Sphingosine 1-phosphate is an important endogenous cardioprotectant released by ischemic pre- and postconditioning

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Vessey DA, Li L, Honbo N, Karliner JS. Sphingosine 1-phosphate is an important endogenous cardioprotectant released by ischemic pre- and postconditioning. Am J Physiol Heart Circ Physiol 297: H1429–H1435, 2009. First published July 31, 2009; doi:10.1152/ajpheart.00358.2009.—Exogenous sphingosine 1-phosphate (S1P) is an effective cardioprotectant against ischemic injury. We have investigated the hypothesis that S1P is also an important endogenous cardioprotectant released during both ischemic preconditioning (IPC) and ischemic postconditioning (IPOST). IPC of ex vivo rat hearts was instituted by two cycles of 3 min ischemia-5 min reperfusion prior to 40 min of index ischemia and then 40 min of reperfusion. IPC resulted in 70% recovery of left ventricular developed pressure (LVDP) upon reperfusion and a small infarct size (10%). VPC23019 (VPC), a specific antagonist of S1P1 and 3 G protein-coupled receptors (GPCRs), when present during preconditioning blocked protection afforded by two cycles of IPC. VPC also blocked preconditioning of isolated rat cardiac myocytes subjected to hypoxia-reoxygenation injury. Increased release of S1P from myocytes in response to IPC was also demonstrated. These data indicate that S1P is released from myocytes in response to IPC and protects by binding to S1P GPCRs. In the ex vivo heart, if a third cycle of IPC was added to increase release of endogenous mediators, then the need for any individual mediator (e.g., S1P) was diminished and VPC had little effect. The adenosine antagonist 8-(p-sulfophenyl)-theophylline (8-SPT) likewise inhibited protection by two cycles but not three cycles of IPC, but VPC plus 8-SPT inhibited protection by three cycles of IPC. Similar to IPC, IPOST induced by four postindex ischemia cycles of 15 s reperfusion-15 s ischemia resulted in 66% recovery of LVDP and a 7% infarct size. When VPC was present during postconditioning and reperfusion, LVDP only recovered by 26% and the infarct size increased to 27%. Adding an additional cycle of IPOST reduced the inhibitory effect of VPC and 8-SPT individually, but not their combined effect. These studies reveal that S1P is an important mediator of both IPC and IPOST that is released along with adenosine during each cycle of IPC or IPOST.

preconditioning; ischemia-reperfusion injury; cardioprotection

MYOCARDIAL ISCHEMIA RESULTS when coronary blood flow to the heart is reduced leading to deficient oxygen and nutrient supply to the heart (2). Restoration of coronary blood flow after extended ischemia, though necessary, actually initiates myocyte damage (7, 16). This is referred to as ischemia-reperfusion injury. However, the heart can be preconditioned by one or more brief nonlethal periods of ischemia instituted prior to the index ischemia with the result that the subsequent damage upon reperfusion is greatly diminished (4, 20). Thus, when a heart exposed to such ischemic preconditioning (IPC) then undergoes an extended period of ischemia and subsequent reperfusion, the loss of myocardial hemodynamic function and subsequent infarct size is substantially reduced (4, 20). Hearts can also be “conditioned” after the index ischemia just prior to full reperfusion (postconditioning). Ischemic postconditioning (IPOST) is accomplished by instituting brief cycles of ischemia-reperfusion after the index ischemia and prior to full reperfusion (30).

The mechanisms involved in the cardioprotective ability of IPC and IPOST to limit ischemia-reperfusion-induced myocardial injury have been the subject of considerable study. Although the mechanisms of IPC have not been clarified in detail, it is apparent that the brief nonlethal ischemic episodes result in the release of endogenous cardioprotectants such as adenosine, opioids, and bradykinin, which then act in parallel to trigger the protective response by binding to specific G protein-coupled receptors (6, 19). IPOST appears to employ a similar mechanism (8, 19, 30) and presumably also activates endogenous mediators that bind to G protein-coupled receptors thereby triggering protection (8, 18, 19).

