Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury

Christopher K. Means, Chun-Yang Xiao, Zhuangjie Li, Tong Zhang, Jeffrey H. Omens, Isao Ishii, Jerold Chun and Joan H. Brown

Am J Physiol Heart Circ Physiol 292:H2944-H2951, 2007. First published 9 February 2007; doi:10.1152/ajpheart.01331.2006

Am J Physiol Heart Circ Physiol 292: H2944–H2951, 2007.
First published February 9, 2007; doi:10.1152/ajpheart.01331.2006.

Sphingosine 1-phosphate $S1P_2$ and $S1P_3$ receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury

Christopher K. Means,[1,2] Chun-Yang Xiao,[1] Zhuangjie Li,[3] Tong Zhang,[2] Jeffrey H. Omens,[3] Isao Ishii,[1] Jerold Chun,[4] and Joan Heller Brown[2]

[1]*Biomedical Sciences Graduate Program and Departments of* [2]*Pharmacology and* [3]*Medicine, University of California, San Diego, La Jolla, California; and* [4]*Department of Molecular Biology, The Scripps Research Institute, La Jolla, California*

Submitted 5 December 2006; accepted in final form 6 February 2007

Means CK, Xiao C-Y, Li Z, Zhang T, Omens JH, Ishii I, Chun J, Brown JH. Sphingosine 1-phosphate $S1P_2$ and $S1P_3$ receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 292: H2944–H2951, 2007. First published February 9, 2007; doi:10.1152/ajpheart.01331.2006.—Sphingosine 1-phosphate (S1P) is released at sites of tissue injury and effects cellular responses through activation of G protein-coupled receptors. The role of S1P in regulating cardiomyocyte survival following in vivo myocardial ischemia-reperfusion (I/R) injury was examined by using mice in which specific S1P receptor subtypes were deleted. Mice lacking either $S1P_2$ or $S1P_3$ receptors and subjected to 1-h coronary occlusion followed by 2 h of reperfusion developed infarcts equivalent to those of wild-type (WT) mice. However, in $S1P_{2,3}$ receptor double-knockout mice, infarct size following I/R was increased by >50%. I/R leads to activation of ERK, JNK, and p38 MAP kinases; however, these responses were not diminished in $S1P_{2,3}$ receptor knockout compared with WT mice. In contrast, activation of Akt in response to I/R was markedly attenuated in $S1P_{2,3}$ receptor knockout mouse hearts. Neither $S1P_2$ nor $S1P_3$ receptor deletion alone impaired I/R-induced Akt activation, which suggests redundant signaling through these receptors and is consistent with the finding that deletion of either receptor alone did not increase I/R injury. The involvement of cardiomyocytes in $S1P_2$ and $S1P_3$ receptor mediated activation of Akt was tested by using cells from WT and S1P receptor knockout hearts. Akt was activated by S1P, and this was modestly diminished in cardiomyocytes from $S1P_2$ or $S1P_3$ receptor knockout mice and completely abolished in the $S1P_{2,3}$ receptor double-knockout myocytes. Our data demonstrate that activation of $S1P_2$ and $S1P_3$ receptors plays a significant role in protecting cardiomyocytes from I/R damage in vivo and implicate the release of S1P and receptor-mediated Akt activation in this process.

cardioprotection; mitogen-activated kinase; G protein-coupled receptors; infarct

SPHINGOSINE 1-PHOSPHATE (S1P) is a bioactive lysophospholipid generated through the breakdown of sphingomyelin. A number of regulated enzymes, including sphingomyelinase and sphingosine kinase, control its formation (40). A role for S1P in regulating cellular responses to injury and inflammation has become increasingly well accepted. In the heart, as in other tissues, sphingomyelinase is activated by ischemia-reperfusion (I/R) (anoxia-reoxygenation) and by cytokines such as TNF-α, suggesting that sphingolipid metabolites (ceramide, sphingosine, and S1P) are generated and may participate in cellular responses to these interventions (5, 8, 12, 23). Sphingosine kinase has also been shown to be activated by I/R in the heart (18). Although intracellular actions of sphingomyelin metabolites had been examined for many years, the cloning of G protein-coupled receptors with specificity for S1P led to recognition that sphingolipid-mediated responses are effected, in large part, through extracellular activation of cell surface receptors (6, 16, 26).

