cardiac sections from SHAM and ISO7d groups (25X) and dot plots (lower panel)
showing the heart weight/body weight (HW/BW) ratio in SHAM and ISO 7d: **B.** Representative
serial M-mode echocardiographic tracings (upper panel) before ISO and following ISO7d and
dot plots (lower panel) showing Septum thickness; **C.** Representative immunoblots (upper
panels) and dot plots (lower panel) of GRK2 levels in ISO7d groups compared to SHAM (GRK2
levels Fold over SHAM mean). **D.** Representative immunoblots (upper panels) and dot plots
(lower panel) of S1PR1 membrane levels in crude LV membrane preparations from SHAM and
ISO7d mice. ACTIN was used as loading control. GAPDH and Na+/K+ATPase were used as
membrane purification control (S1PR1 levels Fold over SHAM mean). Statistical significance
between groups was determined by Mann-Whitney exact test. N= 5 for each group. Barred
diamond represents the median. *p<0.05 vs SHAM.

**Figure 5.** The overexpression of S1PR1 restores the receptor plasma membrane levels impaired
during heart failure. **A.** Representative immunoblots (upper panel) and dot plots (lower panel)
showing S1PR1 membrane density in crude LV membrane preparations from SHAM and HF rats. ACTIN was used as loading control (S1PR1 levels Fold over SHAM mean). Statistical significance between groups was determined by Mann-Whitney exact test. N= 5 for each group. Barred diamond represents the median. *p<0.05 vs SHAM; B. Overall design of 20-week study of S1PR1 gene delivery in HF rats; C. Representative images of S1PR1 and GFP immunohistochemistry stainings on cardiac sections from HF, HF+rAAV6-S1PR1 and HF+rAAV6-GFP rats, performed at the end of the study period; D. Representative images (upper panel) of RT-PCR on cardiac total RNA lysates from HF, HF+rAAV6-S1PR1 and HF+rAAV6-GFP rats, showing the overexpression of transgenic human S1PR1 (hS1PR1) and GFP; E. Representative immunoblots (lower panel) showing the transgenic expression of human S1PR1 (hS1PR1) and GFP in HF, HF+rAAV6-S1PR1 and HF+rAAV6-GFP rats; F. Representative immunoblots (upper panels) and dot plots (lower panel) of S1PR1 membrane density in crude LV membrane preparations from SHAM, HF and HF+rAAV6-S1PR1 rats. ACTIN was used as loading control (S1PR1 Fold over SHAM mean). Statistical significance between groups was determined by exact tests (Mann-Whitney and Kruskal-Wallis) followed by Bonferroni post-hoc correction. N= 5 for each group, except N=7 for HF+rAAV6-S1PR1 group. Barred diamond represents the median. *p<0.05 vs SHAM; †p<0.05 vs HF.

**Figure 6.** rAAV6-S1PR1 gene therapy at 12 weeks after MI ameliorates cardiac function. Bar graphs showing cardiac parameters measurement at 12 weeks after MI and/or after 12 weeks of treatment. A. Data are presented as mean ± SEM; Statistical significance between groups was determined LV internal diameter at diastole (LVIDd) measured by echocardiography 12 weeks after MI and after 12 weeks of treatment; B. Ejection Fraction (EF) as measured by
echocardiography 12 weeks after MI (A) and 12 weeks after in vivo gene delivery; C. Average LV +dP/dt and LV -dP/dt values evaluated under basal conditions and after maximal isoproterenol stimulation; D. β1AR density in cardiac homogenates purified from hearts of all experimental groups performed at the end of the study period. Statistical significance between groups was determined by repeated measure ANOVA with Bonferroni post-hoc correction (panels A, B and C) and, by one-way ANOVA with Bonferroni post-hoc correction (panel D; barred diamond represents the mean). N= 10 for each group. *p<0.05 vs SHAM; †p<0.05 vs HF.

Figure 7. Beneficial effects of S1PR1 gene delivery on cardiac angiogenesis. A. Total myocardial blood flow measured at basal condition and after maximal coronary dilatation; B. Bar graph showing capillary density on mm2 ratio evaluated by Lectin Bandeiraea simplicifolia I (BS-I) staining of capillaries in cardiac section obtained from HF control groups and HF plus rAAV6-S1PR1. Statistical significance between groups was determined by repeated measure ANOVA followed by Bonferroni post-hoc correction. N= 10 for each group. *p<0.05 vs SHAM; †p<0.05 vs HF.
Figure 1
Figure 2

(A) WTβ1AR-FLAG+S1PR1-GFP
IB: GRK2
IB: GAPDH

(B) PKA-β1AR-FLAG+S1PR1-GFP
IB: GRK2
IB: GAPDH

(C) GRK-β1AR-FLAG+S1PR1-GFP
IB: GRK2
IB: GAPDH

Graphs showing the levels of GRK2 under different conditions.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
β1-Adrenergic Receptor and Sphingosine-1-Phosphate Receptor 1 Reciprocal Down-Regulation Influences Cardiac Hypertrophic Response and Progression Toward Heart 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 coniugated with GFP (S1PR1-GFP) while H9c9 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 de-paraffinization 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 de-paraffinization 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**

   Chronic sphingosine 1-phosphate 1 receptor activation attenuates early-stage diabetic nephropathy independent of lymphocytes. Kidney Int. 2011 May;79(10):1090-8
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 1

A

IP: Anti-S1PR1

Total Lysates

IB: β1AR

KS

WT ISO

WT SIP

KS

WT ISO

WT SIP

β1AR
Supplementary Figure 4

A

S1P Blood serum levels (μM)

SHAM  HF  HF+AAV6-S1PR1

B

IB: SphK1

IB: GAPDH

SHAM  HF  HF+AAV6-S1PR1

SK1 levels (Fold over SHAM)