The question we wished to answer in this study is whether there are additional equally potent endogenous mediators of cardioprotection released by IPC and IPOST. The lipid mediator sphingosine 1-phosphate (S1P) is a potent modulator of cell signaling, and these effects are in part the result of S1P binding to specific cell surface G protein-coupled receptors (14, 21). S1P has been found to be effective as an exogenous protectant against ischemic injury in both cardiomyocytes and ex vivo rat and mouse heart (9–11, 13, 15, 22). Preischemia treatment with S1P (pharmacological preconditioning) has been shown to diminish the damage associated with ischemia-reperfusion injury in both isolated perfused hearts andcultured cardiac myocytes (9, 13, 15, 27, 28, 30). This protection is the result of S1P binding to specific G protein-coupled receptors triggering a signaling cascade that leads to cardioprotection (23, 27, 30). Although prior studies employing gene-targeted mice have implicated S1P2 and S1P3 receptors in ischemic injury (17, 23), there are no studies of S1P receptors in either ischemic pre- or postconditioning. In view of the cardioprotective effect of S1P and the fact that myocardium contains significant levels of S1P (25), we hypothesized that S1P is one of the primary endogenous cardioprotectants released during both pre- and postconditioning.

MATERIALS AND METHODS

Materials. d-Erythro-S1P was obtained from Biomol Research Laboratories. VPC23019 (VPC) was obtained from Avanti Polar Lipids. Adenosine, 8-(p-sulfophenyl)theophylline (8-SPT), and triphenyltetrazolium chloride were obtained from Sigma. d-Erythro[3-3H]sphingosine was obtained from American Radiolabeled Chemicals.
Langendorff ex vivo perfused heart. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, Washington, DC, 1996). Hearts from 230- to 250-g rats were removed under pentobarbital anesthesia and mounted on a Langendorff apparatus as described previously (27). Hearts were perfused at a pressure of 90 mmHg with oxygenated (95% O₂-5% CO₂) Krebs-Henseleit solution at 37°C. Although this high oxygen tension predisposes the model to higher levels of activated oxygen species, it provides the most stable hemodynamic function. Left ventricular developed pressure (LVDP) was measured with a Millar micromanometer-tipped catheter. To measure infarct size, hearts were sectioned and stained with triphenyltetrazolium chloride and the infarct area was determined by computer analysis as described (25).

The protocol for nonconditioned hearts consisted of continuous perfusion for 30 min after the heart was mounted on the Langendorff apparatus. Sustained ischemia (index ischemia) was then induced by halting perfusion for 40 min. During the index ischemia the heart was lowered into a thermostated chamber that maintains an ambient temperature of 37°C. This was followed by the reperfusion stage in which flow was again initiated for 40 min. IPC consisted of either two or three cycles of 3 min ischemia-5 min reperfusion just prior to the index ischemia. Ischemic postconditioning consisted of four or five cycles of 15 s reperfusion-15 s ischemia immediately following the 40-min index ischemia at the beginning of reperfusion. To administer S1P, a 2.64 mM stock solution was prepared in DMSO and 91 μl (for 0.4 μM final S1P) was added per 600 ml of perfusion buffer. The S1P receptor, G protein-coupled receptor antagonist VPC was dissolved directly in the perfusion buffer at a concentration of 1 μM. The adenosine receptor antagonist 8-SPT was dissolved directly in perfusion buffer at a concentration of 0.2 μM. The hemodynamic parameters measured were heart rate and left ventricular developed pressure (from left ventricle systolic pressure and left ventricle end-diastolic pressure).