The S1P receptors, originally classified into the edg receptor family, are now referred to as $S1P_1$–$S1P_5$. The $S1P_1$ (edg1), $S1P_2$ (edg5), and $S1P_3$ (edg3) receptors are ubiquitously expressed, whereas the expression of $S1P_4$ and $S1P_5$ receptors is more restricted. The selectivity in coupling of these receptors to specific G proteins and signal-transduction pathways has not been well established because few receptor subtype-selective agonists or antagonists are available. The generation of knockout mice, in which specific S1P receptor genes are deleted by homologous recombination (2, 15, 17, 28), has therefore provided a much-needed means for examining the roles of the different S1P receptor subtypes as well as their downstream targets.

$S1P_1$ receptor knockout mice ($S1P_1^{-/-}$ mice) show embryonic lethality due to the aberrant vasculogenesis that results from loss of S1P receptors in vascular endothelial cells (2, 28). In contrast $S1P_2$, $S1P_3$, or $S1P_{2,3}$ receptor knockout mice ($S1P_2^{-/-}$, $S1P_3^{-/-}$, or $S1P_{2,3}^{-/-}$) are viable and show only modest phenotypic changes (15, 17). Our previous studies examining mouse embryonic fibroblasts (MEF cells) derived from these mice revealed that PLC activation is regulated by $S1P_3$ receptors alone, Rho activation is regulated by both $S1P_2$ and $S1P_3$ receptors, and adenylate cyclase inhibition is regulated by $S1P_1$ receptors, because this response is not lost in MEF cells from $S1P_2^{-/-}$, $S1P_3^{-/-}$, or $S1P_{2,3}^{-/-}$ mice (15, 17).

Sphingolipid metabolites such as S1P and ceramide have been suggested to regulate cell survival. Whereas ceramide is considered to be proapoptotic, S1P can suppress ceramide-mediated apoptosis, providing a yin-yang aspect to sphingomyelinase signaling (9). S1P has been shown to activate Akt (14, 37, 39), which has been associated with cell survival in cardiomyocytes (10, 31, 38). In addition, S1P has been shown to protect neonatal rat cardiomyocytes and perfused rabbit and mouse hearts from ischemic damage (5, 18, 19, 22). However, neither the receptor subtype nor the signal-transduction pathways mediating these effects has been established, nor has an in vivo protective role for endogenously released S1P been demonstrated. Accordingly, we designed experiments to examine the cell-survival pathways regulated by S1P in cardiomy-

Address for reprint requests and other correspondence: J. H. Brown, Dept. of Pharmacology, Univ. of California, San Diego, 9500 Gilman Dr., La Jolla, CA, 92093-0636 (e-mail: jhbrown@ucsd.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ocytes, to determine whether S1P receptor activation participated in the response to I/R injury in vivo, and to identify the S1P receptor subtypes and downstream mediators affording cardioprotection.

The experiments reported here demonstrate that I/R injury is not altered in either $S1P_2^{-/-}$ or $S1P_3^{-/-}$ mice but is markedly increased in $S1P_{2,3}^{-/-}$ mice. Examination of the signal-transduction pathways regulated by S1P in isolated cardiomyocytes and by I/R in WT and S1P receptor knockout mice revealed that activation of Akt in cardiomyocytes, although modestly diminished in $S1P_2^{-/-}$ and $S1P_3^{-/-}$ mice, is severely compromised in cardiomyocytes and in vivo in hearts from $S1P_{2,3}^{-/-}$ mice. We conclude that S1P formation during I/R limits cardiomyocyte damage by stimulating both $S1P_2$ and $S1P_3$ receptors and suggest that the protective effect of $S1P_{2,3}$ receptor stimulation occurs through activation of Akt-mediated survival pathways.

