Life Sciences

Interplay between the SAFE and the sphingolipid pathway for cardioprotection

--Manuscript Draft--

Manuscript Number:	LFS-D-24-05896R2
Article Type:	Research paper
Keywords:	Survivor Activating Factor Enhancement pathway; sphingolipid; Ischemia-reperfusion injury; Mitochondria; Tumor necrosis factor α; Signal Transducer and Activator of Transcription 3
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Abstract:	Aim: Activation of both the Survivor Activating Factor Enhancement (SAFE) pathway (including Tumour Necrosis Factor-alpha (TNF-α) and Signal Transducer and Activator of Transcription-3 (STAT-3)) and the sphingolipid signalling pathway (including sphingosine kinase-1 (SK1) and sphingosine-1 phosphate (S1P)) play a key role in promoting cardioprotection against ischemia-reperfusion injury (IRI). We investigated whether the activation of the SAFE pathway by exogenous S1P is dependent on the activation of SK1 for cardioprotection. Materials and methods: Isolated cardiomyocytes from TNF-α knockout (KO) mice, cardiomyocyte-specific STAT-3KO mice and their wild-type (WT) littermates were exposed to simulated ischemia in the presence of a trigger of the SAFE pathway (S1P) and SK1 inhibitor (SK1-I). Similarly, isolated perfused hearts from adult TNF-αKO, STAT-3KO and WT mice were subjected to IRI with S1P and/or SK1-I. Cell viability, infarct size (IS) and SK1 activity were assessed. Key findings: In isolated cardiomyocytes and in isolated hearts subjected to simulated ischemia/IRI, S1P pretreatment decreased cell death in WT mice, an effect that was abrogated in the presence of SK1-I. S1P failed to reduce cell death after simulated ischemia/IRI in both cardiomyocytes or hearts isolated from TNF-αKO and STAT-3KO mice. Interestingly, S1P pretreatment increased SK1 activity in WT and STAT-3KO mice, with no changes in TNF-αKO mice. Significance: Our data strongly suggest SK1 as a key component to activate STAT-3 downstream of TNF-α in the SAFE pathway, paving the way for the development of novel cardioprotective strategies that may target SK1 to modulate the SAFE pathway and increase cell survival following IRI.
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	Joel Karliner Prof, University of California joel.karliner@va.gov Prof Karliner is an expert in the field of sphingolipid signalling for cardioprotection
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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Cover Letter

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Cape Town, 6th October 2024

Dear Dr. Sheikh,

Manuscript Title: **Interplay between the SAFE and the sphingolipid pathway for cardioprotection**

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We would like to thank the reviewers and the Editorial Board for reviewing our manuscript and for giving us the opportunity to submit a revised version of the manuscript for possible publication in Life Sciences. We have considered all the points highlighted by the reviewer 2 and we have revised the manuscript accordingly. Please accept the enclosed revised manuscript in consideration for publication in *Life Sciences.*

This manuscript has not been published previously, either in whole or in part, and is not under consideration for publication elsewhere. All authors attest to the originality of the text, and the originality of any/all supporting tables and figures. We also hereby affirm that ethical approval for this work was obtained as appropriate to this work.

I confirm that all co-authors have accepted the submission of the manuscript for publication in *Life Sciences*.

Sincerely yours,

Prof Sandrine Lecour, PharmD, PhD

Response to reviewers - Life Sciences

Ref.: Ms. No. LFS-D-24-05896
Interplay between the SAFE and the sphingolipid pathway for cardioprotection
Life Sciences

REVIEWERS' COMMENTS:

Reviewer #2: The authors have responded adequately to most queries. However, although the reviewer understands that the focus was on cell death mechanisms, the reporting of mechanical performance of the hearts with the different treatments, will still be interesting and noteworthy for the field of cardioprotection. Therefore, it is suggested that the authors do report on the cardiac forces for all groups and treatment and provide that data in a supplementary file. It is a waste not reporting on this data actually. And too often the reporting of data is too selective for only reporting positive data, which is becoming a real problem in the field of cardioprotection with its poor translation to clinically effective treatment. The positive bias in reporting could certainly be a cause for this poor translation. So, please, when you indeed have this data, provide it to the scientific community.

We fully agree with the reviewer's comments that reporting cardiac forces and the coronary flow adds value to the manuscript. We have inserted these values in a supplementary table in the revised manuscript. However, we would like to acknowledge some limitations in the reporting of our data. As mentioned previously to the reviewer, we didn't intend to report these data as an outcome (we mistakenly overlooked this aspect) and, after searching through all our records, we could only retrieve data at baseline and at the end of reperfusion (see table in supplementary data).
Nevertheless, we managed to obtain some valuable information from the table. The following sentences have been added in the results section of the revised manuscript (page 10, lines 16-22):

"In WT isolated hearts, the developed tension was decreased following an IRI in the CTRL group ($p<0.001$ reperfusion vs baseline), an effect that was lost when hearts were pretreated with S1P prior to IRI (n.s. reperfusion vs baseline in S1P group) (supplementary table 1). Interestingly, perfusion of SK1-I given together with S1P partially abolished the effect of S1P on the developed tension following IRI ($p<0.05$, reperfusion vs baseline in S1P+SK1-I group). At baseline or at the end of reperfusion, there was no difference in the coronary flow between the different experimental groups and IRI didn't alter the coronary flow at the end of reperfusion (supplementary table 1)."

For the last question, the reviewer was asking for effects of S1P on vascular resistance, not on LV pressure. So, for your constant pressure model, the question is whether S1P altered the coronary flow (=total flow) through the hearts?

We apologize for misinterpreting your query with regards to the coronary flow. Data related to the coronary flow data have been inserted into the supplementary table 1. At baseline or at endpoint, there was no difference in the coronary flow between the different experimental groups. Similarly, an ischemia-reperfusion injury didn't alter the coronary flow at the end of reperfusion. This is now specified in the revised manuscript, as mentioned above.

As we didn't record the haemodynamic parameters during/shortly after the perfusion of the drugs nor at the onset of reperfusion, we feel that our data do not give complete information on the performance of the heart in our setting. We therefore have added the following sentence in the discussion of the revised manuscript to acknowledge the limitation of our data (page 13, lines 12-15):

"*In our experimental model, we explored principally the role of SK1 on the activation of the SAFE pathway to improve cell survival. It would also be of interest to explore* ***in detail*** *the role of the modulation of these signalling events on the haemodynamic parameters of the heart following an ischemia-reperfusion insult."*

Interplay between the SAFE and the sphingolipid pathway for cardioprotection

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Abstract

Aim: Activation of both the Survivor Activating Factor Enhancement (SAFE) pathway (including Tumour Necrosis Factor-alpha (TNF-α) and Signal Transducer and Activator of Transcription-3 (STAT-3)) and the sphingolipid signalling pathway (including sphingosine kinase-1 (SK1) and sphingosine-1 phosphate (S1P)) play a key role in promoting cardioprotection against ischemia-reperfusion injury (IRI). We investigated whether the activation of the SAFE pathway by exogenous S1P is dependent on the activation of SK1 for cardioprotection.

Materials and methods: Isolated cardiomyocytes from TNF-α knockout (KO) mice, cardiomyocyte-specific STAT-3KO mice and their wild-type (WT) littermates were exposed to simulated ischemia in the presence of a trigger of the SAFE pathway (S1P) and SK1 inhibitor (SK1-I). Similarly, isolated perfused hearts from adult TNF-αKO, STAT-3KO and WT mice were subjected to IRI with S1P and/or SK1-I. Cell viability, infarct size (IS) and SK1 activity were assessed.

Key findings: In isolated cardiomyocytes and in isolated hearts subjected to simulated ischemia/IRI, S1P pretreatment decreased cell death in WT mice, an effect that was abrogated in the presence of SK1-I. S1P failed to reduce cell death after simulated ischemia/IRI in both cardiomyocytes or hearts isolated from TNF-αKO and STAT-3KO mice. Interestingly, S1P pretreatment increased SK1 activity in WT and STAT-3KO mice, with no changes in TNF-αKO mice.

Significance: Our data strongly suggest SK1 as a key component to activate STAT-3 downstream of TNF-α in the SAFE pathway, paving the way for the development of novel cardioprotective strategies that may target SK1 to modulate the SAFE pathway and increase cell survival following IRI.

Keywords

Survivor Activating Factor Enhancement pathway,

Sphingolipid,

Ischemia-reperfusion injury,

Mitochondria,

Tumor necrosis factor alpha,

Signal Transducer and Activator of Transcription 3

Introduction

Ischemic heart disease is the leading cause of death worldwide, and its global prevalence rate is on a constant rise, expected to reach more than 1845 per 100,000 population by the year 2030 [1]. Although a large number of cardioprotective strategies have been reported to protect against ischemia-reperfusion injury (IRI) in preclinical studies, very few have been translated into the clinical setting in the past 30 years [2]. Multiple factors may explain this lack of translation and a better understanding of the cardioprotective signalling pathways that may protect against IRI is likely to enhance the design of suitable strategies that could protect the human heart.

The Survivor Activating Factor Enhancement (SAFE) pathway has been proposed as a major cardioprotective pathway activated by multiple putative cardioprotective strategies, such as ischemic pre- and post-conditioning, sphingosine-1 phosphate (S1P), and melatonin to protect against IRI [3–6]. The activation of this cell survival pathway is triggered by the binding of Tumour Necrosis Factor alpha (TNF-α) to its receptor type 2, which activates downstream targets such as Signal Transducer and Activator of Transcription 3 (STAT-3), mitochondria and microRNAs (see reviews [7,8]). However, most of the cascades of mediators implicated in this pathway remain to be delineated.

Sphingolipids (such as ceramide, sphingosine, and S1P) have been proposed as downstream targets of numerous cardioprotective strategies [9–11]. Indeed, inhibition of the sphingolipid signalling attenuates the cardioprotective effect of both ischemic and TNF-α preconditioning in the isolated heart [12]. Similarly, the activation of sphingosine kinase 1 (SK1), which is a key enzyme involved in the endogenous formation of S1P, is critical for the protection against IRI by ischemic pre- and post-conditioning [13]. Although both the SAFE pathway and the sphingolipids signalling are critical for the cardioprotective effect of various strategies, it is yet unclear as to whether components of the sphingolipid signalling, such as SK1, may act as key players in modulating the SAFE pathway in the heart.

In the present study, we therefore used a combination of *ex vivo* and *in vitro* IRI models to investigate whether the activation of the SAFE pathway is dependent on the activation of SK1.