Cultured rat cardiomyocytes. Primary cardiac myocyte cultures were prepared from ventricular tissue of 1-day-old Sprague-Dawley rats as previously described (13). The euthanasia protocol was approved by the Animal Studies Committee of the San Francisco Veterans Affairs Medical Center. Myocytes were obtained by serial trypsinizations, preplated for 30 min to reduce nonmyocardial populations, and seeded on 35-mm culture dishes at a density of 400 cells/mm² in minimum essential medium with Hanks’ balanced salt solution (GIBCO) containing 10% bovine calf serum and 1.5 mM vitamin B₁₂, 50 U/ml penicillin, and 0.1 mM bromodeoxyuridine to prevent low-level nonmyocardial cell proliferation. Cells were incubated overnight at 37°C under normoxic conditions (99% air-1% CO₂). Hypoxia-reoxygenation. All cells were changed to serum-free medium without glucose. For the hypoxia-reoxygenation experiments cells were exposed to 99% N₂-1% CO₂ for 5 h with preequilibrated media without glucose in a hypoxia chamber (Bactron I Anaerobic Chamber, Sheldon Manufacturing, Cornelius, OR). Cells were then reoxygenated with media containing glucose and maintained in normoxia for 20–22 h. Control cells were treated similarly except they remained under normoxia for the entire experiment.

For preconditioning, cells in normoxia were initially changed to media without glucose and then transferred to the hypoxia chamber where cells were changed to media without glucose equilibrated in hypoxia and then maintained in hypoxia for 15 min. Cells were removed and incubated with normoxic equilibrated media with glucose added in a normoxic atmosphere for 30 min. This procedure was repeated for two cycles of preconditioning. After the second cycle of preconditioning, cells were exposed to the hypoxia-reoxygenation protocol as described above. VPC (0.4 μM in 0.002% DMSO final) was added to some cells 15 min before the initiation of preconditioning and maintained throughout the preconditioning procedure. For inhibitor studies some cells received the inhibitor VPC 15 min prior to a 1-h incubation in either vehicle or S1P (0.4 μM in 0.2% ethanol final).

Measurement of cell viability. Survival was determined by counting the percentage of live cells in each culture dish relative to the live cells in the normoxia control by using a two-color fluorescent assay (Molecular Probes, Eugene, OR) as previously described (13). Cells that failed to develop green fluorescence were scored as viable, and cells with red fluorescence were scored as nonviable. The percentage of live cells was determined by computer-assisted analysis as described (25).

Measurement of S1P release from myocytes. Myocyte cultures prepared as discussed above, were switched to media containing 0-erythro[3-3H]-sphingosine (20 Ci/mmol) and incubated for 5 h under normoxic conditions to label the cellular pool of S1P. The cells were then washed two times with cold glucose-free media and changed to either normoxic (plus glucose) or hypoxic conditioned (minus glucose) medium and incubated under normoxic or hypoxic conditions, respectively, for 15 min. Both sets of cells were then incubated in glucose containing normoxic conditions for 30 min. The medium was then removed and saved, and cells were changed to media for another 15 min of either normoxia or hypoxia. At this point, this medium was collected from both sets of cells and combined with the previous collection. An aliquot of the combined media (0.5 ml) from each sample was spotted on silica gel G TLC plates and developed in methanol-chloroform-acetone-acetic acid-water (10:4:3:2:1). This has been shown previously to effectively separate S1P from sphingosine (24). The portion of the TLC plate corresponding to S1P was cut out and analyzed for radioactivity by liquid scintillation counting. The total cellular protein content on each culture dish was determined by the Bio-Rad Bradford assay.

Statistical analysis. Data are expressed as means ± SE and were compared by two-way ANOVA. P < 0.05 was considered significant.

RESULTS

The first system used for the study of ischemia-reperfusion injury was the Langendorff ex vivo rat heart model. The ex vivo hearts were equilibrated for 30 min and then exposed to 40 min of global ischemia followed by 40 min of reperfusion. The recovery of hemodynamic function was followed by continuous monitoring of the pressure developed by contraction of the left ventricle (LVDP) during reperfusion and measurement of the infarct size after 40 min of reperfusion. Figure 1 shows the results of a study of the recovery of LVDP upon reperfusion as a function of the length of the index ischemia. It was found that 20 min of ischemia was well tolerated but, beyond 25 min of ischemia, recovery of LVDP was increasingly compromised. By 40 min of ischemia, there was only 8.6 ± 1.6% recovery of LVDP. Furthermore, hearts exposed to 40 min of ischemia and then 40 min of reperfusion showed infarcts covering 42 ± 1% risk area. By contrast, hearts exposed to two cycles of IPC, consisting of 3 min ischemia-5 min reperfusion just prior to the 40 min of index ischemia (Fig. 2), recovered hemodynamic function (70 ± 1% recovery of LVDP) and had small infarct sizes (10 ± 1%).