EXPERIMENTAL PROCEDURES

Animals. Generation and maintenance of $S1P_2^{-/-}$, $S1P_3^{-/-}$, and $S1P_{2,3}^{-/-}$ mice were previously reported (15, 17). Animals had free access to water and food. All experiments reported here were performed using 24- to 28-wk-old (25–35 g) mice of either sex. Wild-type (WT) littermate animals were used as controls for all experiments with $S1P_2^{-/-}$ or $S1P_3^{-/-}$ mice. For experiments with $S1P_{2,3}^{-/-}$ mice, the low frequency of obtaining double-knockout mice (1/16) and WT mice (1/16) from the same litter (1/256) necessitated the use of age-matched WT mice of the same background as controls. All procedures were performed in accordance with the National Institutes of Health/University of California, San Diego *Guide for the Care and Use of Laboratory Animals* and approved by the UCSD Institutional Animal Care and Use Committee.

RT-PCR. Cultured adult mouse cardiomyocytes and whole mouse hearts were collected and processed by methods described in previous studies (1, 45). Total RNA was isolated using RNeasy (Qiagen), and cDNA was produced by using Superscript reverse transcriptase (Invitrogen). PCR was performed with an initial denaturation step at 94°C for 5 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 2 min and a final extension step at 72°C for 10 min. The following S1P receptor-specific primers were used for PCR amplification: $S1P_1$ 5′-TCATCGTCCGGCATTACAACTA-3′ and 5′-GAGTGAGCTTGTAGGTGGTG-3′; $S1P_2$ 5′-ATGGGCAGCTTGTACTCGGAG-3′ and 5′-CAGCCAGCAGACGATAAAGAC-3′; $S1P_3$ 5′-CTTGGTCATCTGCAGCTTCATC-3′ and 5′-TGCTGATGCAGAAGGCAATGTA-3′; $S1P_4$ 5′-TGAACATCACGCTGAGTGACCT-3′ and 5′-GATCATCAGCACCGTCTTCAGC-3′; $S1P_5$ 5′-ATCTGTGCGCTCTATGCAAGGA-3′ and 5′-GGTGTAGATGATAGGATTCAGCA-3′ (27). Non-reverse-transcribed RNA was also amplified by PCR to check for contaminating genomic DNA.

Isolation of adult mouse cardiomyocytes. Cardiomyocytes were isolated from the hearts of 3-mo-old WT or S1P receptor knockout mice by an adaptation of the method described by the Alliance for Cell Signaling (http://www.afcs.org); based on Ref 46. Briefly, animals were anesthetized with pentobarbital, and hearts were removed, cannulated, and subjected to retrograde aortic perfusion at 37°C at a rate of 3 ml/min. Hearts were perfused for 4 min in Ca^{2+}-free buffer, followed by 8–10 min of perfusion with 0.25 mg/ml collagenase (Blendzyme 1; Roche). Hearts were removed from the cannula, and the ventricle was dissociated at room temperature by pipetting with increasingly smaller transfer pipettes. Collagenase was inactivated, once tissue was thoroughly digested, by resuspending the tissue in medium containing 10% bovine calf serum. Calcium was gradually added back to a final concentration of 1 mM, and cells were plated on laminin-coated dishes in MEM-HBSS medium containing 5% serum. After 1 h, cells were washed and serum-free medium was added back. Cells remained in serum-free medium overnight (35), and cellular responses were measured the next day.

