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A novel role for mitochondrial sphingosine-1-phosphate produced by sphingosine kinase-2 in PTP-mediated cell survival during cardioprotection

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Abstract

Although mitochondria are key determinants of myocardial injury during ischemia–reperfusion (I/R), their interaction with critical cytoprotective signaling systems is not fully understood. Sphingosine-1-phosphate (S1P) produced by sphingosine kinase-1 protects the heart from I/R damage. Recently a new role for mitochondrial S1P produced by a second isoform of sphingosine kinase, SphK2, was described to regulate complex IV assembly and respiration via interaction with mitochondrial prohibitin-2. Here we investigated the role of SphK2 in cardioprotection by preconditioning. Littermate (WT) and *sphk2*$^{-/-}$ mice underwent 45 min of in vivo ischemia and 24 h reperfusion. Mice received no intervention (I/R) or preconditioning (PC) via 5 min I/R before the index ischemia. Despite the activation of PC-cytoprotective signaling pathways in both groups, infarct size in *sphk2*$^{-/-}$ mice was not reduced by PC (42 ± 3% PC vs. 43 ± 4% I/R, p = ns) versus

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WT (24 ± 3% PC vs. 43 ± 3% I/R, $p < 0.05$). *sphk2*$^{-/-}$mitochondria exhibited decreased oxidative phosphorylation and increased susceptibility to permeability transition (PTP). Unlike WT, PC did not prevent ischemic damage to electron transport or the increased susceptibility to PTP. To evaluate the direct contribution to the resistance of mitochondria to cytoprotection, SphK2, PHB2 or cytochrome oxidase subunit IV was depleted in cardiomyoblasts. PC protection was abolished by each knockdown concomitant with decreased PTP resistance. These results point to a new action of S1P in cardioprotection and suggest that the mitochondrial S1P produced by SphK2 is required for the downstream protective modulation of PTP as an effector of preconditioning protection.

Keywords

Mitochondria; Permeability transition pore; Sphingosine kinase-2; Mitochondrial S1P; Oxidative phosphorylation; Ischemic preconditioning

Introduction

Coronary heart disease is the leading cause of death in Western countries. Ischemic preconditioning (PC) reduces infarct size after a prolonged ischemia–reperfusion [47], including in humans [66]. In most species, repeated short episodes of I/R activate intrinsic prosurvival signaling kinases via the reperfusion injury salvage kinases (RISK) pathway, that includes PI3K/Akt and ERK1/2, and the alternative survivor activating factor enhancement (SAFE) pathway [7] which activates the cytokine tumor necrosis factor alpha (TNFα) [42] and the transcription factor signal transducer and activator of transcription-3 (STAT-3) [40, 57] at reperfusion.

These two pathways converge on mitochondria and are proposed to result in inhibition of mitochondrial permeability transition pore (PTP) opening, by distal components of the cascades which include NO and inhibition of glycogen synthase kinase 3β (GSK3β) [21, 23, 37] and likely hexokinase II (HKII) [5, 19]. Mitochondria are centrally involved in the signal transduction of PC [30] which leads to a partial uncoupling of mitochondrial respiration, resulting in decreased mitochondrial injury following subsequent sustained ischemia [9, 10]. Opening of the PTP is a major determinant of cardiomyocyte death and inhibition of the PTP is a key mechanism of protection by ischemic post- and preconditioning [3, 15, 29, 31, 34, 48], including in humans [51]. Opening of the PTP is characterized by a sudden change of inner mitochondrial membrane permeability. However, the mechanism whereby cytoprotective kinase pathways modulate PTP opening remains an area of active investigation.

Previous studies have demonstrated a role of sphingosine-1-phosphate (S1P) in regulation of cardioprotection [38, 53, 61]. Indeed, activation of sphingosine kinase 1 (SphK1) and production of S1P is an important mediator of PC as shown by the use of a SphK inhibitor [35]. It has been suggested that this protection is due to the “inside-out” effect of S1P [61] whereby the S1P formed inside cells by SphK1 is released and activates plasma membrane S1P receptors, either on the same cell or on nearby cells. S1P receptor ligation then triggers signaling cascades that suppress apoptosis and leads to cardioprotection [59, 61, 67]. As a G protein-coupled receptor (GPCR) ligand, S1P produced by SphK1 activates sarcolemmal receptors, which in turn activate the RISK and SAFE pro-survival kinases.

Although there is no doubt regarding the extracellular function of S1P produced by SphK1 during ischemia–reperfusion [36, 59, 60], much less is known regarding the S1P produced by SphK2. Recent studies have elucidated two new intracellular roles of SphK2. First,

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SphK2 localized in the nucleus can produce S1P, which binds to and inhibits histone deacetylases 1 and 2, causing an increase in acetylation of specific histone lysines and an increase of specific gene transcription [25]. Second, our group has recently shown that S1P produced by SphK2 in the mitochondria interacts with prohibitin 2 (PHB2) to control the assembly and function of cytochrome oxidase (COX) in the electron transport chain [56]. SphK2 null mitochondria present a reduced mitochondrial S1P content in the presence of a normal cytosolic S1P content, consistent with the fact that SphK1 activity was unchanged in the SphK2 deficient mice [56]. Furthermore, the absence of SphK2 and thus the decreased mitochondrial S1P content led to a dysfunction in mitochondrial respiration through cytochrome oxidase at baseline in these mice [56].

A recent study on STAT3-deficient mice that contain defects in mitochondrial respiration at baseline showed that these knockout mice were not more sensitive to ischemia–reperfusion but refractory to cardioprotection [6].

Since mitochondria contribute a critical role in determining whether or not the heart recovers during reperfusion [28], we investigated the cardiac role of mitochondrial S1P produced by SphK2 on modulation of PTP opening during ischemia–reperfusion. The present work is the first in vivo study to demonstrate that, due to a preexisting defect in mitochondrial respiration and enhanced susceptibility to PTP opening, SphK2 deficient mice cannot be protected by preconditioning and sustain an increase in myocardial injury in vivo despite activation of PC dependent cytoprotective kinase pathways. The PHB2/COX mechanism of SphK2 action in mitochondria is supported by the attenuation of cardioprotection by the acute downregulation of PHB2 and COX subunit IV. Mitochondrial S1P generated by SphK2 acts at the mitochondrial level via modulation of PTP opening and the regulation of oxidative phosphorylation.