Materials and methods

Animals

All experimental procedures were performed with the approval of the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town. All protocols were performed in compliance with the the Care and Use of Laboratory Animals Guide published by the United States National Institutes of Health in 2011. Cardiomyocyte specific STAT-3 knockout (STAT-3 KO) mice with a C57BL/6 genetic background were created in our facility by crossing homozygous floxed STAT-3 mice with heterozygous ventricular myosin light chain 2 (MLC2v)-driven Cre recombinase mice, as previously described [14]. TNF-α deficient mice (TNF-α KO) with a C57BL/6 genetic background, were generous gifts from Dr Jacobs and Prof Ryffel (Department of Immunology, University of Cape Town) [15]. Male mice aged 12-14 weeks were used in this study.

Perfusion of isolated adult mouse hearts

Mice were anaesthetized with intraperitoneal (i.p.) sodium pentobarbital (60 mg/kg) and heparinized (25 IU, i.p.), and hearts were isolated and perfused on the retrograde Langendorff system as previously described [15].]. Briefly, hearts were perfused with a modified Krebs–Henseleit buffer (NaCl 118.0 mM; $NaHCO_3$ 24.0 mM; KCl 4.0 mM; NaH_2PO_4 1.0 mM; $CaCl_2$ 2.5 mM; $MgCl_2$ 1.2 mM; di-sodium EDTA 0.5 mM; glucose 10 mM; gassed with 95% O_2 / 5%CO_2 at 37ºC) in a retrograde fashion with a constant pressure of 110 cmH_2O. Hearts were fastened, via a rigid lightweight lexan coupling rod, to a force displacement transducer (Grass FT03C, MA, USA) by means of a 4-0 silk (on a 20-mm curved atraumatic needle) placed through the apex of the heart. Diastolic tension was adjusted to 2 g and hearts were paced at 600 bpm. A minimum of 1.5 ml/min and maximum of 5.0 ml/min of coronary flow rate, heart rate between 460 and 600 beats/min and developed force ≥ 4 g was deemed acceptable. After 20 min of a stabilisation period, the hearts were subjected to 35 min of global ischaemia at 37°C followed by 45 min of reperfusion. The experimental protocol is illustrated in Figure 1. S1P (10nM) was administered for 7 min followed by a washout period of 10 min before ischemia induction. SKI-178 (15 μM), a specific SK1 inhibitor (SK1-I) [16] was administered for 15 min with a washout period of 5 min prior to ischemia. At the end of the experimental protocol, infarct size was assessed by triphenyl-tetrazolium chloride staining and analysed using computerized planimetry (Planimetry+, Boreal Software, Norway) [15]. In a separate group of experiments, hearts were freeze clamped in liquid nitrogen at the end of the reperfusion period and stored at -80°C for SK1 activity analysis.

Isolation of adult mouse cardiomyocytes

TNF-α KO (n=8), STAT-3 KO (n=6), and their respective littermate control mice (n=25) were anesthetized via an intraperitoneal injection (i.p.) of sodium pentobarbital (60 mg/kg) and heparinized (25 IU). The heart was rapidly excised and cannulated on the Langendorff perfusion system, and the cardiomyocytes were isolated as previously described [17]. Isolated cardiomyocytes were plated onto 6 well plates precoated with laminin in minimum essential medium containing penicillin, streptomycin and 5% foetal calf serum and incubated for 1 h at 37°C prior to experiments, as previously described [17].

Experimental protocol in isolated adult mouse cardiomyocytes

The experimental protocol conducted in isolated adult cardiomyocytes is shown in Figure 1. Normoxic control cells were maintained under normoxic conditions throughout the protocol (20% O_2, 5% CO_2 and balance N_2). Simulated ischemia control cardiomyocytes were subjected to 2 h of simulated ischemia using a simulated ischemic buffer ($MgCl_2.6H_2O$ 1.2 mM, KCl 16 mM, KH_2PO_4 1 mM, NaCl 74 mM, $CaCl_2$ 1.2 mM, $NaHCO_3$ 10 mM, sodium lactate 20 mM, HEPES 25 mM, pH 6.7) with cells kept in a humidified hypoxic environment (1% O_2, 5% CO_2 and balance N_2) at 37°C. The treated cardiomyocytes were exposed to S1P (10nM) for 30 min followed by a washout period of 30 min before the simulated ischemic insult. The SK1 inhibitor (SKI-178, 15 µM) was given for 1 h, starting 30 min before S1P treatment.

Culture of H9c2 cardiomyocytes

H9c2 cardiomyocyte cell lines were purchased commercially from the European Collection of Cell Cultures (Centre for Applied Microbiology and Research, UK). The cells were stored in cryovials at $1x10^6$ cells/mL in liquid nitrogen (-196˚C). To seed the cells, the cryovial was thawed at room temperature. T25 (25 cm^3) tissue culture flasks were supplemented with 4 - 6 mL Dulbecco's modified Eagle's medium (DMEM) and the contents of the cryovials were transferred into tissue culture flasks. The DMEM contained 4.5 g/L glucose, 0.110 g/L sodium pyruvate and L-glutamine and was supplemented with 10% fetal calf serum (FCS) and 1% (weight/volume) penicillin/streptomycin. When the cells (with a passage number between 15 and 18) reached 80% confluency, they were used for treatment and follow up experiments.

Experimental protocol in H9c2 cardiomyocytes

To further assess the role of SK1 in cardioprotection, H9c2 cells were subjected to simulated ischemia and exposed to melatonin used as an activator of the SAFE pathway, with or without SKI-I (n=5 independent experiments). Briefly, H9c2 cells were pretreated with melatonin (75 ng/L) for 40 minutes prior to 8 h of simulated ischemia with simulated ischemic buffer adjusted at pH 6.4 and hypoxic conditions (5% CO_2, 94% N_2 and 1% O_2), as previously described [18] . SKI-I (15 µM) was given (with or without melatonin) for 55 minutes prior to simulated ischemia. The control group (CTRL) was exposed to simulated ischemia only. The cell viability was measured at the end of the simulated ischemic insult.

Western blotting was used to further explore the role of SK1 in STAT-3 activation with exogenous S1P. Briefly, H9c2 cells were exposed to S1P (10 nM) for 7 min with or without pretreatment with SK1-I (15 µM) for 30 min. Cell lysates were electrophoresed and the levels of phosphorylated (tyr705) and total STAT-3 (Cell Signaling Technology, Inc., Danvers, MA, USA) were quantified, as previously described [19]. Equal loading was verified using β-actin. Densitometric analysis was performed using the ImageJ software. STAT-3 activation was measured as ratio of phosphorylated STAT-3 to total STAT-3.

Measurement of cell viability

To assess cell viability in isolated cardiomyocytes or H9c2 cardiomyocytes, the cells were loaded with 0.04% trypan blue and analysed using a light microscope at 40x magnification. The number of viable (unstained) and nonviable (blue stained) cardiomyocytes in four random microscopic fields was recorded, with at least 100 cells counted in each well.

Analysis of mitochondrial permeability transition

To assess the mitochondrial permeability transition pore (mPTP) opening at the end of 2 h of simulated ischemia, isolated cardiomyocytes were incubated with 20nM dye tetra-methyl-rhodamine-methyl ester (TMRM) for 10 min followed by a 10 min washout period [20]. Cardiomyocytes were then lifted from the wells using a cell scraper and analysed by flow cytometry (FACSCalibur, BD Biosciences, MD, USA) as previously described [21]. The fluorescence intensity was measured and analysed using the FlowJo

software (FlowJo LLC, Ashland, OR, USA) and expressed as arbitrary fluorescence units (AFU). Data were normalized to the normoxic control group.

Measure of SK1 activity

Frozen hearts were pulverized before protein extraction as previously described [15]. SK1 activity was measured using a specific biochemical fluorescence-based assay kit (Gyrasol Technologies, KS, USA) following the manufacturer's instructions. The SK1 activity was normalized to that of the control group.

Chemicals

Unless specified, all chemicals were obtained from Sigma-Aldrich, South Africa.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.0 (GraphPad Software, La Jolla, California, USA). Data are expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were performed using the Student's t-test. For comparisons of three or more groups, one-way ANOVA followed by the Holm-Sidak test for multiple comparisons or Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used, depending on the distribution of data. The Shapiro-Wilk test was used to test the normality of the data. Statistical significance was set at $p<0.05$.

Results

Exogenous S1P improves cell survival via the SAFE pathway.

To determinate the role of the SAFE pathway in S1P induced cardioprotection, isolated adult cardiomyocytes from TNF-α KO, STAT-3 KO, and their wildtype (WT) littermates were exposed to simulated ischemia with or without S1P pretreatment (Figure 2). Cardiomyocyte cell death under normoxic conditions in WT littermates (TNF-α WT and STAT-3 WT) and knockout mice (TNF-α KO and STAT-3 KO) was 21.4±1.2%, 15.7±1.3%, 19.3±1.5% and 21.3±3.3% respectively, indicating that approximately 20% of cardiomyocytes did not survive the isolation and the plating process. Exposure to 2 h of simulated ischemia increased cardiomyocyte cell death to 45.4±2.1% (TNF-α WT) and 39.6±2.1% (STAT-3 WT) ($p<0.05$ vs normoxic group), with comparable effects in TNF-α KO (45.0±1.4%) and STAT-3 KO (39.8±4.8%) ($p<0.05$ vs normoxic group). S1P pretreatment decreased cell death to 25.1±1.6% and 19.6±2.8% in TNF-α WT and STAT-3 WT, respectively ($p<0.01$ vs CTRL). However, S1P pretreatment did not protect cardiomyocytes against simulated ischemia in TNF-α KO (49.2±1.1%) or STAT-3 KO (41.7±3.8%) mice.

Exogenous S1P confers protection by inhibiting mPTP opening via the SAFE pathway.

To delineate the role of mPTP opening in the beneficial effect of S1P, isolated adult cardiomyocytes from TNF-α KO, STAT-3 KO, and their WT littermates were exposed to simulated ischemia with or without S1P pretreatment and TMRM fluorescence, reflecting mPTP opening, was measured (Figure 2). TMRM fluorescence intensity after exposure of the cells to 2 h of simulated ischemia decreased from 100% to 71.3±2.8% and 74.7±6.5% in TNF-α WT and STAT-3 WT, respectively ($p<0.01$ vs normoxic controls), indicative of mPTP opening. The same effect was observed in TNF-α KO and STAT-3 KO cardiomyocytes (77.7±2.0% and 68.1±3.0% respectively, ($p<0.01$ vs normoxic controls). S1P pretreatment restored the TMRM fluorescence intensity to 93.4±2.7% and 95.5±4.3% in TNF-α WT and STAT-3 WT cardiomyocytes, respectively ($p<0.05$ vs simulated ischemic groups). In contrast, S1P pretreatment failed to restore the TMRM fluorescence intensity after simulated ischemia in TNF-α KO (80.3±1.9%) and STAT-3 KO cardiomyocytes (68.4±3.7%).

SK1 is required for exogenous S1P-induced cardioprotection.

