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Sphingosine-1-phosphate (S1P) activates STAT3 to protect against de novo acute heart failure (AHF)

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ABSTRACT

Aims: Acute heart failure (AHF) is a burden disease, with high mortality and re-hospitalisations. Using an ex-vivo model of AHF, we have previously reported that sphingosine-1-phosphate (S1P) confers cardioprotection. However, the mechanisms remain to be elucidated. In the present study, we aimed to examine the role of the cardioprotective signal transducer and activator of transcription 3 (STAT3) in S1P mediated improved functional recovery in AHF.

Material and methods: Isolated hearts from male Long-Evans rats were subjected to hypotensive AHF for 35 min followed by a recovery phase of 30 min ($n \geq 4$/group). S1P (10 nM) was given during either the hypotensive or the recovery phase with/without an inhibitor of STAT3, AG490. Functional parameters were recorded throughout the experiment.

Key findings: Following an AHF insult, S1P, given during the recovery phase, improved the heart rate (HR) compared to the control (175.2 ± 30.7 vs. 71.6 ± 27.4 beats per minute (BPM); $p < 0.05$), with no changes in the left ventricular developed pressure. This effect was associated with an increase in phosphorylated STAT3 levels in the nucleus. Addition of AG490 with S1P abolished the cardioprotective effect of S1P (42.3 ± 17.1 vs. 148.8 ± 26.4 BPM for S1P; $p < 0.05$).

Significance: Our data suggest that S1P protects in an ex-vivo rat heart model of AHF by activation of STAT3 and provide further evidence for the usage of S1P as a potential therapy in patients suffering from AHF.

1. Introduction

Acute heart failure (AHF) is a global burden disease defined as “the rapid onset of, or change in, symptoms and signs of heart failure” [1,2]. It holds the highest post-discharge cardiovascular diagnosis in the elderly (> 65 years), with a third of the patients being re-hospitalised or dead within 90 days of previous discharge [3]. AHF can be widely divided into two sub-types: de novo and “acute-on-chronic” heart failure or acute decompensation of chronic heart failure (ADCHF) [3–5]. Various cardiac complications such as acute pulmonary oedema, hypertensive heart failure, right heart failure, cardiogenic shock and acute coronary syndromes precede AHF. Since the aetiology of AHF is so diverse, it is only logical that the same pattern of treatment doesn't follow the one-size-fits-all ideology and additional therapies are urgently needed. The standard treatment for AHF follows conventional approaches including the use of oxygen, diuretics, opioids, vasodilators, but the hospital admission rates remain high [2].

Sphingosine-1-phosphate (S1P), a sphingolipid well known as a critical component of high density lipoprotein (HDL), exerts multiple cardioprotective effects including vasodilation and inhibition of atherosclerosis [6]. In cells, S1P is formed by phosphorylation of sphingosine in the presence of sphingosine kinase 1 [7,8]. In an ischemia-reperfusion (I/R) setting, both endogenous and exogenous S1P decrease infarct size and improve functional recovery [9]. This protective effect is mediated, at least in part, by the Survivor Activating Factor Enhancement (SAFE) as well as the Reperfusion Injury Salvage Kinase (RISK) pathways, which involve the activation of signal transducer and activator of transcription 3 (STAT3) and Akt, respectively [6,10,11]. Despite our knowledge of cardioprotective effects of S1P in the I/R setting, very little is known about effects of S1P in AHF. Knapp et al. report reduction in sphinganine and free sphingosine in plasma from patients with systolic failure, regardless of consistent S1P levels between patients and controls [12]. In our preliminary study, we successfully demonstrated that administration of S1P in an isolated rat heart model of de novo AHF improves the recovery of the heart [13]. However, the mechanisms of protection with S1P remain to be elucidated. In the

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present study, we therefore explored the mechanisms of protection of S1P in this model of de novo AHF and we hypothesized that STAT3 may be an important mediator by which S1P exerts its benefit.

2. Materials and methods

2.1. Animals

Male Long Evans rats (250–320 g) were obtained from the animal unit at the University of Cape Town, Cape Town, South Africa. The rats received standard chow and water ad libitum and were housed in cages at 22 °C in a 12 h day/night cycle. The procedures were approved by the University of Cape Town animal ethics committee (project number: 011/038). All animal experiments were performed in accordance with the National Institutes of health guide for the care and use of laboratory animals.