Methods

Protocols and studies involving animals were performed in accordance with the Virginia Commonwealth University Institutional Animal Care and Use Committee guidelines. SphK2 knockout mice were obtained from Dr. Richard Proia (NIH).

Murine left anterior descending coronary artery occlusion model

WT littermates and transgenic male mice (8–10 weeks old) were anesthetized by intraperitoneal injection of pentobarbital sodium (90 mg/kg) and buprenorphine (0.01 mg/kg SC). Animals were orally intubated and ventilated by a rodent ventilator (minivent, Harvard Apparatus).

A left thoracotomy was performed in the fourth intercostal space. A small curved needle was passed around the left anterior descending coronary artery to induce ischemia reperfusion. Mice were subjected to 45 min of regional myocardial ischemia followed by either 10 min (for isolation of mitochondria, see below) or 24 h of reperfusion. Myocardial infarct size was then determined by triphenyltetrazolium staining [20–22, 64]. Mice were randomly assigned to receive no additional treatment (ischemia–reperfusion, I/R) or ischemic preconditioning (PC), induced by one cycle of 5 min ischemia and 5 min of reperfusion before the index ischemia.

Isolation of a mixed population of murine cardiac mitochondria

Heart mitochondria were isolated at 10 min reperfusion. Myocardial area at risk was removed and placed into buffer A (100 mM KCl, 50 mM 3-(*N*-morpholino) propanesulfonic acid, 1 mM EGTA, 5 mM $MgSO_4$, and 1 mM ATP, pH 7.4 at 4°C) containing 0.2% bovine serum albumin. Cardiac tissue was homogenized with a polytron tissue processor

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(Kinematica GmbH, Switzerland) for 2.5 s at a rheostat setting of 10,000 rpm. The homogenate was centrifuged at 3,000*g* to collect the crude cytosolic fraction. The pellet was resuspended in buffer A and incubated with 5 mg/g trypsin for 15 min at 4°C. After homogenization with a Teflon pestle at 600 rpm, the nuclear fraction was pelleted at 500*g*. The remaining supernatant was then centrifuged at 3,000*g* to pellet the mitochondria. After washing, the mitochondria were resuspended in 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA, pH 7.4. Protein content was measured by Lowry determination. Mitochondria were kept on ice and used within 4 h.

Mitochondrial oxidative phosphorylation

Oxygen consumption in mitochondria was measured using a Clark-type oxygen electrode at 30°C. Mitochondria were incubated in buffer containing 80 mM KCl, 50 mM MOPS, pH 7.4, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg/ml BSA. Glutamate/malate (complex I substrate, 20/5 mM) and the complex IV substrate TMPD (1 mM)-ascorbate (10 mM) plus rotenone (7.5 μM) were used as electron donors. Maximal rate of state 3 respiration (2 mM ADP) was measured as previously described [13]. The net reactive oxygen species (ROS) production was measured as net H_2O_2 production (pmol/30 min/mg protein).

Calcium retention capacity (CRC)

CRC is defined as the amount of Ca^{2+} required to trigger a massive Ca^{2+} release by isolated cardiac mitochondria. It is used as an indicator of the PTP sensitivity to Ca^{2+} and expressed as nmol $CaCl_2$/mg mitochondrial protein [21, 32]. CRC was evaluated in medium containing 150 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinic acid in 20 mM Tris/HCl, pH 7.4 by gradual addition to fresh mitochondria (125 μg/ml at 25°C) of a known amount of calcium (5 nmol). Extramitochondrial Ca^{2+} concentration was recorded with 0.5 μM Calcium Green-5N and fluorescence monitored with excitation and emission wavelengths set at 500 and 530 nm, respectively. Assessment of the CRC was performed in each experimental group (n = 4/group).

Analysis of ERK1/2, Akt, GSK3*β* and STAT3 phosphorylation by western blot

After the preconditioning stimulus (5 min ischemia followed by 5 min reperfusion), the area at risk was removed and homogenized in buffer A supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Meylan, France). A total of 50 μg of each sample was separated by SDS-PAGE on 10% gels. The phosphorylation state and the total protein of ERK1/2, Akt, STAT3 and GSK3*β* were determined by immunoblotting with antibodies from Cell Signaling Technology (Danvers, MA) (n = 4/group). Relative levels were determined by densitometry using ImageJ (NIH, USA; http://rsb.info.nih.gov/ij/).

Cell culture and transfection

H9c2 cardiomyoblasts were issued to Centre National de la Recherche Scientifique (CNRS) (C. Kieda, patent 99-16169, France). All cell culture reagents were obtained from Invitrogen (Cergy Pontoise, France). Cells were cultured under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mM glucose and supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were plated at a density of 15,000 cells/cm^2 and passaged when they were 70–80% confluent. Specific siRNAs targeted to SphK2, PHB2 or COX IV were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were grown to 80% confluence and transfected with 100 nM each siRNA using DharmaFECT 1 siRNA transfection reagent (Fisher-Bio-block, Illkirch, France). 24 h later, transfection mixtures were replaced with complete regular medium antibiotic-free. 48 h after transfection, cells were lysed and proteins analyzed by western blotting.

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Cellular model of hypoxia–reoxygenation (H/R)

H9c2 cardiomyoblasts at 37°C were subjected to 180 min hypoxia followed by 60 min reoxygenation. siRNA transfected cells were randomized to receive no further intervention (H/R) or preconditioning (PC) performed by 20 min hypoxia followed by 20 min reoxygenation before the long period of hypoxia. During hypoxia, the cell culture medium was replaced with an acidic medium containing (in mM): 118 NaCl, 2.6 KCl, 14.5 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 at pH 6.2, and cardiomyoblasts were exposed to hypoxia in a controlled hypoxic chamber (Adelbio®, Clermont-Ferrand, France) by 95% nitrogen and 5% CO_2 gas mixture flushing up to partial O_2 pressure of 1–2%. Reoxygenation was conducted in a normoxic incubator at 37°C, by replacing the acidic medium for 1 h with a pH 7.4 Krebs–Henseleit buffer containing 11 mM glucose and 2% BSA.