To evaluate whether the cardioprotective effect of exogenous S1P is dependent on SK1, isolated adult cardiomyocytes from WT mice were exposed to simulated ischemia in the presence of S1P with or without a specific SK1 inhibitor and cell viability was assessed (Figure 3A). Exposure to 2 h of simulated ischemia increased cardiomyocyte cell death from 23.9±0.5% to 48.4±0.7% ($p<0.0001$ vs normoxic control). S1P pretreatment decreased cell death to 25.4±1.0% ($p<0.0001$ vs simulated ischemia only). However, the beneficial effect of S1P was inhibited in the presence of SK1-I (46.4±2.0%) ($p<0.05$ vs S1P group). Similar findings were observed with exogenous melatonin as a cardioprotective agent known to activate the SAFE pathway (Figure 3B). In H9c2 cells subjected to simulated ischemia, a pretreatment with melatonin decreased cell death from 40.4±1.0% to 15.2±2.6 ($p<0.05$) but this cardioprotective effect was inhibited in the presence of SK1-I (39.8±6.1%).

Similar findings were also reported in an *ex vivo* model (Figure 4). In WT isolated hearts (figure 4A), S1P pretreatment reduced infarct size following IRI from 49.8±1.4% to 30.6±2.1% ($p<0.05$ vs CTRL). This effect was associated with an increase in SK1 activity (2.4±0.5 folds vs CTRL). The addition of SK1-I abolished both the infarct sparing and the increase in SK1 activity observed with S1P treatment (Figure 4A). In WT isolated hearts, the developed tension was decreased following an IRI in the CTRL group ($p<0.001$ reperfusion vs baseline), an effect that was lost when hearts were pretreated with S1P prior to IRI (n.s. reperfusion vs baseline in S1P group) (supplementary table 1). Interestingly, perfusion of SK1-I given together with S1P partially abolished the effect of S1P on the developed tension following IRI ($p<0.05$, reperfusion vs baseline in S1P+SK1-I group). At baseline or at the end of reperfusion, there was no difference in the coronary flow between the different experimental groups and IRI didn't alter the coronary flow at the end of reperfusion (supplementary table 1).

SK1 interacts with the SAFE pathway for cardioprotection

To better understand how SK1 interacts with the SAFE pathway, SK1 activity was measured in isolated hearts of both TNF-α KO and STAT-3 KO mice exposed to IRI with/without S1P pretreatment. In TNF-α KO mice, exogenous S1P failed to increase the activity of SK1 and reduce infarct size. Despite the increase in SK1 activity (3.1±0.9 fold) with S1P, no infarct size reduction was observed in STAT-3 KO mice. In addition, an increase in STAT-3 activation (1.7±0.1 fold) was observed after S1P stimulation (Figure 5), an effect that was prevented in the presence of SK1-I.

Discussion

The present findings highlight an interplay between the SAFE pathway and the sphingolipid pathway, where SK1 acts as a key element to activate STAT-3 downstream of TNF-α for cardioprotection. First, S1P-induced cardioprotection in isolated WT cardiomyocytes was associated with an increase in SK1 activity. Second, the presence of a specific SK1 inhibitor abolished STAT-3 activation and the cytoprotective effects of S1P. Third, the absence of S1P-induced cardioprotection in TNF-α or STAT-3 KO isolated hearts was associated with an increase in SK1 activity in STAT-3 KO hearts only, suggesting that SK1 functions as an intermediate component between TNF-α and STAT-3 to activate the SAFE pathway.

S1P is a well-known pharmacological conditioning agent that confers protection against IRI. Multiple signaling cascades have been proposed, including the Reperfusion Injury Salvage Kinase (RISK) pathway, the endogenous sphingolipid pathway and the activation of the SAFE pathway, with possible cross-talk among these various pathways [22]. Although mPTP is known as a key end-target of the RISK and Sphingolipid pathways to promote cell survival, its role downstream of the SAFE pathway remains poorly studied [23–25]. Using TNF-α and STAT-3 KO animals, we demonstrated that S1P-induced protection against IRI was dependent on a decrease in susceptibility to mPTP opening downstream of the SAFE pathway (TNF-α/STAT-3). The fact that mPTP is a common downstream effector of the Reperfusion Injury Salvage Kinase (RISK), sphingolipid and SAFE pathways to limit cell death raises the question of whether these different pathways interact with each other. Previous research has provided some insights into the interaction between the RISK and the SAFE pathways, demonstrating that Akt, a major kinase of the RISK pathway, modulates the activation of STAT-3, and vice versa [5,8]. However, there is little information on the possible interplay between components of the sphingolipid network, such as SK1, and the SAFE pathway for cardioprotection.

SK1 is an enzyme present in cells that converts sphingosine into S1P [22]. As such, it is an essential component of the sphingolipid pathway. SK1 activity decreases following IRI, an effect that can be mitigated by cardioprotective strategies such as ischemic conditioning [26]. Our findings demonstrate that SK1 is required to limit cell death caused by IRI in both isolated cardiomyocytes and isolated hearts. Given that S1P is a product of SK1, one could speculate that providing exogenous S1P, might compensate the absence of production of cardioprotective intracellular S1P formation by SK1 inhibitors.

However, this hypothesis was not supported by our experiments, as the cardioprotective effect of exogenous S1P was lost in the presence of an SK1 inhibitor. Furthermore, we confirmed that the cardioprotective effect of exogenous melatonin, another cardioprotective agent that does not belong to the sphingolipid pathway, was also lost in the presence of the SK1 inhibitor. Given that both melatonin and exogenous S1P provide cardioprotection through activation of the SAFE pathway [5–7], it can be inferred that SK1 activation may be linked to the activation of the SAFE pathway.

Upon stimulation with exogenous S1P, TNF-α binds to its receptor 2, triggering the phosphorylation of Janus kinase (JAK), which in turn, phosphorylates cytosolic STAT-3 [7]. Once phosphorylated, STAT-3 can translocate to the nucleus and mitochondria to promote cell survival [7,8]. In the present study, the absence of both cardioprotection and SK1 activation with exogenous S1P stimulation in TNF-α KO mice suggests that SK1 activation is dependent on TNF-α. This conclusion aligns with previous research that documented a rapid increase in SK1 activity following TNF-α stimulation of human endothelial cells [27]. It is also supported by experimental data demonstrating that TNF-α can bind to TNF receptor 2, activating TNF receptor-associated factor 2 (TRAF2), which subsequently enables the binding (and activation) of SK1 to its TRAF2-binding motif [28,29]. Even though exogenous S1P also failed to limit infarct size in STAT-3 KO hearts subjected to IRI, it induced an increase in SK1 activity that was comparable to that observed in WT hearts. This finding aligns with previous research that concluded that STAT-3 activation is required to provide an S1P-induced reduction in infarct size [5]. More interestingly, this suggests that not only SK1 activation is not dependent on STAT-3 activation (contrary to what has been proposed in colorectal cancer [30]) but also that SK1 activation cannot provide cytoprotection against IRI in the absence of STAT-3, which may thus act as a downstream mediator of cardioprotection. It would be of interest to explore whether TRAF2 is the sole activator of SK1 in our model. Indeed, other stimuli known to activate SK1 include extracellular regulated kinase (ERK) which can be activated after binding of S1P to S1P receptors may possibly also contribute to activate SK1 in our setting [31,32]. Additionally, our results in H9c2 cells indicate that inhibition of SK1 prevents S1P-induced STAT-3 activation, further suggesting a role for SK1 in modulating STAT-3 activation, and thus, the SAFE pathway. This raises the question of how SK1 can activate STAT-3. A potential explanation may come from a previous study in cancerous lymphocytes reporting that the specific SK1 inhibitor SKI-178 can prevent phosphorylation (i.e., activation) of both JAK and STAT-3, leading to

massive cell death [16]. Although the cellular behaviors and mechanisms of cancer cells and cardiomyocytes may differ, our data also suggest that SK1 activates JAK/STAT3 for cardioprotection.

This study has some limitations. The primary role of SK1 is the formation of S1P from sphingosine, and it is possible that some mechanisms of the cardioprotective effect of SK1 involve further formation of endogenous S1P and the possible binding of S1P to its receptors. Indeed, exogenous S1P fails to protect in S1P-Receptor-2 and S1P-Receptor-3 KO mice [33] and activation of S1P Receptors can lead to STAT-3 phosphorylation [19,34]. Therefore, it would be important to further study the cellular dynamic of S1P and the possible interaction of S1P receptors/other sphingolipid components with the SAFE pathway in future studies. Another limitation is that we focused our investigations on S1P-induced cardioprotection only. It would also be interesting to also assess the role of ceramide (a precursor to sphingolipids and S1P) in the interplay between the SAFE and the sphingolipid pathways in IRI. Indeed, previous studies have suggested that TNF-induced cardioprotection is mediated by ceramide [35]. In our experimental model, we explored the role of SK1 on the activation of the SAFE pathway to improve cell survival. It would also be of interest to explore in detail the role of the modulation of these signalling events on the haemodynamic parameters of the heart following an ischemia-reperfusion insult. In addition, no in vivo models were used in any of our investigations, which means that the potential role of plasma high-density lipoprotein content was not considered. High-density lipoproteins contain a considerable amount of sphingolipids (including S1P), which may affect the response to the interventions [10,36]. However, several models have been employed, including isolated hearts from KO animals, which have been extensively validated to evaluate signalling pathways, particularly because they enable the use of inhibitors without causing toxicity [3,5,12,19,37]. Finally, It would be interesting to assess whether

Conclusion

This study suggests that the interplay between the SAFE and sphingolipid pathways is essential for effective cardioprotection with S1P. SK1 emerges as a crucial factor in activating STAT-3 through TNF-α in the SAFE pathway (see Figure 6). Our findings provide a basis for innovative cardioprotective approaches that could target SK1 to regulate the activation of the SAFE pathway and enhance cell survival after IRI.

Abbreviations

AFU	Arbitrary fluorescence units
CTRL	Control group
DMEM	Dulbecco's Modified Eagle Serum
IRI	Ischemia reperfusion injury
KO	knockout
MLC2v	ventricular myosin light chain 2
mPTP	Mitochondrial permeability transition pore
SAFE	Survivor Activating Factor Enhancement
SEM	Standard error of the mean
SK1	Sphingosine kinase 1
SK1-I	Sphingosine kinase 1 inhibitor
S1P	Sphingosine 1 phosphate
STAT-3	Signal Transducer and Activator of Transcription 3
TMRM	Tetra-methyl-rhodamine-methyl ester
TNF-α	Tumor necrosis factor alpha
TRAF2	TNF receptor-associated factor 2
WT	Wild type

Acknowledgements

The authors would like to thank Ms Nolize Dietrich for her assistance in data capturing.