To examine the role of endogenous S1P in ischemic preconditioning of the ex vivo heart, we made use of VPC, an antagonist of cell surface S1P1 and 3 G protein-coupled receptors (3). We have previously shown that VPC blocks cardioprotection by exogenously added S1P (27). VPC by itself has no effect on the extent of ischemia-reperfusion injury (Fig. 2). To study the role of endogenous S1P in IPC, VPC was added at a concentration of 1 μM to the perfusion buffer during the...
cycles of ischemic preconditioning. The presence of VPC greatly reduced the effectiveness of two cycles of IPC (Fig. 2). The recovery of LVDP was reduced to 27 ± 6% and the infarct size was increased to 26 ± 4%. This indicates that S1P release is an important contributor to the overall cardioprotective effect of two cycles of IPC.

However, this ability of VPC to reduce cardioprotection by IPC could be overridden by adding an additional cycle of preconditioning (Fig. 2), which is expected to promote release of additional cardioprotectants. Thus, after three cycles of IPC, even in the presence of 1 μM VPC the recovery of LVDP was 72 ± 6% and the infarct size was small (5.8 ± 1.1%). This reveals that in the presence of increased levels of release of endogenous mediators the requirement for any one individual mediator, such as S1P, is reduced. These data also suggest that there may be a hierarchy of mediator release, with S1P among the foremost.

To rule out nonspecific effects of VPC on signaling pathways other than antagonism of S1P1 and 3 G protein-coupled receptors, we looked at the effect of VPC on pharmacological preconditioning by adenosine. Adenosine, at a concentration of 0.2 μM prior to index ischemia, supported 70 ± 8% recovery of LVDP with a 9 ± 3% infarct size, and this was unaffected by cotreatment with 1 μM VPC (68 ± 5% recovery of LVDP and 9 ± 2% infarct size). Also, when VPC was not present during IPC but was added to preconditioned hearts at the time of reperfusion, there was no effect on LVDP recovery (74 ± 8%) or on infarct size (9 ± 2%). This demonstrates that VPC action is related to the preconditioning step and that the further release of S1P during the reperfusion stage is not required.

To demonstrate that the basis for three IPC cycles attenuating the impact of VPC on cardioprotection is due to enhanced release of another endogenous cardioprotectant, we conducted the same set of experiments with the broad spectrum adenosine antagonist 8-SPT. As shown in Fig. 2, 8-SPT at a concentration...

Fig. 1. Effect of ischemia duration on recovery of hemodynamic function. Ex vivo hearts were equilibrated for 30 min and then exposed to periods of ischemia of different duration. This was followed by 40 min of reperfusion during which the recovery of left ventricular developed pressure (LVDP) was measured. Recovery is reported as the maximum LVDP obtained post-ischemia as a percent of the preischemic value. Data are presented as means ± SE (n ≥ 4).

Fig. 2. Effect of the D-erythro-sphingosine-1-phosphate (S1P) receptor antagonist VPC23019 (VPC) and the adenosine receptor antagonist 8-(p-sulfophenyl)-theophylline (8-SPT) on cardioprotection by 2 vs. 3 cycles of ischemic preconditioning. Isolated perfused hearts were preconditioned in the presence or absence of VPC or 8-SPT with either 2 cycles (2×P) or 3 cycles (3×P) of 3 min of ischemia-5 min of reperfusion prior to 40 min of index ischemia and 40 min of reperfusion. The maximum recovery of LVDP during reperfusion is reported as a percent of the preischemic value and the infarct size was determined at the end of the 40 min of reperfusion. The control (Con) was exposed to equilibration (±VPC and/or 8-SPT in the absence of preconditioning), index ischemia, and reperfusion. Data are reported as means ± SE. *Each of these conditions (which are not significantly different from one another) is significantly different (P < 0.05) from all other conditions without an asterisk. **Controls (which are not significantly different from one another) are significantly different from all other conditions. The sample size is n = 4–7 for LVDP and 4–5 for infarct size.
of 0.2 μM had no effect by itself but inhibited the cardioprotection associated with two cycles of IPC (40 ± 2% recovery of LVDP and 26 ± 3% infarct size). Thus VPC and 8-SPT had an identical effect on infarct size and a similar effect on LVDP. As with VPC, this antagonism of IPC by 8-SPT could be eliminated by adding a third cycle of IPC (recovery of LVDP of 78 ± 2 and 9.0 ± 0.4% infarct size). However, protection by three cycles of IPC was greatly diminished in the presence of both VPC plus 8-SPT (28 ± 7% recovery of LVDP and 31 ± 2% infarct size), indicating their additive effect.