Measurement of H9c2 cell death after H/R

To determine cell death, cells were loaded with 5 μM propidium iodide (PI) which only permeates damaged cells. Cardiomyoblasts were imaged with an Olympus IX-50 (Japan) inverted microscope equipped with a 100 W mercury lamp (HBO) as a source of epifluorescence illumination and with a 12-bit cooled digital CCD camera (ORCA R2, Hamamatsu). Propidium iodide (PI) fluorescence was excited at 520–550 nm and recorded at 580 nm. Images (4 fields of 250–300 cells each) were acquired after an illumination time of 20 ms per image using digital epifluorescence imaging software (ImageJ, NIH, USA; http://rsb.info.nih.gov/ij). The cell death percentage after the reoxygenation period was calculated from the number of cells stained with PI divided by the total number of cells.

Measurement of PTP opening in cells

Direct assessment of PTP opening in H9c2 cells was performed with calcein acetoxymethyl ester (calcein-AM) loading and $CoCl_2$, resulting in mitochondrial localization of calcein fluorescence [39, 50]. Cells were loaded with 1 μM calcein-AM for 20 min at 37°C in 1 ml, pH 7.4, reoxygenation buffer supplemented with 1 mM $CoCl_2$. They were then washed free of calcein and $CoCl_2$ and incubated in reoxygenation medium. PTP opening was determined from the reduction in mitochondrial calcein signal (expressed as the percentage of the baseline value) after injection of H_2O_2 (100 μM). Fluorescence was measured in cells every 10 s after an illumination time of 15 ms per image (emitting from 460 to 490 nm and detecting at 515 nm) using digital epifluorescence imaging software (ImageJ, NIH, USA; http://rsb.info.nih.gov/ij).

Immunoblotting studies of H9c2 cells

After transfection, cells were lysed in buffer containing 20 mM Tris pH 8.0, 138 mM NaCl, 1% NP40, 1 mM DTT, 2.7 mM KCl, 1 mM MgCl, 2.5% glycerol, 5 mM EDTA supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cells were then centrifuged to remove insoluble material, and the protein concentration was determined using the Bradford assay. Equal amounts of protein (50 μg) were separated by SDS-PAGE, and immunopositive bands (antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were visualized by enhanced chemiluminescence as previously described [25, 56].

Statistical analysis

Data are expressed as the means ± SEM. Differences in the relationship between infarct size and area at risk were evaluated by analysis of covariance (ANCOVA) and post hoc Tukey's test, with infarct size as the dependent variable and area at risk as the covariant. Statistical calculations were performed using Statview® 5.0 Power PC version (SAS Institute Inc., Cary, NC). For other analyses, differences between groups were compared by one-way

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analysis of variance (ANOVA). When a significant F value was obtained, means were compared using a Tukey's test. Statistical significance was defined as a value of $p < 0.05$.

Results

Infarct size is not reduced by ischemic preconditioning in *sphk2*$^{-/-}$ mice

A genetic approach combined with an in vivo model of ischemia–reperfusion was used to assess the involvement of decreases in mitochondrial S1P in myocardial reperfusion injury. Areas at risk (AAR) were comparable among groups, ranging from 41 ± 1 to 43 ± 2% of the left ventricle (p = ns among groups). When infarct size was plotted versus its major determinant in this mouse model, AAR, wild type mice exhibited a significant linear relationship between infarct size and AAR in the untreated I/R group (Fig. 1a) with an infarct size averaging 43 ± 3% of AAR (Fig. 1b). As expected [24, 47], most data points for preconditioned hearts were below the regression line for the untreated I/R group (Fig. 1a), indicating that for any size of AAR, PC hearts developed significantly smaller infarcts, averaging 24 ± 3% of AAR (Fig. 1b). Ischemic *sphk2*$^{-/-}$ mice developed similar infarct size compared to WT averaging 43 ± 4% of AAR (p = ns, Fig. 1b). In contrast, PC failed to induce protection in *sphk2*$^{-/-}$ mice, with an infarct size averaging 42 ± 3% of AAR (Fig. 1b) and a comparable slope of the regression line compared to ischemic *sphk2*$^{-/-}$ group (p = ns, Fig. 1c). These data suggest that SphK2, although not contributing to infarct size in our model, is required for effective protection by ischemic preconditioning.

Deletion of SphK2 does not affect preconditioning cytoprotective pathways

In order to address the mechanism of a resistant phenotype to cardioprotection in *sphk2*$^{-/-}$ mice, signaling pathways that mediate cytoprotection were assessed. Following the PC stimulus, phosphorylated forms of ERK1/2, Akt, STAT3 and GSK3β were significantly increased in both WT and *sphk2*$^{-/-}$ groups compared to sham-WT ($p < 0.05$, Fig. 2). Surprisingly, although there is no reduction of infarct size in *sphk2*$^{-/-}$ mice, protective signaling pathways are activated. In the basal condition (sham), phosphorylated forms of Akt, GSK3β and STAT3 were slightly increased in *sphk2*$^{-/-}$ compared to the WT group ($p < 0.05$, Fig. 2). Thus, in the absence of SphK2, cardioprotection is ineffective despite the activation of cytoprotective signaling pathways, including those known to be activated by SphK1 [35].

Preconditioning failed to protect mitochondrial function in *sphk2*$^{-/-}$ *mice*

Since cytoprotective signaling pathways were activated by PC, we next focused on the effector of protection, the mitochondria. In the baseline state (sham groups), respiration measured with substrate donors to complex I was lower in mitochondria isolated from *sphk2*$^{-/-}$ compared to WT mice (Fig. 3a, [56]). A similar decrease was observed with an electron donor to COX (Fig. 3b), localizing the defect to respiration through this complex [56]. One consequence of these basal defects is that *sphk2*$^{-/-}$ mitochondria exhibit an increased capacity for maximal production of ROS with glutamate/malate as complex I substrates averaging 62 ± 8 pmol/30 min/mg protein versus 37 ± 7 pmol/30 min/mg WT group ($p < 0.05$). Sham mitochondria isolated from *sphk2*$^{-/-}$ hearts showed a significant decrease in CRC compared to WT (Fig. 4). In concert with our previous study [56], these results indicate that deletion of SphK2 altered mitochondrial function in the baseline state, underlined by a sensitization of the PTP opening and a lower respiratory capacity.