Declaration of interests

The authors declare that they have no conflict of interest related to the present study.

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Funding sources

Part of the study was funded by the University of Cape Town, the South African Medical Research Council and the South African National Research Foundation. Sarah Pedretti and Martin Cour received a Fellowship from the University of Cape Town, South Africa. Sandrine Lecour received funding from the Oppenheimer Memorial trust. Derek J Hausenloy is supported by the Duke-NUS Signature Research Programme funded by the Ministry of Health, Singapore Ministry of Health's National Medical Research Council under its Singapore Translational Research Investigator Award (MOH-STaR21jun-0003), Centre Grant scheme (NMRC CG21APR1006), and Collaborative Centre Grant scheme (NMRC/CG21APRC006).

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Figures Legends

Figure 1 - Experimental protocol in isolated hearts and cardiomyocytes

WT: wild type; KO: knockout; TNF-α: Tumor Necrosis Factor α; STAT-3: Signal Transducer and Activator of Transcription 3; CTRL: Control; S1P: Sphingosine 1 phosphate; SK1: Sphingosine kinase 1, SK1-I: SK1 inhibitor.

Figure 2 - Cytoprotective effect of S1P in TNF-α KO and STAT-3 KO isolated cardiomyocytes following simulated ischemia

Cell death (left panels) and mitochondrial permeability transition pore (mPTP) opening (right panels) were assessed in cardiomyocytes isolated from wild-type (WT) TNF-α mice (panel A; n=4 -9/group), TNF-α knockout (KO) mice (panel B; n=4-8/group), WT STAT-3 mice (panel C; n=4-15/group), and STAT-3 KO mice (panel D; n=4-6/group). Cardiomyocytes were exposed to simulated ischemia (CTRL) with or without pretreatment with sphingosine 1 phosphate (S1P). Cell death was assessed by microscopy after staining with tryptan blue, and mPTP opening was assessed by fluorescence intensity using tetra-methyl-rhodamine-methyl (expressed as arbitrary fluorescence units, AFU).

* $p<0.05$; ** $p<0.01$; **** $p<0.001$

Figure 3 - Effect of SK1 inhibitor on the cytoprotective effect of S1P or melatonin in cardiomyocytes exposed to simulated ischemia

Cell death was assessed in A) isolated cardiomyocytes (n=6-20/group) exposed to simulated ischemia (CTRL) with sphingosine 1 phosphate (S1P) and/or sphingosine kinase 1 inhibitor (SK1-I), and B) H9c2 cells (n=5/group) exposed to simulated ischemia (CTRL) with melatonin (Mel) and/or SK1-I.

* $p<0.05$; **** $p<0.0001$

Figure 4 - Interplay between the SAFE pathway and SK1 in S1P-induced cardioprotection in isolated hearts subjected to ischemia-reperfusion injury.

Infarct size and sphingosine kinase 1 (SK1) activity were assessed in isolated hearts of wildtype (panel A; n=6-15/group), TNF-α knockout (KO) (panel B; n=4-7/group) and STAT-3 KO (panel C; n=4-6/group) mice subjected to ischemia-reperfusion injury (CTRL) and treated with sphingosine 1 phosphate (S1P). SK1-I: sphingosine kinase inhibitor.

* p<0.05; ** p<0.01; *** p<0.001

Figure 5 - Effect of SK1 on S1P-induced STAT-3 activation in H9c2 cells

H9c2 cells were exposed to sphingosine 1 phosphate (S1P) in the presence or absence of sphingosine kinase 1 inhibitor (SK1-I). Phosphorylated STAT-3 (Phospho-STAT-3) and total STAT-3 and were assessed by western blotting (panel B). Phospho-STAT-3/STAT-3 in S1P and S1P+SK1-I groups (n=6/group) were compared to the control (CTRL) group (expressed as fold increase).

* p<0.05

Figure 6 - Proposed interplay between Sphingosine Kinase 1 and the SAFE pathway for cardioprotection.

Abbreviations: JAK: Janus Kinase; mPTP: Mitochondrial Permeability Transition Pore; SK1: Sphingosine Kinase 1; STAT-3: Signal Transducer and Activator of Transcription 3; TNF-α: Tumour Necrosis Factor alpha; TNFR2: TNF receptor 2; TRAF2: TNF receptor-associated factor 2.

Interplay between the SAFE and the sphingolipid pathway for cardioprotection

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Abstract

Aim: Activation of both the Survivor Activating Factor Enhancement (SAFE) pathway (including Tumour Necrosis Factor-alpha (TNF-α) and Signal Transducer and Activator of Transcription-3 (STAT-3)) and the sphingolipid signalling pathway (including sphingosine kinase-1 (SK1) and sphingosine-1 phosphate (S1P)) play a key role in promoting cardioprotection against ischemia-reperfusion injury (IRI). We investigated whether the activation of the SAFE pathway by exogenous S1P is dependent on the activation of SK1 for cardioprotection.

Materials and methods: Isolated cardiomyocytes from TNF-α knockout (KO) mice, cardiomyocyte-specific STAT-3KO mice and their wild-type (WT) littermates were exposed to simulated ischemia in the presence of a trigger of the SAFE pathway (S1P) and SK1 inhibitor (SK1-I). Similarly, isolated perfused hearts from adult TNF-αKO, STAT-3KO and WT mice were subjected to IRI with S1P and/or SK1-I. Cell viability, infarct size (IS) and SK1 activity were assessed.

Key findings: In isolated cardiomyocytes and in isolated hearts subjected to simulated ischemia/IRI, S1P pretreatment decreased cell death in WT mice, an effect that was abrogated in the presence of SK1-I. S1P failed to reduce cell death after simulated ischemia/IRI in both cardiomyocytes or hearts isolated from TNF-αKO and STAT-3KO mice. Interestingly, S1P pretreatment increased SK1 activity in WT and STAT-3KO mice, with no changes in TNF-αKO mice.

Significance: Our data strongly suggest SK1 as a key component to activate STAT-3 downstream of TNF-α in the SAFE pathway, paving the way for the development of novel cardioprotective strategies that may target SK1 to modulate the SAFE pathway and increase cell survival following IRI.

Keywords

Survivor Activating Factor Enhancement pathway,

Sphingolipid,

Ischemia-reperfusion injury,

Mitochondria,

Tumor necrosis factor alpha,

Signal Transducer and Activator of Transcription 3

Introduction

Ischemic heart disease is the leading cause of death worldwide, and its global prevalence rate is on a constant rise, expected to reach more than 1845 per 100,000 population by the year 2030 [1]. Although a large number of cardioprotective strategies have been reported to protect against ischemia-reperfusion injury (IRI) in preclinical studies, very few have been translated into the clinical setting in the past 30 years [2]. Multiple factors may explain this lack of translation and a better understanding of the cardioprotective signalling pathways that may protect against IRI is likely to enhance the design of suitable strategies that could protect the human heart.

The Survivor Activating Factor Enhancement (SAFE) pathway has been proposed as a major cardioprotective pathway activated by multiple putative cardioprotective strategies, such as ischemic pre- and post-conditioning, sphingosine-1 phosphate (S1P), and melatonin to protect against IRI [3–6]. The activation of this cell survival pathway is triggered by the binding of Tumour Necrosis Factor alpha (TNF-α) to its receptor type 2, which activates downstream targets such as Signal Transducer and Activator of Transcription 3 (STAT-3), mitochondria and microRNAs (see reviews [7,8]). However, most of the cascades of mediators implicated in this pathway remain to be delineated.

Sphingolipids (such as ceramide, sphingosine, and S1P) have been proposed as downstream targets of numerous cardioprotective strategies [9–11]. Indeed, inhibition of the sphingolipid signalling attenuates the cardioprotective effect of both ischemic and TNF-α preconditioning in the isolated heart [12]. Similarly, the activation of sphingosine kinase 1 (SK1), which is a key enzyme involved in the endogenous formation of S1P, is critical for the protection against IRI by ischemic pre- and post-conditioning [13]. Although both the SAFE pathway and the sphingolipids signalling are critical for the cardioprotective effect of various strategies, it is yet unclear as to whether components of the sphingolipid signalling, such as SK1, may act as key players in modulating the SAFE pathway in the heart.

In the present study, we therefore used a combination of *ex vivo* and *in vitro* IRI models to investigate whether the activation of the SAFE pathway is dependent on the activation of SK1.

Materials and methods

Animals

All experimental procedures were performed with the approval of the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town. All protocols were performed in compliance with the the Care and Use of Laboratory Animals Guide published by the United States National Institutes of Health in 2011. Cardiomyocyte specific STAT-3 knockout (STAT-3 KO) mice with a C57BL/6 genetic background were created in our facility by crossing homozygous floxed STAT-3 mice with heterozygous ventricular myosin light chain 2 (MLC2v)-driven Cre recombinase mice, as previously described [14]. TNF-α deficient mice (TNF-α KO) with a C57BL/6 genetic background, were generous gifts from Dr Jacobs and Prof Ryffel (Department of Immunology, University of Cape Town) [15]. Male mice aged 12-14 weeks were used in this study.

Perfusion of isolated adult mouse hearts

Mice were anaesthetized with intraperitoneal (i.p.) sodium pentobarbital (60 mg/kg) and heparinized (25 IU, i.p.), and hearts were isolated and perfused on the retrograde Langendorff system as previously described [15].]. Briefly, hearts were perfused with a modified Krebs–Henseleit buffer (NaCl 118.0 mM; $NaHCO_3$ 24.0 mM; KCl 4.0 mM; NaH_2PO_4 1.0 mM; $CaCl_2$ 2.5 mM; $MgCl_2$ 1.2 mM; di-sodium EDTA 0.5 mM; glucose 10 mM; gassed with 95% O_2 / 5%CO_2 at 37ºC) in a retrograde fashion with a constant pressure of 110 cmH_2O. Hearts were fastened, via a rigid lightweight lexan coupling rod, to a force displacement transducer (Grass FT03C, MA, USA) by means of a 4-0 silk (on a 20-mm curved atraumatic needle) placed through the apex of the heart. Diastolic tension was adjusted to 2 g and hearts were paced at 600 bpm. A minimum of 1.5 ml/min and maximum of 5.0 ml/min of coronary flow rate, heart rate between 460 and 600 beats/min and developed force ≥ 4 g was deemed acceptable. After 20 min of a stabilisation period, the hearts were subjected to 35 min of global ischaemia at 37°C followed by 45 min of reperfusion. The experimental protocol is illustrated in Figure 1. S1P (10nM) was administered for 7 min followed by a washout period of 10 min before ischemia induction. SKI-178 (15 μM), a specific SK1 inhibitor (SK1-I) [16] was administered for 15 min with a washout period of 5 min prior to ischemia. At the end of the experimental protocol, infarct size was assessed by triphenyl-tetrazolium chloride staining and analysed using computerized planimetry (Planimetry+, Boreal Software, Norway) [15]. In a separate group of experiments, hearts were freeze clamped in liquid nitrogen at the end of the reperfusion period and stored at -80°C for SK1 activity analysis.