2.2. Isolated rat heart model

Rats were anaesthetised with sodium pentabarbitone (60 mg/kg intraperitoneally) and heparin, as previously described [14]. In absence of the pedal reflex, a sufficient degree of anaesthesia was established and the skin was incised at the xyphoid-sternum before incision of the ribs to open the thoracic cavity. The heart was rapidly excised and transferred to a Petri dish containing ice cold Krebs-Henseleit buffer [15]. The buffer consisted of sodium chloride (NaCl) (118.5 mM, Sigma), sodium hydrogen carbonate ($NaHCO_3$) (25 mM, Merck), potassium chloride (KCl) (4.7 mM, Sigma), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) (1.2 mM, Merck), potassium di-hydrogen phosphate (KH_2PO_4) (1.2 mM, Sigma), glucose (11.1 mM, Merck) and calcium chloride dihydrate ($CaCl_2.2H_2O$) (1.3 mM, Sigma). The aorta was cannulated and the heart was mounted on the Langendorff retrograde perfusion system, as previously described [14]. A pressure transducer (AD Instruments) was attached to a balloon inserted into the left ventricle (LV) of the heart and the pulmonary artery was snipped to release the pressure in the heart caused by retrograde perfusion. The balloon was inflated and hearts were perfused at constant pressure while the temperature was maintained at 37 °C. Standard baseline parameters for every heart were: HR between 240 and 420 beats per minute (BPM) and diastolic pressure between 4 and 12 mm Hg. The acceptable baseline left ventricular developed pressure was > 80 mm Hg while the coronary flow of the heart was between 8 and 12 ml per minute.

2.3. Experimental protocol

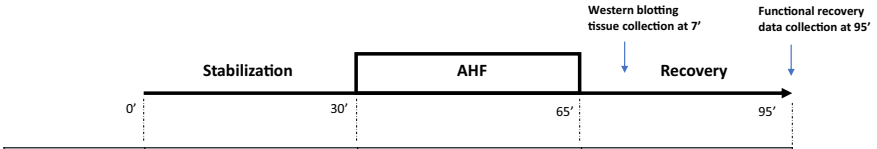
The protocol of the isolated heart study (Fig. 1) was divided into three phases: stabilization, AHF and recovery. Stabilization phase (30′) was characterised by normotensive perfusion (100 cm H_2O) with only glucose (11.1 mmol/L) as the substrate in the perfusate. This was followed by the AHF phase (35′) where the perfusion pressure was dropped to 20 cm H_2O. Concentration of glucose was dropped to 2.5 mmol/L and free fatty acids (1.3 mmol/L) were introduced in the perfusate, as previously described [13]. AHF is accompanied with increased neurohormonal stimulus. To better mimic the clinical setting of AHF, we added adrenaline during the AHF phase of the already established isolated heart model of de novo AHF [13]. We therefore administered adrenaline (10–8 M) to induce a severe model of AHF. Previous work done in our lab by Horak and Opie [16] showed reduced efficiency of pressure work or "oxygen wastage" at this concentration of adrenaline in catecholamine induced myocardial injury. In the recovery phase, the perfusion pressure was returned to normotensive conditions (100 cm H_2O) while the substrate concentrations of glucose and FFA were maintained as per the AHF phase. Hearts were treated with S1P (10 nM) [13] during either the AHF phase or the recovery phase as shown in Fig. 1. AG490 (100 nM), an inhibitor of STAT3 pathway, was perfused during the AHF phase.

2.4. Western blot analysis

For Western blot analysis, tissue was collected at 7′ during the recovery phase, as previously described by Somers et al. [6] (see Fig. 1). Hearts (n = 4 per group) were snap frozen in liquid nitrogen and stored at −80 °C until analysis. Mitochondrial, cytosolic and nuclear fractions were extracted as previously described [14]. Levels of phosphorylated and total STAT3 were analysed by sodium dodecyl sulphate (SDS) gel electrophoresis [6]. Voltage dependent anion channel (VDAC), β-actin and Lamin were used as loading markers for mitochondrial, cytosolic and nuclear fractions, respectively. Densitometric analysis was performed using ImageJ software.

2.5. Statistical analysis

All data are expressed as mean ± standard error of the mean. For multiple comparisons, one or two way Anova and Bonferroni post tests were used and $p < 0.05$ was considered statistically significant.