Next, the impact of these mitochondrial alterations was assessed during I/R. In WT animals, I/R resulted in a significant reduction in CRC that averaged 588 ± 36 nmol $CaCl_2$/mg protein ($p < 0.05$ vs. sham-WT, Fig. 4), concomitant with a decrease in respiration with electron donors to complex I as well as to COX (Fig. 3a, b). As expected, PC significantly

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improved CRC when compared to I/R alone (Fig. 4) and preserved oxidative phosphorylation measured with complex I and IV substrates in the WT group (Fig. 3a, b). In contrast to WT mice, although I/R exhibited a similar alteration in CRC that averaged 550 ± 54 nmol $CaCl_2$/mg protein (p = ns vs. I/R-WT, Fig. 4), PC failed to improve CRC in *sphk2*$^{-/-}$ mitochondria (Fig. 4). Moreover, PC also failed to protect mitochondrial respiration in *sphk2*$^{-/-}$ mitochondria (Fig. 3a, b).

Inhibition of the mS1P–PHB2–COX complex prevents preconditioning protection

We previously demonstrated that deficiency in mitochondrial S1P in *sphk2*$^{-/-}$ mice led to a PHB2-dependent aberrant assembly of the COX complex [56]. In order to examine whether PHB2 and possibly COX is involved in the ischemia-tolerant phenotype, we investigated the extent of cell death observed in cardiomyoblasts submitted to hypoxia–reoxygenation following depletion of these proteins. Specific siRNAs targeted to SphK2, PHB2 or the nuclear-encoded COX subunit IV markedly decreased the respective protein expression in cells (Fig. 5a). In line with the previous study performed on HeLa cells [56], depletion of PHB2 induced a decrease of SphK2 expression on H9c2 cells (Fig. 5a).

In the siControl group, 180 min hypoxia followed by 60 min reoxygenation induced cell death averaging 49 ± 3% (Fig. 5b, c). Preconditioning significantly reduced cell death by approximately half, averaging 26 ± 2% ($p < 0.05$ vs. H/R group, Fig. 5b, c). Cell death after hypoxia–reoxygenation was similar to the siControl group for each knockdown. However, in contrast to the siControl group, preconditioning failed to reduce cell death when SphK2, PHB2, or COX IV was down regulated (Fig. 5c). Taken together, these data suggest that mS1P–PHB2–COX complex [56] contributes to the protection elicited by preconditioning.

Down-regulation of SphK2, PHB2 or COX IV sensitizes PTP opening

Since PTP resistance represents a key role in preconditioning protection [27], in order to further delineate the mechanism involved in the preconditioning-resistant phenotype, the effect of the knockdown of SphK2, PHB2, and COX IV on PTP resistance was studied. H_2O_2 stress of calcein-loaded siControl cardiomyoblasts resulted in a reduction in mitochondrial calcein fluorescence up to 82 ± 6% of the initial value (Fig. 6a). This effect was abolished by cyclosporine A treatment (1 μM), a potent PTP inhibitor, confirming that the reduction in mitochondrial calcein fluorescence was due to PTP opening (siControl + CsA, Fig. 6a). Interestingly, PTP opening was sensitized when SphK2, PHB2 or COX IV were down regulated, characterized by a significant reduction of mitochondrial calcein fluorescence compared to siControl (Fig. 6b). Thus, the deletion of SphK2, PHB2 or COX subunit IV lowers the resistance to PTP opening.

Discussion

The potent lipid mediator S1P is recognized as a critical cardioprotective molecule [38]. S1P produced inside the cell is released and binds to specific G protein-coupled cell surface receptors that activate a signaling cascade that leads to cardioprotection [59, 63, 68]. The majority of research to date has focused on the activation of these receptors, but there is now evidence that S1P exerts intracellular functions independently of cell surface receptors, notably in the mitochondria [56]. Utilizing genetic in vivo and in vitro models, the present study demonstrates that due to a preexisting defect in mitochondrial respiration, SphK2 deficient mice cannot be protected by preconditioning (PC). This work suggests that SphK2 is downstream of the SphK1-cytoprotective pathway and, that presence of SphK2 is required to maintain a functional COX and normal mitochondrial respiration to modulate PTP opening during preconditioning.

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Indeed, even if cytosolic signaling kinase pathways are activated in *sphk2*$^{-/-}$ mice, shown by the phosphorylation of ERK1/2, Akt, STAT3 and GSK3β, preconditioning fails to afford protection in vivo. The recent finding of Vessey et al. [62] in the isolated perfused heart and several previous reports suggest a crucial role for SphK2 in ischemic protection. Wacker et al. [65] reported that the expression and activity level of the SphK2 isoform in the cerebral micro-vasculature is increased after preconditioning, whereas SphK1 is unchanged. These authors further demonstrated that dimethylsphingosine treatment, an inhibitor of SphK2, completely abolished the reduction of infarct volume provided by the cerebral hypoxic preconditioning stimulus [65]. In the current model, activity of SphK2 and consequently the mitochondrial S1P content were decreased in transgenic mice despite a normal activity of SphK1 and a normal cytosolic S1P content. Taken together, these results suggest first, that mitochondrial S1P constitutes a distinct pool of S1P which acts independently of the cytosolic S1P pool generated via SphK1; and second, that mitochondrial S1P produced by mitochondrial SphK2 was responsible for inducing an ischemia-tolerant phenotype. In this way, it is known that preconditioning is not effective in the aged heart and a recent study demonstrated that the activity of SphK2 is reduced with aging [60], supporting the relevance of SphK2 activity in cardioprotection.

The current study subsequently focused on the mitochondrial-based mechanism since the *sphk2*$^{-/-}$ mitochondrial phenotype is refractory to protection via the activation of upstream mediators including GSK3β. Preconditioning failed to attenuate the ischemia-induced defects in mitochondrial respiration in *sphk2*$^{-/-}$ mice. However, since the mitochondrial respiratory function is already altered in the baseline state, we hypothesized that the preexisting mitochondrial defect was responsible for the inability to protect the heart by preconditioning. Indeed, the sole known role of mitochondrial S1P involves its interaction with the mitochondrial chaperone PHB2 that is required for the proper assembly of complex IV, resulting in a decrease in mitochondrial respiration [56]. In the present study, in vitro results clearly demonstrated that reduction of mitochondrial respiration at baseline, even by indirect (SphK2 or PHB2) or direct (COX subunit IV) knockdown, prevented the protective effect of preconditioning, consistent with the phenomenon of aging where cardioprotection from preconditioning is ineffective [1] and perhaps in line with the presence of a cytochrome oxidase defect in the aged heart [17, 49]. Future work is required to understand the relative contributions of defects in cytoprotective kinase cascades [58] in relation to the relative refractoriness of the mitochondria to undergo protective modulation following the activation of cytoprotective networks [46].