Isolation of adult mouse cardiomyocytes

TNF-α KO (n=8), STAT-3 KO (n=6), and their respective littermate control mice (n=25) were anesthetized via an intraperitoneal injection (i.p.) of sodium pentobarbital (60 mg/kg) and heparinized (25 IU). The heart was rapidly excised and cannulated on the Langendorff perfusion system, and the cardiomyocytes were isolated as previously described [17]. Isolated cardiomyocytes were plated onto 6 well plates precoated with laminin in minimum essential medium containing penicillin, streptomycin and 5% foetal calf serum and incubated for 1 h at 37°C prior to experiments, as previously described [17].

Experimental protocol in isolated adult mouse cardiomyocytes

The experimental protocol conducted in isolated adult cardiomyocytes is shown in Figure 1. Normoxic control cells were maintained under normoxic conditions throughout the protocol (20% O_2, 5% CO_2 and balance N_2). Simulated ischemia control cardiomyocytes were subjected to 2 h of simulated ischemia using a simulated ischemic buffer ($MgCl_2.6H_2O$ 1.2 mM, KCl 16 mM, KH_2PO_4 1 mM, NaCl 74 mM, $CaCl_2$ 1.2 mM, $NaHCO_3$ 10 mM, sodium lactate 20 mM, HEPES 25 mM, pH 6.7) with cells kept in a humidified hypoxic environment (1% O_2, 5% CO_2 and balance N_2) at 37°C. The treated cardiomyocytes were exposed to S1P (10nM) for 30 min followed by a washout period of 30 min before the simulated ischemic insult. The SK1 inhibitor (SKI-178, 15 µM) was given for 1 h, starting 30 min before S1P treatment.

Culture of H9c2 cardiomyocytes

H9c2 cardiomyocyte cell lines were purchased commercially from the European Collection of Cell Cultures (Centre for Applied Microbiology and Research, UK). The cells were stored in cryovials at $1x10^6$ cells/mL in liquid nitrogen (-196˚C). To seed the cells, the cryovial was thawed at room temperature. T25 (25 cm^3) tissue culture flasks were supplemented with 4 - 6 mL Dulbecco's modified Eagle's medium (DMEM) and the contents of the cryovials were transferred into tissue culture flasks. The DMEM contained 4.5 g/L glucose, 0.110 g/L sodium pyruvate and L-glutamine and was supplemented with 10% fetal calf serum (FCS) and 1% (weight/volume) penicillin/streptomycin. When the cells (with a passage number between 15 and 18) reached 80% confluency, they were used for treatment and follow up experiments.

Experimental protocol in H9c2 cardiomyocytes

To further assess the role of SK1 in cardioprotection, H9c2 cells were subjected to simulated ischemia and exposed to melatonin used as an activator of the SAFE pathway, with or without SKI-I (n=5 independent experiments). Briefly, H9c2 cells were pretreated with melatonin (75 ng/L) for 40 minutes prior to 8 h of simulated ischemia with simulated ischemic buffer adjusted at pH 6.4 and hypoxic conditions (5% CO_2, 94% N_2 and 1% O_2), as previously described [18] . SKI-I (15 µM) was given (with or without melatonin) for 55 minutes prior to simulated ischemia. The control group (CTRL) was exposed to simulated ischemia only. The cell viability was measured at the end of the simulated ischemic insult.

Western blotting was used to further explore the role of SK1 in STAT-3 activation with exogenous S1P. Briefly, H9c2 cells were exposed to S1P (10 nM) for 7 min with or without pretreatment with SK1-I (15 µM) for 30 min. Cell lysates were electrophoresed and the levels of phosphorylated (tyr705) and total STAT-3 (Cell Signaling Technology, Inc., Danvers, MA, USA) were quantified, as previously described [19]. Equal loading was verified using β-actin. Densitometric analysis was performed using the ImageJ software. STAT-3 activation was measured as ratio of phosphorylated STAT-3 to total STAT-3.

Measurement of cell viability

To assess cell viability in isolated cardiomyocytes or H9c2 cardiomyocytes, the cells were loaded with 0.04% trypan blue and analysed using a light microscope at 40x magnification. The number of viable (unstained) and nonviable (blue stained) cardiomyocytes in four random microscopic fields was recorded, with at least 100 cells counted in each well.

Analysis of mitochondrial permeability transition

To assess the mitochondrial permeability transition pore (mPTP) opening at the end of 2 h of simulated ischemia, isolated cardiomyocytes were incubated with 20nM dye tetra-methyl-rhodamine-methyl ester (TMRM) for 10 min followed by a 10 min washout period [20]. Cardiomyocytes were then lifted from the wells using a cell scraper and analysed by flow cytometry (FACSCalibur, BD Biosciences, MD, USA) as previously described [21]. The fluorescence intensity was measured and analysed using the FlowJo

software (FlowJo LLC, Ashland, OR, USA) and expressed as arbitrary fluorescence units (AFU). Data were normalized to the normoxic control group.

Measure of SK1 activity

Frozen hearts were pulverized before protein extraction as previously described [15]. SK1 activity was measured using a specific biochemical fluorescence-based assay kit (Gyrasol Technologies, KS, USA) following the manufacturer's instructions. The SK1 activity was normalized to that of the control group.

Chemicals

Unless specified, all chemicals were obtained from Sigma-Aldrich, South Africa.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.0 (GraphPad Software, La Jolla, California, USA). Data are expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were performed using the Student's t-test. For comparisons of three or more groups, one-way ANOVA followed by the Holm-Sidak test for multiple comparisons or Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used, depending on the distribution of data. The Shapiro-Wilk test was used to test the normality of the data. Statistical significance was set at $p<0.05$.

Results

Exogenous S1P improves cell survival via the SAFE pathway.

To determinate the role of the SAFE pathway in S1P induced cardioprotection, isolated adult cardiomyocytes from TNF-α KO, STAT-3 KO, and their wildtype (WT) littermates were exposed to simulated ischemia with or without S1P pretreatment (Figure 2). Cardiomyocyte cell death under normoxic conditions in WT littermates (TNF-α WT and STAT-3 WT) and knockout mice (TNF-α KO and STAT-3 KO) was 21.4±1.2%, 15.7±1.3%, 19.3±1.5% and 21.3±3.3% respectively, indicating that approximately 20% of cardiomyocytes did not survive the isolation and the plating process. Exposure to 2 h of simulated ischemia increased cardiomyocyte cell death to 45.4±2.1% (TNF-α WT) and 39.6±2.1% (STAT-3 WT) ($p<0.05$ vs normoxic group), with comparable effects in TNF-α KO (45.0±1.4%) and STAT-3 KO (39.8±4.8%) ($p<0.05$ vs normoxic group). S1P pretreatment decreased cell death to 25.1±1.6% and 19.6±2.8% in TNF-α WT and STAT-3 WT, respectively ($p<0.01$ vs CTRL). However, S1P pretreatment did not protect cardiomyocytes against simulated ischemia in TNF-α KO (49.2±1.1%) or STAT-3 KO (41.7±3.8%) mice.

Exogenous S1P confers protection by inhibiting mPTP opening via the SAFE pathway.

To delineate the role of mPTP opening in the beneficial effect of S1P, isolated adult cardiomyocytes from TNF-α KO, STAT-3 KO, and their WT littermates were exposed to simulated ischemia with or without S1P pretreatment and TMRM fluorescence, reflecting mPTP opening, was measured (Figure 2). TMRM fluorescence intensity after exposure of the cells to 2 h of simulated ischemia decreased from 100% to 71.3±2.8% and 74.7±6.5% in TNF-α WT and STAT-3 WT, respectively ($p<0.01$ vs normoxic controls), indicative of mPTP opening. The same effect was observed in TNF-α KO and STAT-3 KO cardiomyocytes (77.7±2.0% and 68.1±3.0% respectively, ($p<0.01$ vs normoxic controls). S1P pretreatment restored the TMRM fluorescence intensity to 93.4±2.7% and 95.5±4.3% in TNF-α WT and STAT-3 WT cardiomyocytes, respectively ($p<0.05$ vs simulated ischemic groups). In contrast, S1P pretreatment failed to restore the TMRM fluorescence intensity after simulated ischemia in TNF-α KO (80.3±1.9%) and STAT-3 KO cardiomyocytes (68.4±3.7%).

SK1 is required for exogenous S1P-induced cardioprotection.

To evaluate whether the cardioprotective effect of exogenous S1P is dependent on SK1, isolated adult cardiomyocytes from WT mice were exposed to simulated ischemia in the presence of S1P with or without a specific SK1 inhibitor and cell viability was assessed (Figure 3A). Exposure to 2 h of simulated ischemia increased cardiomyocyte cell death from 23.9±0.5% to 48.4±0.7% ($p<0.0001$ vs normoxic control). S1P pretreatment decreased cell death to 25.4±1.0% ($p<0.0001$ vs simulated ischemia only). However, the beneficial effect of S1P was inhibited in the presence of SK1-I (46.4±2.0%) ($p<0.05$ vs S1P group). Similar findings were observed with exogenous melatonin as a cardioprotective agent known to activate the SAFE pathway (Figure 3B). In H9c2 cells subjected to simulated ischemia, a pretreatment with melatonin decreased cell death from 40.4±1.0% to 15.2±2.6 ($p<0.05$) but this cardioprotective effect was inhibited in the presence of SK1-I (39.8±6.1%).

Similar findings were also reported in an *ex vivo* model (Figure 4). In WT isolated hearts (figure 4A), S1P pretreatment reduced infarct size following IRI from 49.8±1.4% to 30.6±2.1% ($p<0.05$ vs CTRL). This effect was associated with an increase in SK1 activity (2.4±0.5 folds vs CTRL). The addition of SK1-I abolished both the infarct sparing and the increase in SK1 activity observed with S1P treatment (Figure 4A). In WT isolated hearts, the developed tension was decreased following an IRI in the CTRL group ($p<0.001$ reperfusion vs baseline), an effect that was lost when hearts were pretreated with S1P prior to IRI (n.s. reperfusion vs baseline in S1P group) (supplementary table 1). Interestingly, perfusion of SK1-I given together with S1P partially abolished the effect of S1P on the developed tension following IRI ($p<0.05$, reperfusion vs baseline in S1P+SK1-I group). At baseline or at the end of reperfusion, there was no difference in the coronary flow between the different experimental groups and IRI didn't alter the coronary flow at the end of reperfusion (supplementary table 1).