IPOST was also studied in the ex vivo heart model. Hearts were first equilibrated for 30 min and then exposed to 40 min of index ischemia. Full reperfusion was preceded by multiple cycles of 15 s reperfusion-15 s ischemia and then 40 min of full reperfusion. LVDP was measured throughout reperfusion and infarct size was determined at the end of 40-min reperfusion. Our standard IPOST protocol consisted of four cycles of 15 s reperfusion-15 s ischemia. This resulted in 66 ± 2% recovery of LVDP and a 7.0 ± 0.4% infarct size (Fig. 3). When 1 μM VPC was added to the buffer during IPOST and reperfusion, the recovery of LVDP was reduced to 26 ± 17% and the infarct size enlarged to 27 ± 3% (Fig. 3). Clearly VPC reduced the effectiveness of IPOST. This indicates that S1P is an important endogenous mediator of IPOST. We increased the number of cycles of IPOST in an attempt to increase the release of endogenous mediators. With five cycles of IPOST the effect of VPC was markedly impaired (63 ± 12% recovery of LVDP and 7.7 ± 0.8% infarct size, Fig. 3) relative to four cycles. This is consistent with the data obtained from the above study of IPC, which suggests that any one mediator is essential only when the conditioning response is suboptimal and therefore the overall level of each mediator is suboptimal.

We also compared the effects of a blockade of S1P receptors with that of adenosine receptors and examined the effect of combined S1P/adenosine blockade. As with S1P, antagonism of adenosine receptor binding with 8-SPT reduced the protection by four cycles of IPOST (only 11 ± 2% recovery of LVDP and 37 ± 5% infarct size). This effect could be reduced by adding an additional cycle of IPOST. However, the protection against S1P or adenosine blockade achieved by an extra cycle of IPOST could be diminished by blockade of both S1P and adenosine receptors (Fig. 3), indicating that the extra cycle had released additional quantities of both of these cardioprotectants.

The heart contains other cell types (fibroblasts, coronary arterial endothelial cells, and smooth muscle cells) that could be responsible for the release of S1P. In an effort to determine whether the source of the endogenous S1P released by preconditioning is at least in part directly from myocytes, we studied the effect of VPC on preconditioning in cultured neonatal rat cardiac myocytes exposed to 5 h of hypoxia-glucose deprivation and 20–22 h of reoxygenation with glucose. Cell injury was quantitated by counting live cells with the calcein-ethidium homodimer-1 fluorescence assay. As shown in Fig. 4.