Since *sphk2*$^{-/-}$ mitochondria presented an increase in PTP sensitivity at baseline, we propose that SphK2 could modulate the PTP through an interaction between mitochondrial S1P–PHB2–COX complex and components of the PTP. Generally, myocardial cardioprotection studies correlated the reduction of infarct size with the resistance of PTP opening [2, 4, 16, 18, 26, 43]. Here we investigated whether basal altered PTP sensitivity may cause the observed failure of preconditioning. We tested the effect of the acute knockdown of each constituent in the interaction (SphK2, PHB2 and COX IV) on PTP sensitivity and hypoxia–reoxygenation stress. Interestingly, knockdown of each potential component led to a failure of preconditioning and to a clear decrease in the resistance to PTP opening in response to cell stress. In contrast, the extent of cell death in the absence of preconditioning was unaltered. Hence, our results support the notion that mitochondrial S1P (produced by SphK2) is required for preconditioning by regulating the mitochondrial respiratory chain and that the respiratory chain function can modulate the PTP. The enhanced calcium sensitivity to PTP seems to be the mitochondrial mechanism responsible for the mitochondrial phenotype that is resistant to cardioprotection, perhaps related to PHB2 interactions or alternatively via the COX defect. It is interesting to note that blockade of the electron transport chain at proximal sites protects mitochondrial function [10] and

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attenuates the susceptibility to PTP [11], whereas blockade at COX does not protect [14]. Thus, *sphk2*$^{-/-}$ mitochondria with a defect in COX (and an increased net ROS production from the electron transport chain) may offer a relative resistance to cardioprotection due to the distal site of the defect in the chain. Defects in electron transport, especially distal in the chain at COX [12, 41], favor increased ROS production.

It has been proposed that SphK1 mediates prosurvival effects whereas SphK2 is proapoptotic [33, 44, 45, 52]. In the current study, SphK2 deletion did not induce protection following I/R, inconsistent with a proapoptotic role of SphK2 in the heart during the stress of ischemia and reperfusion. The observed difference could be explained by the fact that the proapoptotic effect of SphK2 was only demonstrated following overexpression in different models [44, 45, 52].

Although SphK2 deficient mice present mitochondrial defects at baseline, we were surprised to find that there was no difference in infarct size between *sphk2*$^{-/-}$ and WT mice. However, this finding is consistent with the fact that our SphK2 knockout mice exhibit no evident cardiac phenotype, with no differences in baseline cardiac function including left ventricle mass index, development of fibrosis or impairment of diastolic function (supplemental data Figure S and Table 1). In the recent study in a non blood perfused system, Vessey et al. [62] observed an increased infarct size after ischemia–reperfusion in their SphK2 transgenic mice accompanied by an increase in phosphorylated p38. The discrepancy with our study could be explained by the different transgenic model and also by the fact that our in vivo model is a more complex integrative setting. Thus, it appears that the modest increase in susceptibility to ischemic injury, evident in the isolated perfused organ, is not so severe as to emerge in the setting of the more complex, in vivo model of cardiac injury. Furthermore, our observations are confirmed by the in vitro analysis in H9c2 cells, where knockdown of SphK2 did not affect cell death following hypoxia-reoxygenation. Thus, baseline mitochondrial defects appear to contribute to the effectiveness of cytoprotective intervention and less so to the extent of ischemic injury in the unprotected state (since infarct sizes were comparable between ischemic WT and *sphk2*$^{-/-}$ mice). This concept has been previously observed in other transgenic models including the STAT3-KO mice and the connexin 43-deficient mice, where cardioprotection is lost but without increased infarct size after ischemia–reperfusion compared to the WT [8, 55]. Indeed, although these mice displayed mitochondrial defects at baseline with decreased respiration and resistance of PTP, they did not show greater ischemic insults [8, 55].

In summary, the current study in concert with previous work [62] points to a new role for mitochondrial S1P generated by mitochondrial SphK2 in cardioprotection. Based on these studies and the previous study of mitochondrial function [56], we present a working hypothesis regarding the involvement of the different subcellular S1P pools in cardioprotection (Fig. 7): despite a normal SphK1 activity, the absence of SphK2 induces a reduction of mitochondrial S1P content which in turn alters mitochondrial respiration and enhances PTP susceptibility. This mitochondrial phenotype is refractory to the protection of PC. Thus, the COX defect and the S1P–PHB2–COX interaction seem to contribute to PTP sensitivity; its regulation suggests that in addition to regulation by GSK3β [37] and HKII [5], PTP is responsive to peptide modulators including STAT-3 [8], connexin 43 [54] and likely PHB2. To conclude, SphK2 is required downstream of the SphK1-activated signaling pathways for PTP-dependent preconditioning protection. In other words, preconditioning first activates SphK1 which recruits the cytosolic S1P pool to trigger the cytoprotective signaling pathways by the inside-out effect; second, the mitochondrial pool of S1P is activated to regulate oxidative phosphorylation (through mS1P–PHB2–COX complex) and thus the ROS production, leading to an inhibition of PTP opening. Deficiencies in SphK2 or

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PHB2 may contribute to mitochondrial phenotypes relatively refractory to preconditioning in the presence of co-morbid cardiac conditions including aging and diabetes.