SK1 interacts with the SAFE pathway for cardioprotection

To better understand how SK1 interacts with the SAFE pathway, SK1 activity was measured in isolated hearts of both TNF-α KO and STAT-3 KO mice exposed to IRI with/without S1P pretreatment. In TNF-α KO mice, exogenous S1P failed to increase the activity of SK1 and reduce infarct size. Despite the increase in SK1 activity (3.1±0.9 fold) with S1P, no infarct size reduction was observed in STAT-3 KO mice. In addition, an increase in STAT-3 activation (1.7±0.1 fold) was observed after S1P stimulation (Figure 5), an effect that was prevented in the presence of SK1-I.

Discussion

The present findings highlight an interplay between the SAFE pathway and the sphingolipid pathway, where SK1 acts as a key element to activate STAT-3 downstream of TNF-α for cardioprotection. First, S1P-induced cardioprotection in isolated WT cardiomyocytes was associated with an increase in SK1 activity. Second, the presence of a specific SK1 inhibitor abolished STAT-3 activation and the cytoprotective effects of S1P. Third, the absence of S1P-induced cardioprotection in TNF-α or STAT-3 KO isolated hearts was associated with an increase in SK1 activity in STAT-3 KO hearts only, suggesting that SK1 functions as an intermediate component between TNF-α and STAT-3 to activate the SAFE pathway.

S1P is a well-known pharmacological conditioning agent that confers protection against IRI. Multiple signaling cascades have been proposed, including the Reperfusion Injury Salvage Kinase (RISK) pathway, the endogenous sphingolipid pathway and the activation of the SAFE pathway, with possible cross-talk among these various pathways [22]. Although mPTP is known as a key end-target of the RISK and Sphingolipid pathways to promote cell survival, its role downstream of the SAFE pathway remains poorly studied [23–25]. Using TNF-α and STAT-3 KO animals, we demonstrated that S1P-induced protection against IRI was dependent on a decrease in susceptibility to mPTP opening downstream of the SAFE pathway (TNF-α/STAT-3). The fact that mPTP is a common downstream effector of the Reperfusion Injury Salvage Kinase (RISK), sphingolipid and SAFE pathways to limit cell death raises the question of whether these different pathways interact with each other. Previous research has provided some insights into the interaction between the RISK and the SAFE pathways, demonstrating that Akt, a major kinase of the RISK pathway, modulates the activation of STAT-3, and vice versa [5,8]. However, there is little information on the possible interplay between components of the sphingolipid network, such as SK1, and the SAFE pathway for cardioprotection.

SK1 is an enzyme present in cells that converts sphingosine into S1P [22]. As such, it is an essential component of the sphingolipid pathway. SK1 activity decreases following IRI, an effect that can be mitigated by cardioprotective strategies such as ischemic conditioning [26]. Our findings demonstrate that SK1 is required to limit cell death caused by IRI in both isolated cardiomyocytes and isolated hearts. Given that S1P is a product of SK1, one could speculate that providing exogenous S1P, might compensate the absence of production of cardioprotective intracellular S1P formation by SK1 inhibitors.

However, this hypothesis was not supported by our experiments, as the cardioprotective effect of exogenous S1P was lost in the presence of an SK1 inhibitor. Furthermore, we confirmed that the cardioprotective effect of exogenous melatonin, another cardioprotective agent that does not belong to the sphingolipid pathway, was also lost in the presence of the SK1 inhibitor. Given that both melatonin and exogenous S1P provide cardioprotection through activation of the SAFE pathway [5–7], it can be inferred that SK1 activation may be linked to the activation of the SAFE pathway.

Upon stimulation with exogenous S1P, TNF-α binds to its receptor 2, triggering the phosphorylation of Janus kinase (JAK), which in turn, phosphorylates cytosolic STAT-3 [7]. Once phosphorylated, STAT-3 can translocate to the nucleus and mitochondria to promote cell survival [7,8]. In the present study, the absence of both cardioprotection and SK1 activation with exogenous S1P stimulation in TNF-α KO mice suggests that SK1 activation is dependent on TNF-α. This conclusion aligns with previous research that documented a rapid increase in SK1 activity following TNF-α stimulation of human endothelial cells [27]. It is also supported by experimental data demonstrating that TNF-α can bind to TNF receptor 2, activating TNF receptor-associated factor 2 (TRAF2), which subsequently enables the binding (and activation) of SK1 to its TRAF2-binding motif [28,29]. Even though exogenous S1P also failed to limit infarct size in STAT-3 KO hearts subjected to IRI, it induced an increase in SK1 activity that was comparable to that observed in WT hearts. This finding aligns with previous research that concluded that STAT-3 activation is required to provide an S1P-induced reduction in infarct size [5]. More interestingly, this suggests that not only SK1 activation is not dependent on STAT-3 activation (contrary to what has been proposed in colorectal cancer [30]) but also that SK1 activation cannot provide cytoprotection against IRI in the absence of STAT-3, which may thus act as a downstream mediator of cardioprotection. It would be of interest to explore whether TRAF2 is the sole activator of SK1 in our model. Indeed, other stimuli known to activate SK1 include extracellular regulated kinase (ERK) which can be activated after binding of S1P to S1P receptors may possibly also contribute to activate SK1 in our setting [31,32]. Additionally, our results in H9c2 cells indicate that inhibition of SK1 prevents S1P-induced STAT-3 activation, further suggesting a role for SK1 in modulating STAT-3 activation, and thus, the SAFE pathway. This raises the question of how SK1 can activate STAT-3. A potential explanation may come from a previous study in cancerous lymphocytes reporting that the specific SK1 inhibitor SKI-178 can prevent phosphorylation (i.e., activation) of both JAK and STAT-3, leading to

massive cell death [16]. Although the cellular behaviors and mechanisms of cancer cells and cardiomyocytes may differ, our data also suggest that SK1 activates JAK/STAT3 for cardioprotection.

This study has some limitations. The primary role of SK1 is the formation of S1P from sphingosine, and it is possible that some mechanisms of the cardioprotective effect of SK1 involve further formation of endogenous S1P and the possible binding of S1P to its receptors. Indeed, exogenous S1P fails to protect in S1P-Receptor-2 and S1P-Receptor-3 KO mice [33] and activation of S1P Receptors can lead to STAT-3 phosphorylation [19,34]. Therefore, it would be important to further study the cellular dynamic of S1P and the possible interaction of S1P receptors/other sphingolipid components with the SAFE pathway in future studies. Another limitation is that we focused our investigations on S1P-induced cardioprotection only. It would also be interesting to also assess the role of ceramide (a precursor to sphingolipids and S1P) in the interplay between the SAFE and the sphingolipid pathways in IRI. Indeed, previous studies have suggested that TNF-induced cardioprotection is mediated by ceramide [35]. In our experimental model, we explored the role of SK1 on the activation of the SAFE pathway to improve cell survival. It would also be of interest to explore in detail the role of the modulation of these signalling events on the haemodynamic parameters of the heart following an ischemia-reperfusion insult. In addition, no in vivo models were used in any of our investigations, which means that the potential role of plasma high-density lipoprotein content was not considered. High-density lipoproteins contain a considerable amount of sphingolipids (including S1P), which may affect the response to the interventions [10,36]. However, several models have been employed, including isolated hearts from KO animals, which have been extensively validated to evaluate signalling pathways, particularly because they enable the use of inhibitors without causing toxicity [3,5,12,19,37]. Finally, It would be interesting to assess whether

Conclusion

This study suggests that the interplay between the SAFE and sphingolipid pathways is essential for effective cardioprotection with S1P. SK1 emerges as a crucial factor in activating STAT-3 through TNF-α in the SAFE pathway (see Figure 6). Our findings provide a basis for innovative cardioprotective approaches that could target SK1 to regulate the activation of the SAFE pathway and enhance cell survival after IRI.

Abbreviations

AFU	Arbitrary fluorescence units
CTRL	Control group
DMEM	Dulbecco's Modified Eagle Serum
IRI	Ischemia reperfusion injury
KO	knockout
MLC2v	ventricular myosin light chain 2
mPTP	Mitochondrial permeability transition pore
SAFE	Survivor Activating Factor Enhancement
SEM	Standard error of the mean
SK1	Sphingosine kinase 1
SK1-I	Sphingosine kinase 1 inhibitor
S1P	Sphingosine 1 phosphate
STAT-3	Signal Transducer and Activator of Transcription 3
TMRM	Tetra-methyl-rhodamine-methyl ester
TNF-α	Tumor necrosis factor alpha
TRAF2	TNF receptor-associated factor 2
WT	Wild type

Acknowledgements

The authors would like to thank Ms Nolize Dietrich for her assistance in data capturing.

Declaration of interests

The authors declare that they have no conflict of interest related to the present study.

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Funding sources

Part of the study was funded by the University of Cape Town, the South African Medical Research Council and the South African National Research Foundation. Sarah Pedretti and Martin Cour received a Fellowship from the University of Cape Town, South Africa. Sandrine Lecour received funding from the Oppenheimer Memorial trust. Derek J Hausenloy is supported by the Duke-NUS Signature Research Programme funded by the Ministry of Health, Singapore Ministry of Health's National Medical Research Council under its Singapore Translational Research Investigator Award (MOH-STaR21jun-0003), Centre Grant scheme (NMRC CG21APR1006), and Collaborative Centre Grant scheme (NMRC/CG21APRC006).

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Figures Legends

Figure 1 - Experimental protocol in isolated hearts and cardiomyocytes

WT: wild type; KO: knockout; TNF-α: Tumor Necrosis Factor α; STAT-3: Signal Transducer and Activator of Transcription 3; CTRL: Control; S1P: Sphingosine 1 phosphate; SK1: Sphingosine kinase 1, SK1-I: SK1 inhibitor.

Figure 2 - Cytoprotective effect of S1P in TNF-α KO and STAT-3 KO isolated cardiomyocytes following simulated ischemia

Cell death (left panels) and mitochondrial permeability transition pore (mPTP) opening (right panels) were assessed in cardiomyocytes isolated from wild-type (WT) TNF-α mice (panel A; n=4 -9/group), TNF-α knockout (KO) mice (panel B; n=4-8/group), WT STAT-3 mice (panel C; n=4-15/group), and STAT-3 KO mice (panel D; n=4-6/group). Cardiomyocytes were exposed to simulated ischemia (CTRL) with or without pretreatment with sphingosine 1 phosphate (S1P). Cell death was assessed by microscopy after staining with tryptan blue, and mPTP opening was assessed by fluorescence intensity using tetra-methyl-rhodamine-methyl (expressed as arbitrary fluorescence units, AFU).