![Fig. 3. Effect of the S1P receptor antagonist VPC23019 and the adenosine receptor antagonist 8-SPT on cardioprotection by 4 vs. 5 cycles of ischemic postconditioning. Isolated perfused hearts were exposed to 40 min of index ischemia and then postconditioned in the presence or absence of 1 μM VPC with either 4 cycles (4×POST) or 5 cycles (5×POST) of 15 s of reperfusion-15 s of ischemia prior to full reperfusion in the presence or absence of VPC and/or 8-SPT for 40 min. The maximum recovery of LVDP during reperfusion is reported as a percent of the preischemic value and the infarct size was determined at the end of the full 40 min of reperfusion. The controls were exposed to equilibration, index ischemia, and then reperfusion in the presence or absence of VPC and/or 8-SPT without any postconditioning treatment. The data are reported as means ± SE *These conditions (which are not significantly different from one another) are statistically different (P < 0.05) from all the other conditions; **conditions are statistically different (P < 0.05) from all other conditions. †Treatment was statistically different (P < 0.05) from all other 5×POST treatments. The sample size is n = 4–7 for LVDP and n = 4 for infarct size.](http://ajpheart.physiology.org/)}
hypoxia-reoxygenation decreased cell viability by 35% (65 ± 5% of normoxic control, P < 0.05). Preconditioning myocytes with two cycles of 15 min hypoxia followed by 30 min of reoxygenation prior to hypoxia-reoxygenation led to significantly improved cell survival (93 ± 2% of normoxic control, P < 0.05). When 0.4 μM VPC was added to the culture medium 15 min prior to hypoxia-reoxygenation, cell survival was 75 ± 6%, P < 0.05. These results indicate that cardiac myocytes can respond to preconditioning by exporting S1P in an autocrine manner.

Similar results were obtained when we pharmacologically preconditioned myocytes by adding S1P exogenously. When S1P was added to the culture medium at a concentration of 0.4 μM for 1 h prior to hypoxia-reoxygenation, cell survival was 70.5 ± 8.3% recovery of viability (P < 0.05).

The myocyte culture system was also used to test for IPC-induced release of S1P from myocytes. To accomplish this, myocytes were first incubated for 5 h with d-erythro-[3-3H]-sphingosine. This resulted in the labeling of the intracellular pool of S1P (48 ± 1% of the intracellular label was present as S1P). The cells were then divided into two groups. One set was maintained in normoxia for 60 min. The second set went through an IPC protocol that consisted of 15 min of hypoxia, 30 min of normoxia, and finally a second 15 min of hypoxia. The cell culture medium was then collected and analyzed for S1P by TLC/liquid scintillation counting (see MATERIALS AND METHODS). The IPC-treated cells contained significantly (P < 0.05) more S1P in the cell culture medium (9.00 ± 0.72 cpm/mg protein) than the medium from normoxia controls (6.93 ± 0.99 cpm/mg protein).

DISCUSSION

It is generally accepted that the initial event in IPC is the ischemia-induced release of the endogenous mediators adenosine, bradykinin, and opioids, which then bind to G protein-coupled receptors, triggering cell signaling pathways that lead to cardioprotection (6, 8, 18, 19, 30). Using an antagonist of S1P binding to the S1P1 and 3 G protein-coupled receptors (VPC), we examined the hypothesis that S1P is an equally important endogenous cardioprotectant, synthesized in myocytes and released in response to brief nonlethal bouts of IPC. In support of this hypothesis, VPC was found to inhibit the protection afforded by two cycles of IPC. This effect of VPC on preconditioning indicates that, after two cycles of IPC, S1P is one of the major contributors to cardioprotection. Using an adenosine receptor antagonist, 8-SPT (1), we also obtained a
similar inhibition of IPC protection revealing the equivalence of the two cardioprotectants. The capacity of VPC to reduce cardioprotection seen with two cycles of IPC was overridden if a third cycle of preconditioning was added to promote further release of cardioprotectants. A third cycle of IPC also eliminated inhibition of protection by the adenosine antagonist 8-SPT. Thus with each cycle of IPC there is additional release of these and other cardioprotectants, revealing that the loss of any one protectant can be overridden by the others reaching levels that promote maximum protection. Thus extensive IPC obviates the need for the contribution of any one cardioprotectant. That individual mediators can induce IPC is shown by the fact that exogenously added adenosine, bradykinin, opioids, or S1P in sufficient concentration induce equivalent cardioprotection individually. The cumulative effect of multiple endogenous cardioprotectants is also suggested by studies of a bradykinin antagonist and multiple cycles of IPC (5).