Supplementary Material

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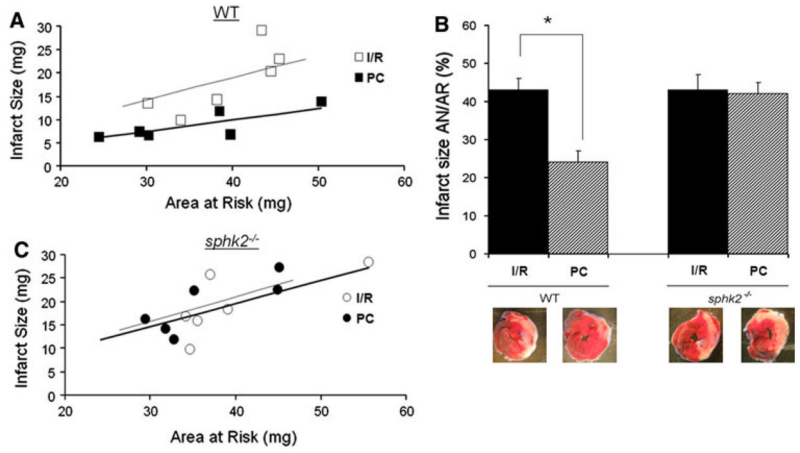

Fig. 1.

sphk2$^{-/-}$ mice are not protected by preconditioning. Scatterplots of relation between infarct size (IS) and area at risk (AAR) in ischemic (I/R, $n = 6$) versus preconditioned (PC, $n = 6$) groups of WT (**a**) or *sphk2*$^{-/-}$ mice (**b**). There is a close linear correlation between IS and AAR in all four groups. In WT but not in *sphk2*$^{-/-}$mice, the slope of the regression line is significantly lower in the PC compared to I/R group. **c** Infarct size expressed as a % of the area at risk in WT or *sphk2*$^{-/-}$ mice. *$p < 0.05$

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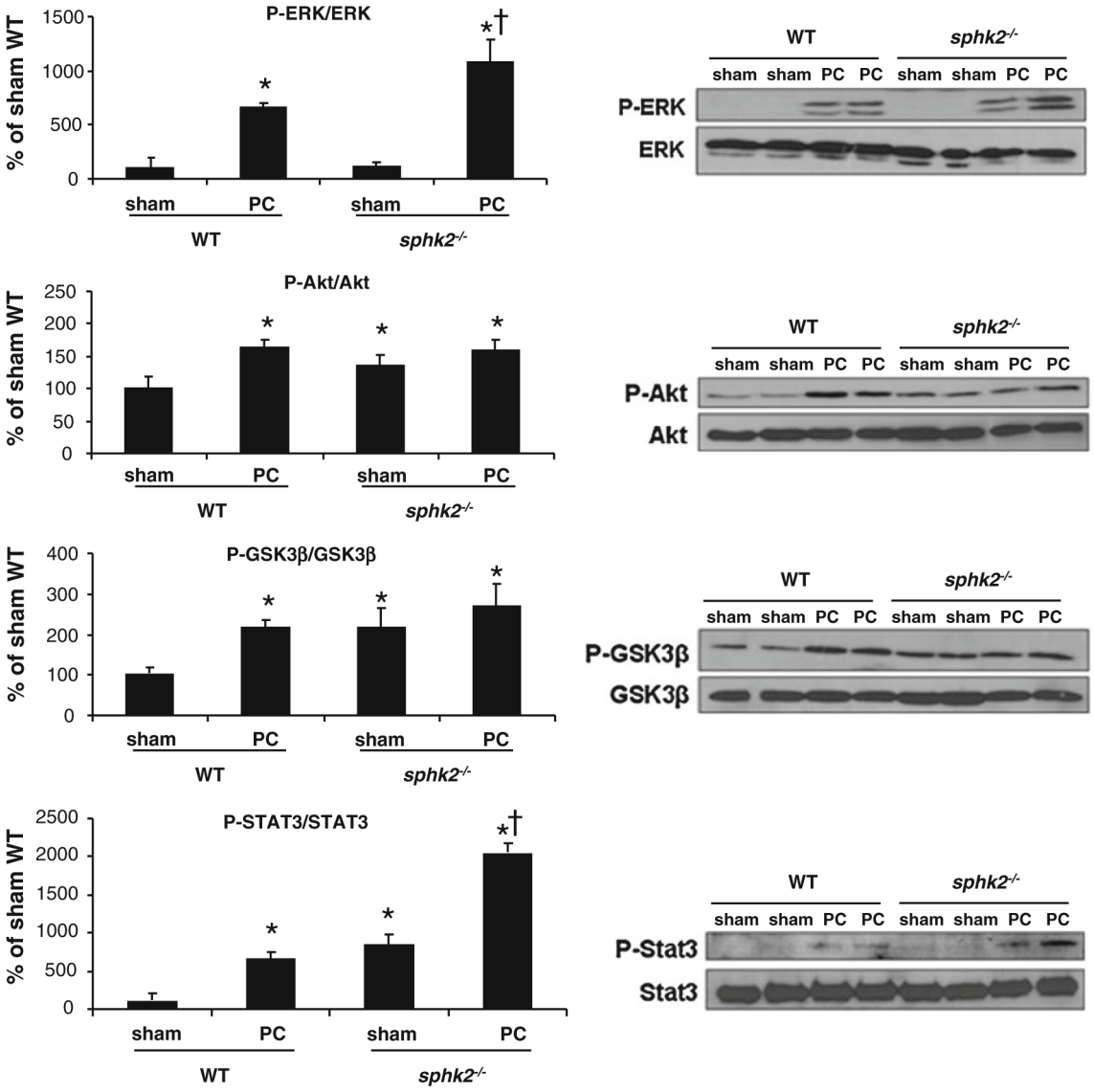

Fig. 2.

Cardioprotective kinase signaling pathways are preserved in *sphk2*$^{-/-}$ mice. *Right panel* Typical immunoblots are shown. *Left panel* Analysis of ERK1/2, Akt, GSK3β and STAT3 phosphorylation in sham and preconditioned (PC) WT and *sphk2*$^{-/-}$ mice. Results are expressed as percentage of sham-WT. *$p < 0.05$ versus sham-WT. $^{<}p < 0.05$ versus sham-*sphk2*$^{-/-}$ ($n = 4$/group)