* $p<0.05$; ** $p<0.01$; **** $p<0.001$

Figure 3 - Effect of SK1 inhibitor on the cytoprotective effect of S1P or melatonin in cardiomyocytes exposed to simulated ischemia

Cell death was assessed in A) isolated cardiomyocytes (n=6-20/group) exposed to simulated ischemia (CTRL) with sphingosine 1 phosphate (S1P) and/or sphingosine kinase 1 inhibitor (SK1-I), and B) H9c2 cells (n=5/group) exposed to simulated ischemia (CTRL) with melatonin (Mel) and/or SK1-I.

* $p<0.05$; **** $p<0.0001$

Figure 4 - Interplay between the SAFE pathway and SK1 in S1P-induced cardioprotection in isolated hearts subjected to ischemia-reperfusion injury.

Infarct size and sphingosine kinase 1 (SK1) activity were assessed in isolated hearts of wildtype (panel A; n=6-15/group), TNF-α knockout (KO) (panel B; n=4-7/group) and STAT-3 KO (panel C; n=4-6/group) mice subjected to ischemia-reperfusion injury (CTRL) and treated with sphingosine 1 phosphate (S1P). SK1-I: sphingosine kinase inhibitor.

* p<0.05; ** p<0.01; *** p<0.001

Figure 5 - Effect of SK1 on S1P-induced STAT-3 activation in H9c2 cells

H9c2 cells were exposed to sphingosine 1 phosphate (S1P) in the presence or absence of sphingosine kinase 1 inhibitor (SK1-I). Phosphorylated STAT-3 (Phospho-STAT-3) and total STAT-3 and were assessed by western blotting (panel B). Phospho-STAT-3/STAT-3 in S1P and S1P+SK1-I groups (n=6/group) were compared to the control (CTRL) group (expressed as fold increase).

* p<0.05

Figure 6 - Proposed interplay between Sphingosine Kinase 1 and the SAFE pathway for cardioprotection.

Abbreviations: JAK: Janus Kinase; mPTP: Mitochondrial Permeability Transition Pore; SK1: Sphingosine Kinase 1; STAT-3: Signal Transducer and Activator of Transcription 3; TNF-α: Tumour Necrosis Factor alpha; TNFR2: TNF receptor 2; TRAF2: TNF receptor-associated factor 2.