	Stabilization (30′; 100 cm H_2O)	AHF (35′; 20 cm H_2O)	Recovery (30′; 100 cm H_2O)
Control (n=11)	Glucose (11.1mmol); no FFA	Glucose (2.5mmol); FFA (1.3mmol); Adr 10^{-8}M)	Glucose (2.5mmol); FFA (1.3mmol)
S1P (in AHF) (n=4)	Glucose (11.1mmol); no FFA	Glucose (2.5mmol); FFA (1.3mmol); Adr 10^{-8}M); S1P (10nM)	Glucose (2.5mmol); FFA (1.3mmol)
S1P (in recovery) (n=11)	Glucose (11.1mmol); no FFA	Glucose (2.5mmol); FFA (1.3mmol); Adr 10^{-8}M)	Glucose (2.5mmol); FFA (1.3mmol); S1P (10nM)
AG490+S1P (n=6)	Glucose (11.1mmol); no FFA	Glucose (2.5mmol); FFA (1.3mmol); Adr 10^{-8}M); S1P (10nM); AG490 (100nM)	Glucose (2.5mmol); FFA (1.3mmol)
AG490 (n=6)	Glucose (11.1mmol); no FFA	Glucose (2.5mmol); FFA (1.3mmol); Adr 10^{-8}M); AG490 (100nM)	Glucose (2.5mmol); FFA (1.3mmol)

Fig. 1. Experimental protocol. Abbreviations: FFA: free fatty acids; AHF: acute heart failure; S1P: sphingosine-1-phosphate.

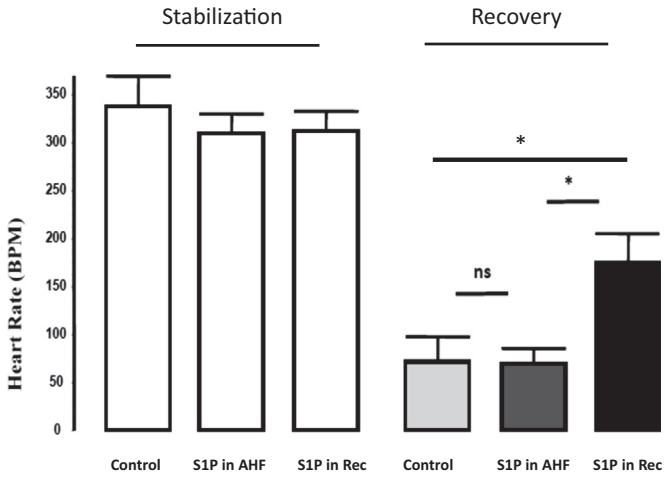


Fig. 2. Heart rate (at the end of stabilization and at end of recovery phase) of isolated hearts subjected to AHF with S1P (10 nM) given during the insult (S1P in AHF) or recovery phase (S1P in rec). BPM: beats per minute; ns: non-significant. *p < 0.05 vs. S1P given during the recovery phase.

3. Results

3.1. Sphingosine-1-phosphate improves recovery after an episode of hypotensive de novo AHF

In control hearts, an AHF insult significantly reduced the HR from 338.2 ± 31.0 BPM at baseline to 71.6 ± 27.4 BPM at the end of the recovery phase ($p < 0.001$) (Fig. 2).

The hearts treated with S1P during the AHF phase did not improve the HR compared to the control group (Fig. 2). However, S1P given during the recovery phase improved the HR significantly (*p < 0.05 vs. S1P in AHF). The stabilization heart rate values for S1P in AHF and S1P in recovery were: 310.4 ± 20.0 and 313.0 ± 20.2 BPM, respectively. At the end of recovery phase, these values for the respective groups were: 69.6 ± 15.7 and 175.2 ± 30.7 BPM (*p < 0.05 vs. control).

3.2. S1P treatment has no effect on the LV developed pressure or rate pressure product

In control hearts, an AHF insult significantly reduced the LV developed pressure from 84.8 ± 1.6 at baseline to 39.9 ± 23.1 mm Hg at the end of the recovery phase ($p < 0.001$) (Table 1).

The hearts treated with S1P exhibited no significant difference in the LV developed pressure irrespective of its phase of administration (see Table 1). The values for stabilization phase were 84.3 ± 1.0 and 86.7 ± 1.9 mm Hg for S1P in AHF and S1P in recovery, respectively. The values at the end of recovery for the same groups were: 49.0 ± 16.9 and 59.3 ± 8.5 mm Hg, respectively.

Similarly, the rate pressure product (RPP = HR × LVDP) did not differ between groups (see Table 1).

Table 1
Systolic, diastolic pressures and rate pressure product of isolated heart subjected to an acute heart failure (AHF) insult with/without sphingosine-1-phosphate (S1P; 10 nM) given during the insult or the recovery phase.