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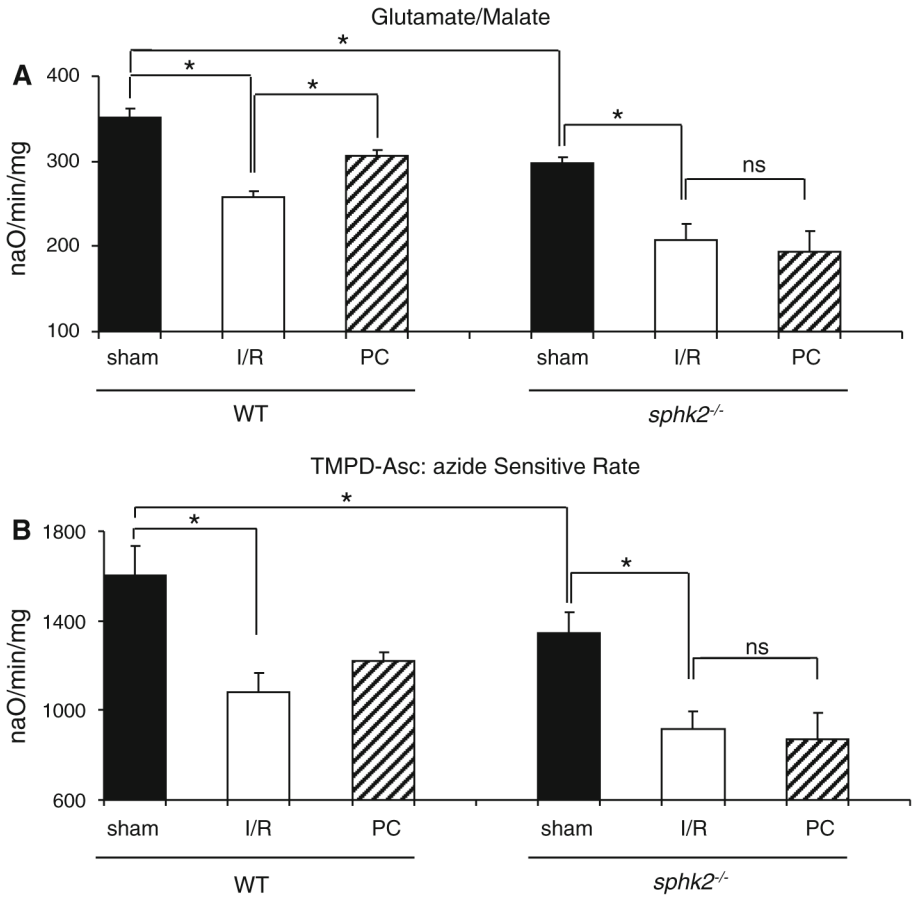

Fig. 3.

Preconditioning failed to rescue OXPHOS in *sphk2*$^{-/-}$isolated mitochondria. Oxidative phosphorylation was measured in isolated mitochondria (after 45 min index ischemia followed by 10 min reperfusion) with glutamate/malate as complex I substrate (**a**) and TMPD-ascorbate as substrate of complex IV via cytochrome *c* (**b**). Maximal state 3 rates were expressed in naO/min/mg (n = 4–5/group). Preconditioning failed to rescue oxidative phosphorylation dependent upon complex I substrates in mitochondria isolated from *sphk2*$^{-/-}$ hearts

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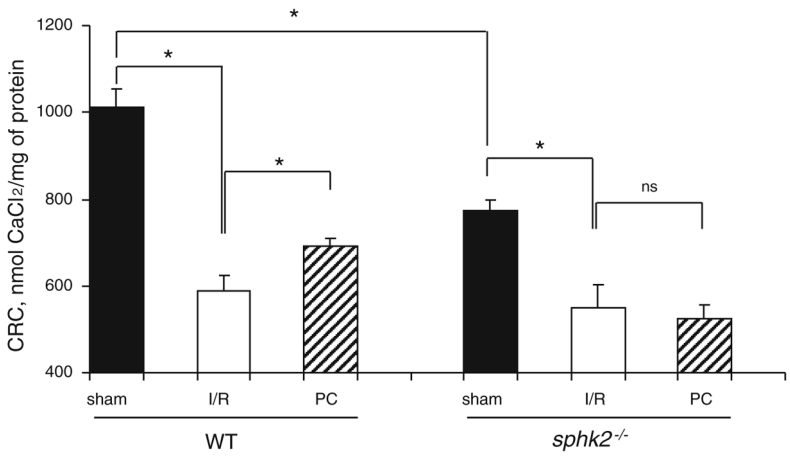

Fig. 4.

Isolated cardiac mitochondria from *sphk2*$^{-/-}$mice have increased susceptibility to PTP despite ischemia preconditioning. Calcium retention capacity (CRC) in baseline (sham), ischemic (I/R) and preconditioned (PC) WT and transgenic mitochondria (n = 4–5/group). The reduction of CRC induced by I/R was reversed by preconditioning in WT but not in *sphk2*$^{-/-}$ mice

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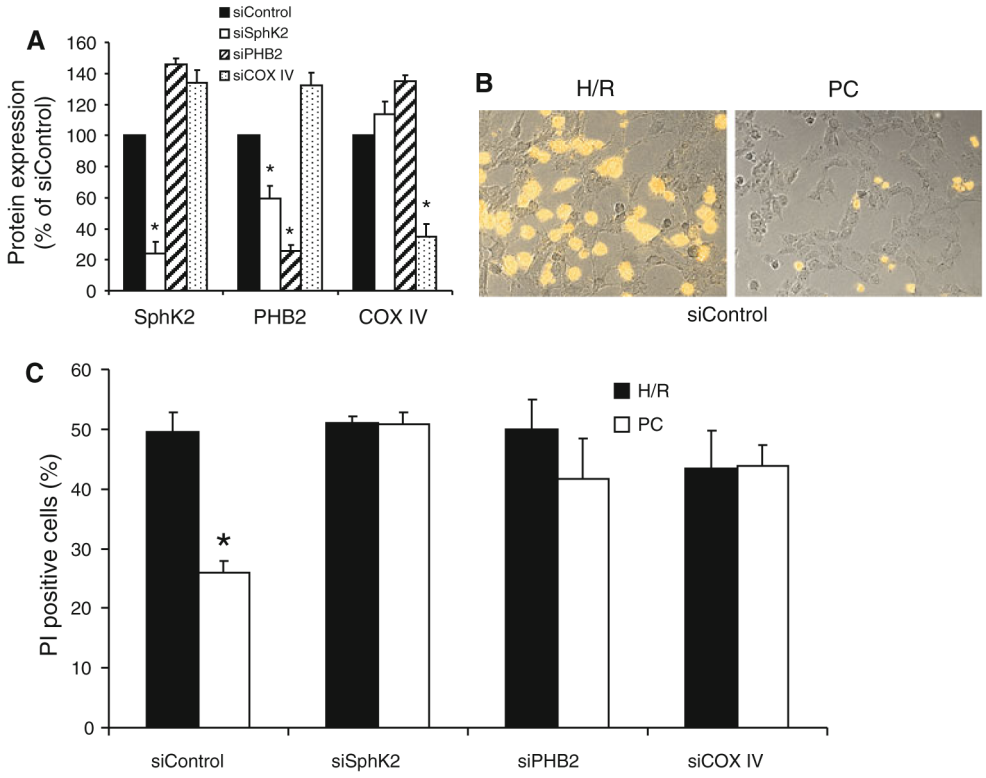

Fig. 5.