Figure 1

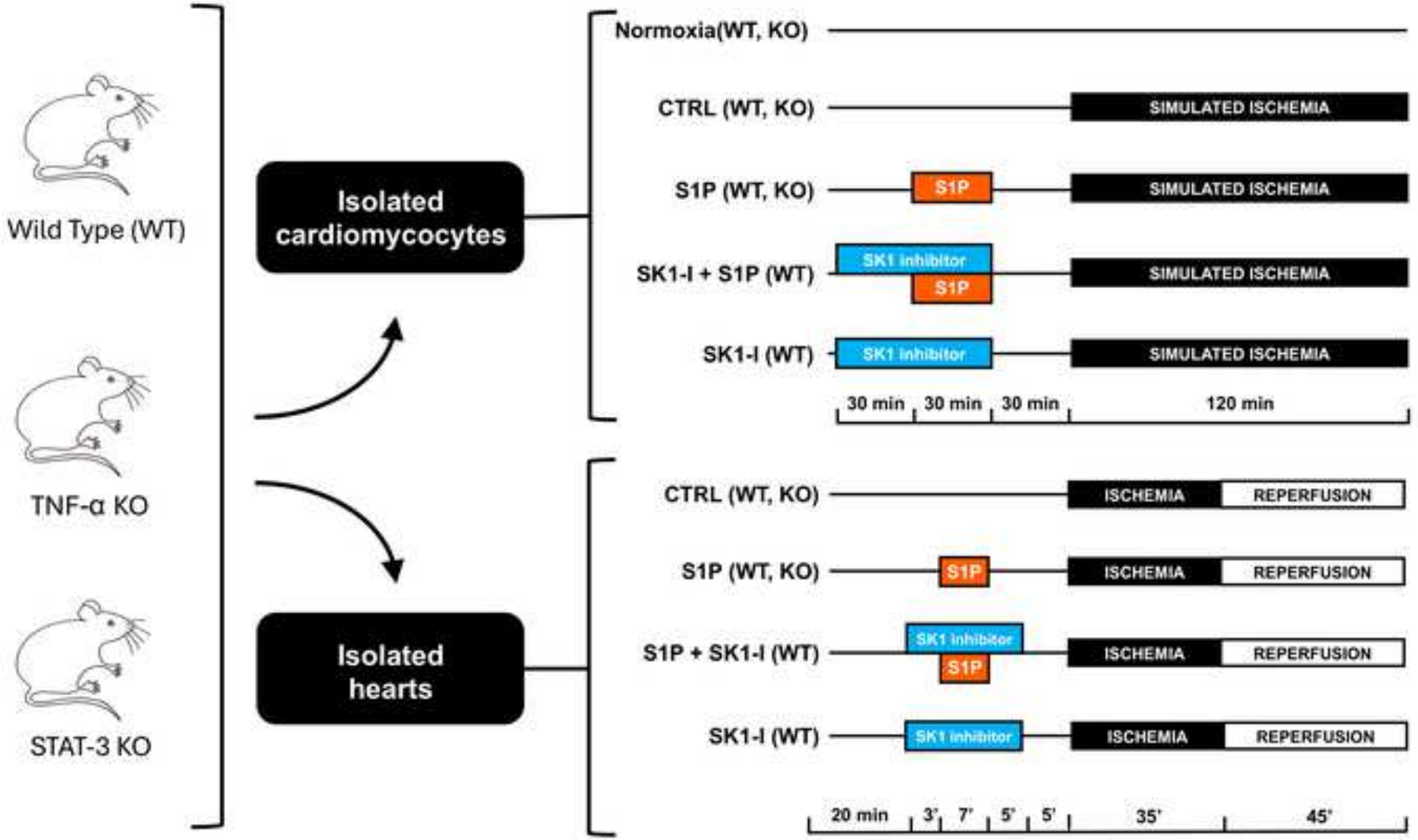

Figure 2

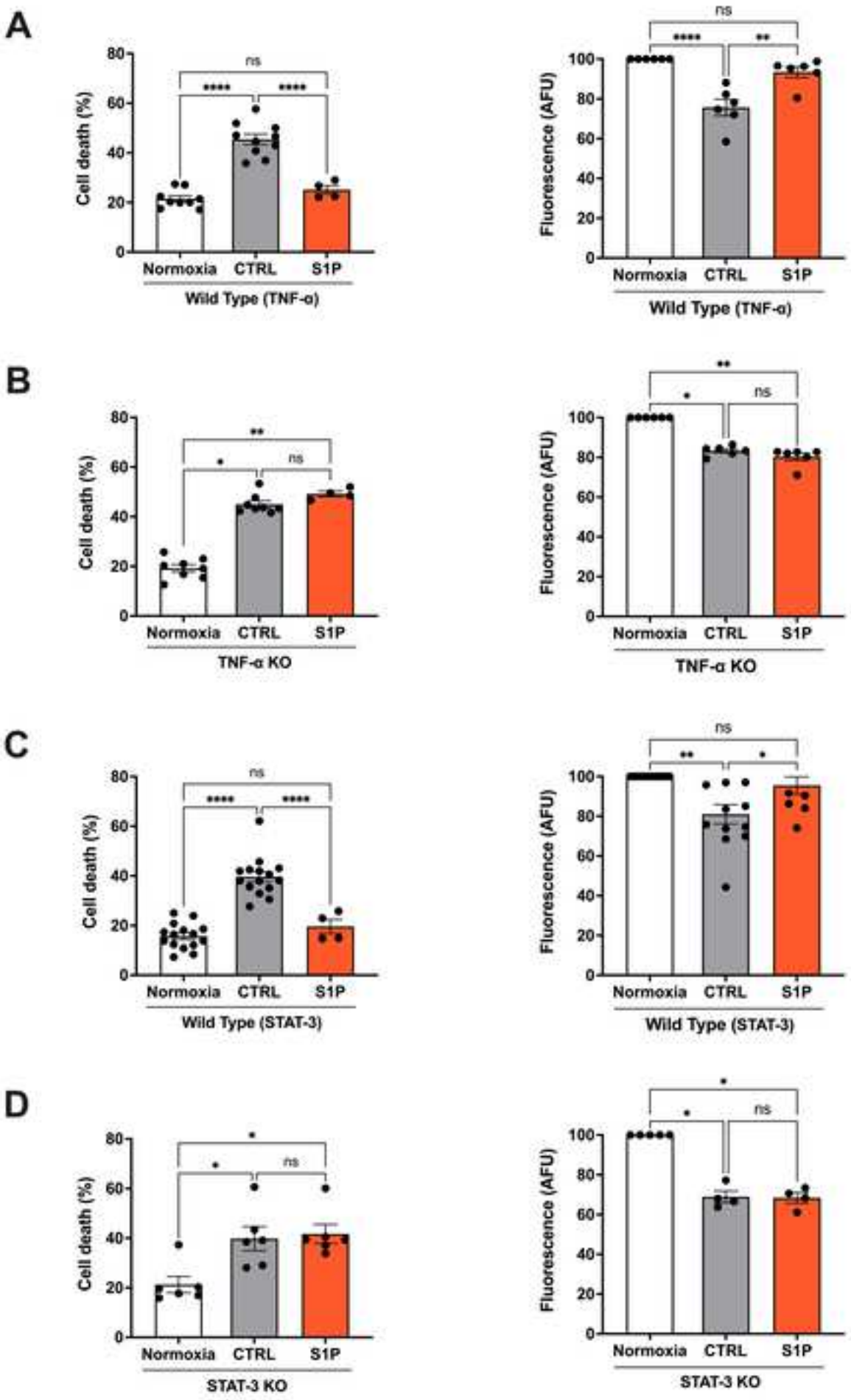

Figure 3

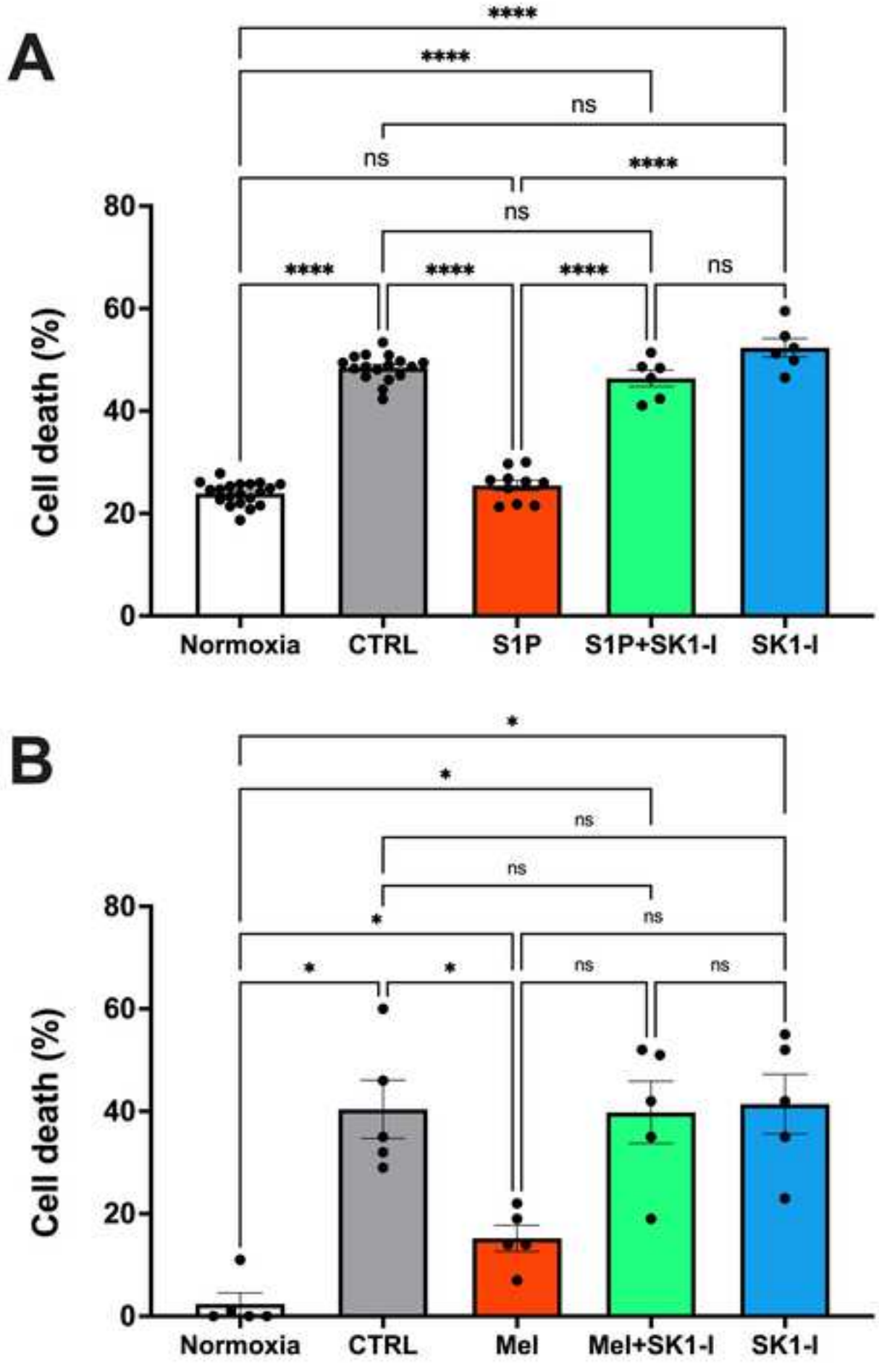

Figure 4

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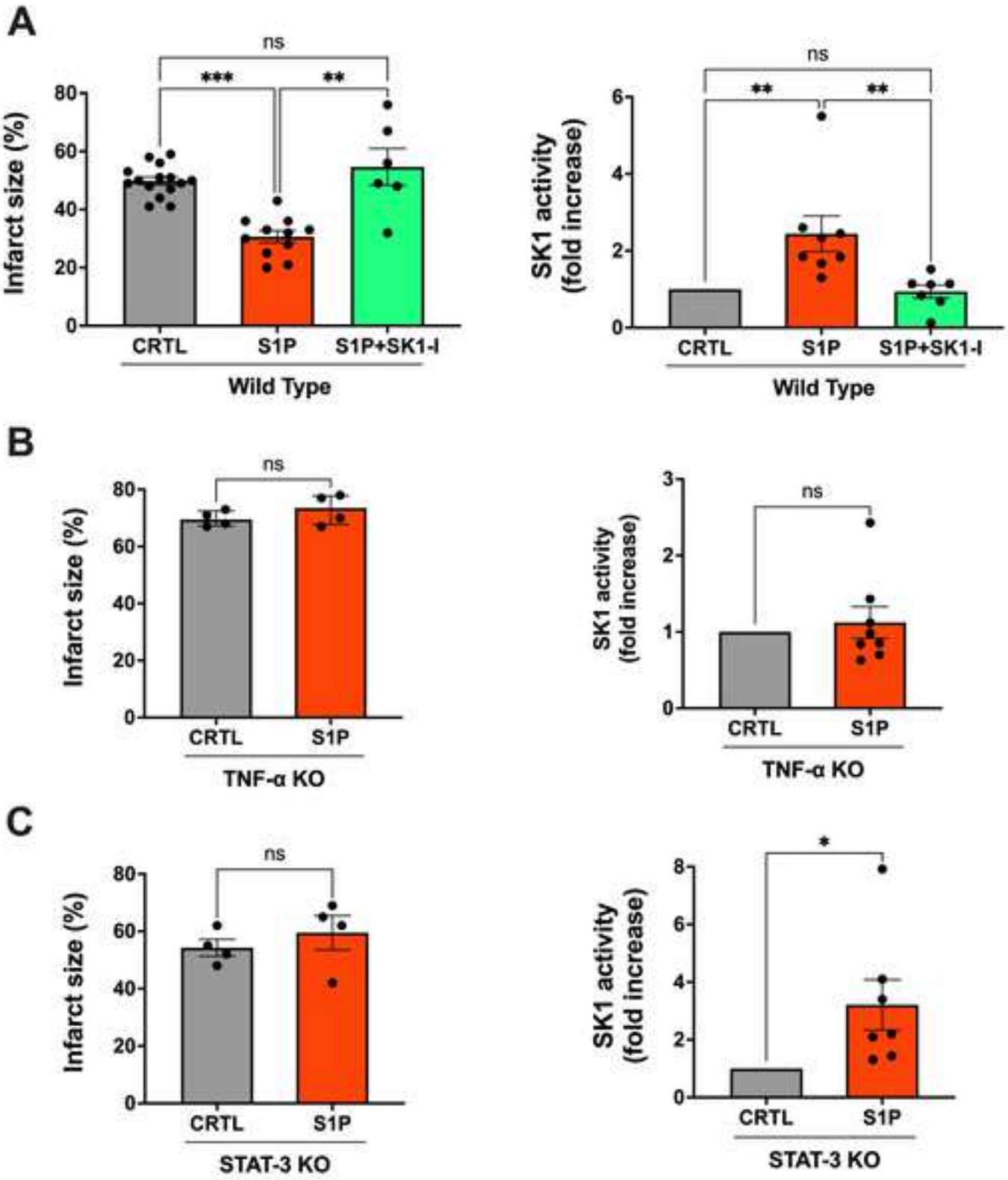
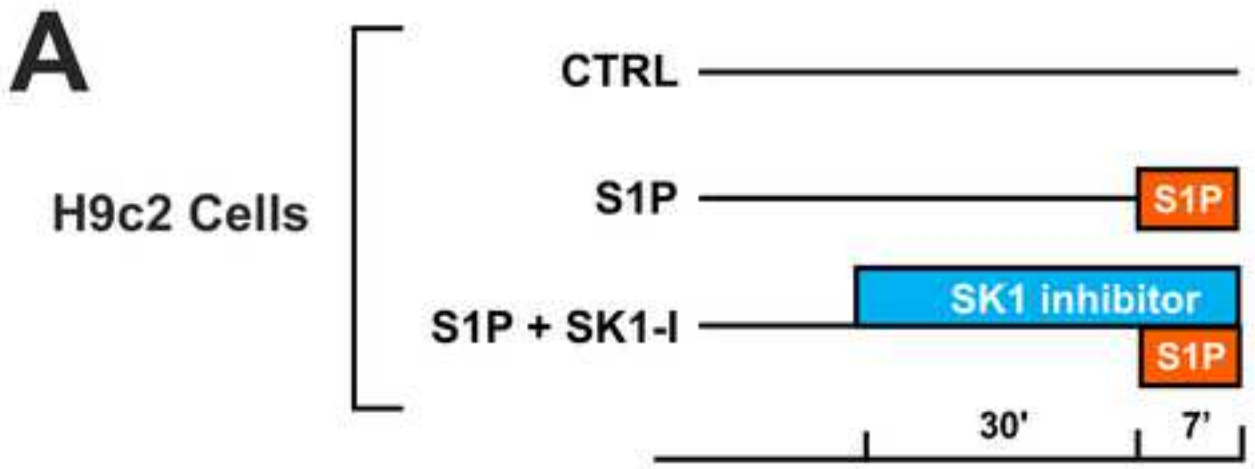


Figure 5

Click here to access/download;Figure;FIGURE 5_H9C2 S1P V3.jpg



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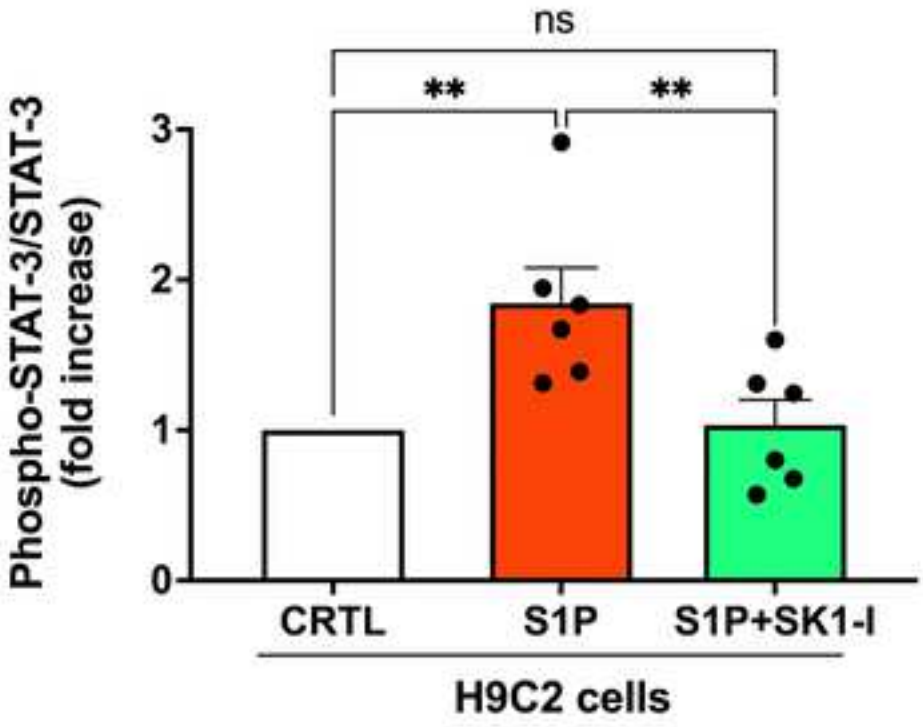

Figure 6

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