	Control	S1P in AHF	S1P in recovery
Systolic pressure (mm Hg)			
Stabilization	84.8 ± 1.1	88.1 ± 2.0	93.2 ± 2.8
Recovery	118.6 ± 31.8	113.0 ± 10.7	104.9 ± 12.1
Diastolic pressure (mm Hg)			
Stabilization	0.0 ± 1.5	3.8 ± 1.7	6.5 ± 1.8
Recovery	78.7 ± 23.7	63.9 ± 14.1	45.6 ± 7.2
Coronary flow (ml/min)			
Stabilization	9.5 ± 0.5	8.5 ± 0.2	9.2 ± 0.4
Recovery	10.6 ± 0.6	10.3 ± 0.3	11.0 ± 0.3
Rate pressure product			
Stabilization	30,597 ± 3488	26,656 ± 2384	27,134 ± 1807
Recovery	5603 ± 2901	5125 ± 1611	9979 ± 1765

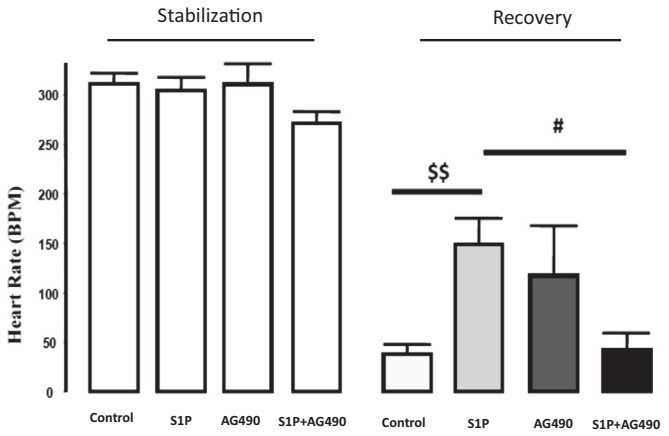


Fig. 3. Heart rate (at the end stabilization and at end of recovery phase) of isolated hearts subjected to AHF with S1P (10 nM) given during the recovery phase with or without the presence of AG490, an inhibitor of STAT3. $$p < 0.01 vs. control; #p < 0.05 vs. recovery S1P + AG490.

3.3. Effect of AG490 in S1P-induced protection in AHF

To test for activation of the STAT3 pathway by S1P, we treated hearts with AG490 during the AHF phase.

The presence of AG490 with S1P significantly reduced the benefit of S1P on the HR (Fig. 3) (42.3 ± 17.1 vs. 148.8 ± 26.4 BPM for S1P only; $p < 0.05$). AG490, when given alone, did not affect the HR compared to control group.

With regards to LVDP, addition of AG490 to the heart had no effect (Table 2). The recovery values for controls, S1P, AG490 and S1P + AG490 hearts were 30.4 ± 12.6, 55.6 ± 7.7, 22.3 ± 9.0 and 52.3 ± 21.1 mm Hg, respectively. Similar findings were observed for systolic pressure and rate pressure product.

3.4. S1P activates phosphorylation of STAT3 in the nucleus

Expression of STAT3 was studied in the nuclear, cytosolic and mitochondrial protein fractions of the heart. S1P treatment resulted in an increase in phosphorylated STAT3 following AHF insult in the nuclear fraction (Fig. 4). The mitochondrial and cytosolic fractions showed no significant changes in the expression of STAT3 following S1P treatment (data not shown). Addition of AG490 to the S1P treatment abolished phosphorylation of STAT3 in the nucleus (Fig. 4).

4. Discussion

Our study aimed to explore the mechanisms involved in the cardioprotective effect of sphingosine-1-phosphate using an ex vivo heart model of de novo AHF. Our data strongly suggest that S1P, given during the recovery phase following AHF, improves the pacemaker activity of the heart, an effect which is mediated via the activation of the STAT3 pathway. Indeed, S1P activates phosphorylation of STAT3 in the nucleus during the recovery phase and its protective effect is abolished in the presence of a STAT3 inhibitor.

We have previously described and validated, for the first time to our knowledge, a novel ex vivo heart model of AHF [13]. This model represents the advantage to be less invasive and more reproducible than an in vivo model of acute heart failure and it represents the ideal model to pre-screen novel putative novel cardioprotective therapies. It is also a

Table 2
Systolic, diastolic pressures and rate pressure product of isolated heart subjected to an acute heart failure (AHF) insult with S1P (10 nM) given during the recovery phase with/without AG490 (inhibitor of STAT3).