Down-regulation of SphK2, PHB2 or COX IV inhibits preconditioning protection. **a–c** H9c2 cells were transfected with siControl, siSphK2, siPHB2 or siCOX IV, as indicated. **a** Protein expression was determined and quantified by immunoblotting of cell lysates. **b** Example of a typical cell image obtained after hypoxia–reoxygenation (H/R) or preconditioning (PC) in the siControl group. **c** Preconditioning significantly reduced cell death in the siControl group ($p < 0.05$). In contrast, preconditioning failed to reduce cell death in transfected cells treated with siRNA targeted to SphK2, PHB2 and COX IV

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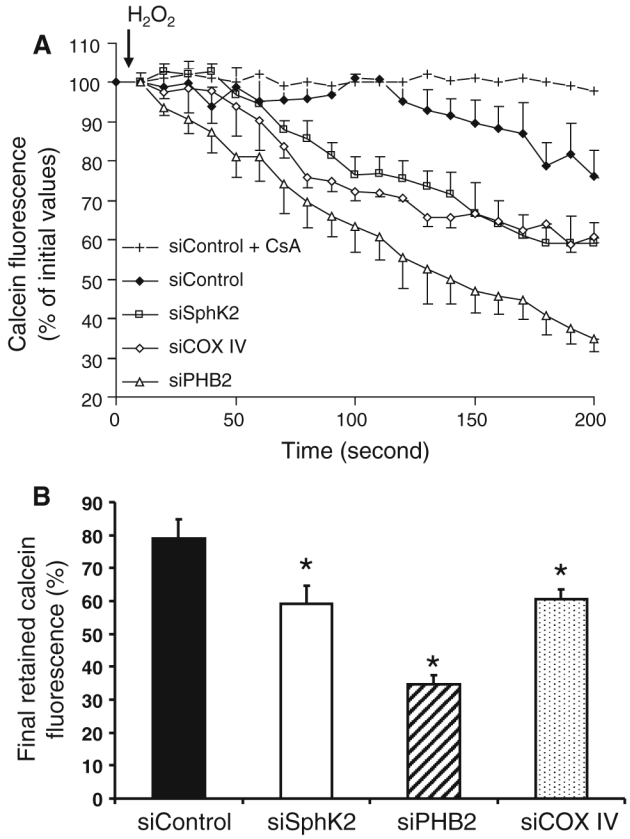

Fig. 6.

Depletion of SphK2, PHB2 or COX IV altered PTP opening in cells. Measurement of calcein fluorescence in H9c2 cells. **a** Residual mitochondrial calcein fluorescence (expressed as percentage of baseline fluorescence) after activation of PTP opening induced by 100 μM H_2O_2. **b** Threshold of PTP opening was reduced when the expression of SphK2, PHB2 or COX IV was downregulated. *$p < 0.05$ versus siControl

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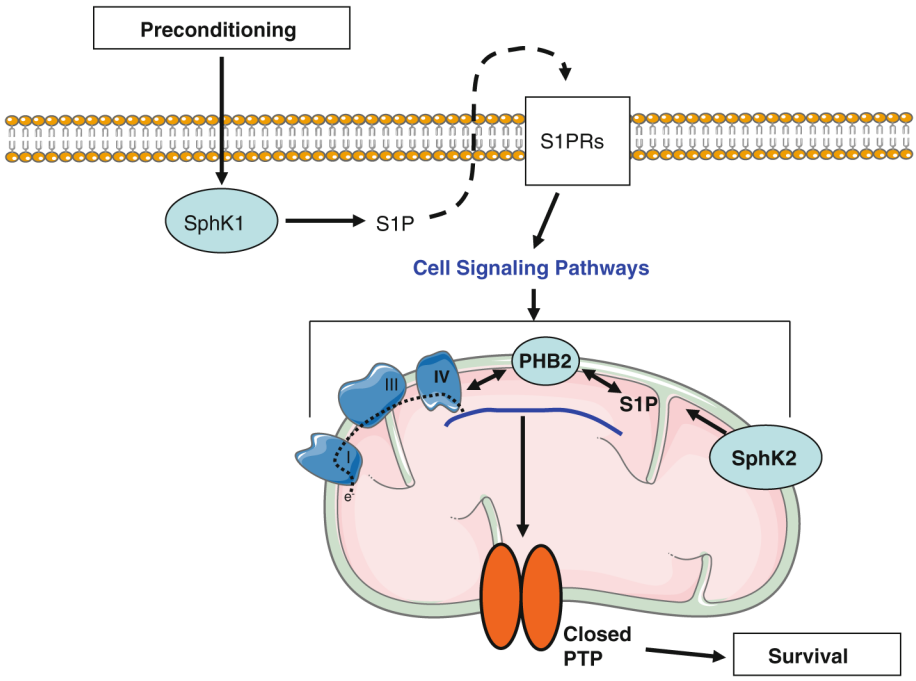

Fig. 7.

Proposed involvement of different subcellular S1P pools in cardioprotection. Preconditioning activates SphK1 which increases the cytosolic pool of S1P leading to cellular release and the activation of S1P receptors on the cell surface. Receptor activation activates cytoprotective kinase cascades that converge on mitochondria as the effector of protection. The mitochondrial pool of S1P regulates oxidative phosphorylation (via the S1P–PHB2–COX complex) and ROS production, leading to an inhibition of PTP opening and cell survival. A deficiency in SphK2 contributes to a reduction of mitochondrial S1P content leading to a mismatch of the respiratory chain responsible for a defect in mitochondrial respiration and dysfunctional PTP regulation