We also examined the question of whether the source of the mediators released by IPC is at least in part the cardiac myocytes. Working with isolated rat myocytes, we were able to demonstrate the same VPC effect. Thus myocytes can be protected against hypoxia-reoxygenation injury by hypoxic preconditioning, and this preconditioning is blocked by VPC. This indicates that S1P is released from myocytes in sufficient amount to induce an autocrine cardioprotective response that is the result of S1P binding to myocyte S1P1 and S3 G protein-coupled receptors, triggering a cell-signaling pathway that results in cardioprotection (Fig. 4). This is consistent with previous work in adult mouse myocytes showing that the cardioprotection produced by monoganglioside GM-1 appeared to be mediated by endogenous S1P release and binding to G protein-coupled receptors (22). It is also consistent with work that showed that S1P can effectively induce cardioprotection when added exogenously (10, 13, 15, 27, 29). However, we cannot exclude the possibility that S1P may also be acting as a regulator or modulator of cardioprotection in addition to its demonstrated function as a mediator of this process.

The myocyte culture system was used to verify that IPC induces S1P release from these cells. Myocytes were labeled with d-erythro[3-3H]-sphingosine, 48% of which was converted to [3H]S1P. Labeled myocytes exposed to an IPC protocol of 15 min of hypoxia, 30 min of normoxia, and finally a second 15 min of hypoxia had significantly more S1P in their media compared with normoxia controls. This increased level of S1P in the dilute media is considered to result from a greater concentration in the unstirred water layer next to the membrane surface. On the basis of all the work in this paper and previous inhibitor studies (27), we envision the scheme shown in Fig. 5 for IPC.

Examination of postconditioning in the ex vivo heart revealed its similarity to preconditioning. VPC was found to block the ability of our normal IPOST protocol to postcondition ex vivo hearts. This effect can again be overridden by adding an additional cycle of postconditioning. Thus S1P is also an important endogenous cardioprotectant released during postconditioning. This adds further support to our previous work (26, 27, 28) that revealed the similarity of the preconditioning and postconditioning mechanisms of cardioprotection.

Using 8-SPT, we also obtained reduction of protection by four cycles of IPOST, which indicates the similarity of the response to adenosine and S1P. The capacity of VPC to reduce cardioprotection associated with four cycles of IPOST was eliminated if a fifth cycle of IPOST was added to promote further release of other cardioprotectants. A fifth cycle of IPOST also reduced inhibition of protection by 8-SPT. Thus with each cycle of IPOST there is additional release of S1P and adenosine (and possibly other cardioprotectants, see Fig. 5) until a level of cardioprotectants is reached such that the loss of any one protectant is insignificant owing to the presence of a sufficient level of other protectants to promote myocyte survival. Thus extensive IPOST obviates the need for the contribution of any one cardioprotectant. This combined IPOST protection by S1P and adenosine is no different from that seen with IPC, revealing the similarity of the mechanisms for the two regimens.

Previous work from our laboratory has identified the importance of the sphingosine kinase/S1P axis in pre- and postconditioning (11, 12, 25). However, the basis for this cardioprotection was not established. There are no previous studies by us or by others in which inhibition of S1P receptors has been shown to abolish or severely impair ischemic pre- or postconditioning effects in the heart. Thus this is the first demonstration that S1P is an important autocrine mediator of ischemic pre- and postconditioning in heart. We were able to verify this by directly measuring S1P release in response to IPC in a myocyte model. Furthermore, support for this role of S1P is provided by our studies of a sphingosine kinase form 1 knockout. We have shown that the knockout out of sphingosine kinase form 1 resulted in a reduction in overall cardiac sphingosine kinase activity and a reduced cellular concentration of S1P and that this is accompanied by a diminished capacity for both pre- and postconditioning (11, 12).

In conclusion, these data provide solid evidence that S1P is an endogenous cardioprotectant. The data also indicate that S1P is one of several known endogenous cardioprotective mediators released by pre- and postconditioning that work in concert via distinct G protein-coupled receptors and that myocytes are a source of this S1P. We have previously also shown that S1P is also capable of working in concert with non-G protein-coupled receptors related cardioprotectants, e.g., sphingosine, to enhance the power of the conditioning effect (26).

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