	Control	S1P	AG490	S1P + AG490
Systolic pressure (mm Hg)				
Stabilization	88.7 ± 2.6	89.8 ± 2.0	92.2 ± 3.1	94.3 ± 2.5
Recovery	96.5 ± 16.5	100.2 ± 7.6	110.1 ± 9.6	105.7 ± 17.6
Diastolic pressure (mm Hg)				
Stabilization	3.7 ± 1.4	2.6 ± 1.8	2.5 ± 2.9	5.3 ± 2.1
Recovery	66.0 ± 12.7	44.5 ± 5.1	87.7 ± 10.5	53.4 ± 9.8
Coronary flow (ml/min)				
Stabilization	8.7 ± 0.2	9.0 ± 0.2	8.6 ± 0.3	9.1 ± 0.3
Recovery	10.9 ± 0.3	10.9 ± 0.3	10.1 ± 0.3	11.0 ± 0.6
Rate pressure product				
Stabilization	26,433 ± 1112	26,508 ± 1273	27,752 ± 1604	24,013 ± 535
Recovery	1941 ± 987	8461 ± 1350	3975 ± 1832	2871 ± 1268

very convenient model to better understand the signalling mechanisms involved in AHF and cardioprotection. Previous work conducted by our group during the AHF insult demonstrated that addition of S1P in the absence of adrenaline improved recovery against AHF [13]. In the present study, adrenaline was given with the aim to better mimic the clinical situation of AHF where neurohormonal activation occurs [16]. The addition of adrenaline during the AHF phase, along with increased FFA and reduced glucose oxidation and expectedly led to a worse haemodynamic outcome of the heart with reduction of the heart rate, contractility and an increase in LV-end diastolic pressure. Unfortunately, we did not measure markers of injury such as troponin I, lactate dehydrogenase levels or other metabolic substrates in our model. The rationale for establishing an hypotensive model stemmed from a clinical study showing an increase in mortality in patients with hypotensive AHF vs hypertensive AHF [17]. The lack of infarct in our isolated hearts (data not shown) suggests that the heart suffers from contractile failure rather than ischemia. Addition of adrenaline in the AHF phase along with increased FFA and reduced glucose oxidation led to worse outcomes vs hearts perfused without adrenaline [13].

Addition of S1P in our AHF model improved the cardiac function by

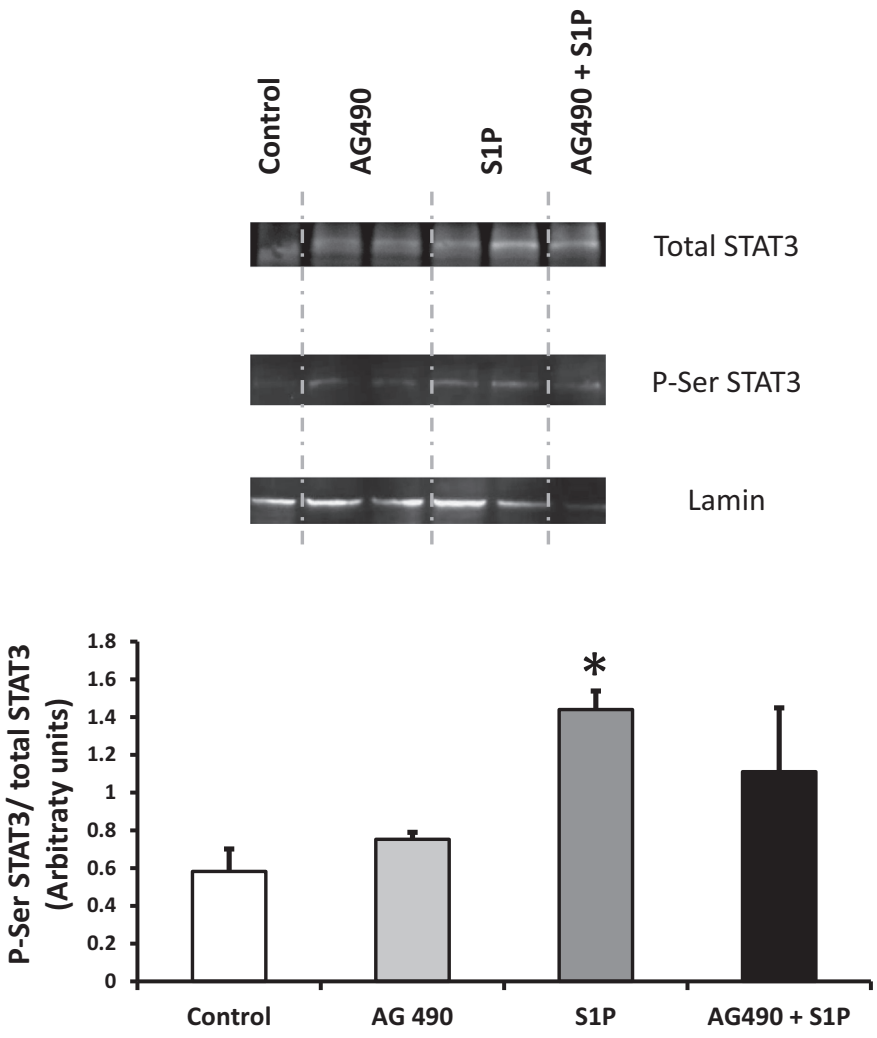


Fig. 4. Western blots representing nuclear expression of total STAT3, serine phosphorylated STAT3 and lamin in hearts subjected to AHF with S1P with/without AG490, an inhibitor of STAT3. *p < 0.05 vs. control.

improving the heart rate without any change in the contractility of the heart nor the diastolic pressure. These data suggest that the protective effect of S1P may act by targeting the sinus node activity. S1P asserts its activity by binding to one of the three receptors [18]. The three receptors are present in the heart, playing a major role in cardiomyocyte survival, inflammatory pathways, vascular resistance and heart rate regulation. Previous literature suggests that the control of the sinus rhythm by S1P is mediated via the activation of the S1P receptor type 3, but further work will be required to confirm which type of receptor may be involved in the protective effect of S1P in our model of AHF [19]. It will also be critical to confirm our findings using an in vivo model of AHF as our ex vivo model does not take into account the multiple systemic effects which may interfere with the protective effect of S1P.

S1P is well known as a pre- and post-conditioning agent [6,9,10]. It reduces infarct size and it improves functional recovery in isolated hearts subjected to ischemia-reperfusion injury [6]. This protective effect is mediated by partial activation of the protective RISK and SAFE pathways [6,20]. Our data suggest that the SAFE pathway is also involved in the protection observed by S1P in a model of AHF. In our model, S1P activates STAT3 in the nucleus only. Considering the short time points during which a protective effect of STAT3 is observed in our system, it is unlikely that this effect results from a transcriptional activity of STAT3. Although STAT3 is well known as a transcription factor, there is now clear evidence in the literature suggesting non-transcriptional activities of STAT3 (see review) [20]. Hence, activated STAT3 can translocate into the mitochondria where it regulates the opening of the mitochondrial permeability transition pore [21,22]. Despite multiple attempts, we did not observe any activation of STAT3 within the mitochondria (data not shown). However, this activation of STAT3 within the mitochondria cannot be excluded as the collection of the tissue samples may not coincide with the time of translocation of STAT3 within the mitochondria. Indeed, the activation of STAT3 within the mitochondria is transient and previous work conducted in our laboratory has highlighted that mitochondrial phosphorylated STAT3 is only present for few minutes in an isolated heart [6]. Whether the activation of STAT3 with S1P requires the binding of S1P onto its receptors is unclear. It is also highly possible that S1P may act as an intracellular agent without the necessity for receptor binding. Indeed, intracellular endogenous S1P is known to be involved in the regulation of cell survival paths, ion channels and calcium activity within the cell, independently of its specific cell binding receptors [18,23]. It is also unclear how exogenous S1P may interact the endogenous levels of S1P present within the cell. We anticipate that further experiments at different time points will help elucidate the mechanism of protection by STAT3 and its various cellular fractions. As mentioned above, S1P can activate both the SAFE and RISK pathways in the heart. It would therefore be of interest to explore whether the activation of the RISK path may also contribute to the beneficial effect of S1P in our model of AHF.

In conclusion, our present data highlight a beneficial effect of S1P against hypotensive AHF, as observed with an improvement in the pacemaker activity of the heart. This effect was mediated, at least in part, by the activation of STAT3 in the nuclear fraction but further work will be required to understand the exact mechanisms. Our data here strongly suggest that S1P could be a potential therapy to consider for patients suffering from de novo hypotensive AHF.

Abbreviations

S1P	Sphingosine-1-phosphate
LVDP	Left ventricular developed pressure
AHF	Acute Heart Failure
STAT3	Signal transducer and activator of transcription 3
FFA	free fatty acids

Conflict of interest

The authors declare no conflict of interest.

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