Immunoblot analysis. For Western blotting, adult mouse cardiomyocytes or cardiac homogenates were prepared as described previously (45). Equal amounts of total protein were loaded. The antibodies used for immunoblotting were as follows: rabbit anti phospho-Akt (Ser^{473}), rabbit total Akt, rabbit anti phospho-ERK1/2, rabbit total ERK1/2, rabbit anti phospho-p38, rabbit total p38, mouse anti phospho-JNK, or rabbit total JNK (Cell Signaling Technology).

Animal model. Occlusion and reperfusion of the coronary artery were performed as previously reported (44). Briefly, mice were anesthetized with an intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (5 mg/kg) and were placed in a supine position under body-temperature control. Each animal was endotracheally intubated and ventilated with a tidal volume of 0.5 ml at a rate of 120 strokes/min by using a rodent respirator (model no. 683; Harvard Apparatus). After left thoracotomy, a 7-0 surgical suture was passed underneath the left anterior descending coronary artery (LAD) at a position 2 mm from the tip of the left auricle under the aid of a stereoscope (Nikon). PE-10 tubing (1–2 mm in length) was placed along the vessel as a cushion and was secured around the tubing to occlude the LAD. For the sham-operated control mice, the procedure was performed as above except that the suture was not secured around the LAD to occlude the vessel. Myocardial ischemia was verified by blanching of the left ventricle (LV) and by change in electrocardiogram. Blood flow was restored after 1 h of occlusion by removing the ligature and PE tubing.

Assessment of area at risk and infarct size. Following 2 h of reperfusion, the LAD was reoccluded and 5% Evans blue dye (0.2 ml) was injected into the LV cavity with a 27-gauge needle to define the nonischemic zone. The heart was excised immediately and rinsed in saline to remove excess dye, and the LV was frozen and cut transversely into five slices of equal thickness. These samples were incubated in 1% 2,3,5-triphenyltetrazolium chloride-containing Tris-HCl buffer (pH 7.8) at 37°C for 10 min to stain the viable myocardium (brick red) and then were fixed in 10% formalin-phosphate buffered saline for 24 h. Each slice was weighed and photographed from both sides by using a microscope equipped with a high-resolution digital camera (COOLPIX 990; Nikon). The area at risk (AAR), infarcted

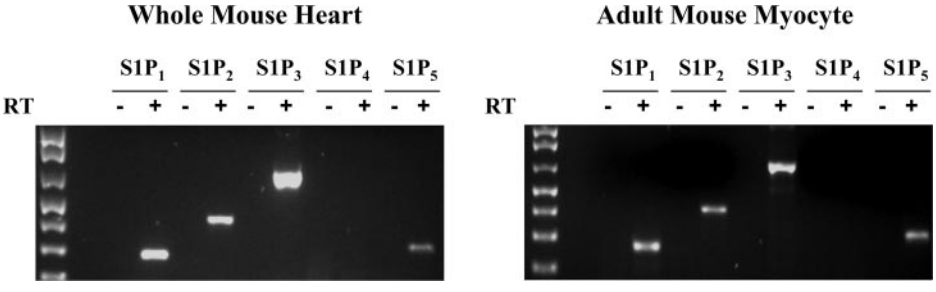

Fig. 1. Expression of sphingosine 1-phosphate (S1P) receptor mRNA in adult mouse cardiomyocytes and whole mouse heart. RT-PCR was carried out on RNA isolated from cultured mouse cardiomyocytes or total mouse heart homogenates. S1P receptor expression was analyzed by PCR in reverse-transcribed (+) and non-reverse-transcribed (−) cDNA by using receptor subtype-specific primers. Sizes of PCR products are 270 ($S1P_1$), 415 ($S1P_2$), 617 ($S1P_3$), and 305 bp ($S1P_5$).

tissue, and the total LV area were measured by digital planimetry using NIH Image.

Statistical analysis. All values are expressed as means ± SE of *n* independent experiments. Statistical analysis was performed with unpaired *t*-test for two groups and one-way ANOVA followed by Dunnett's test for three or more groups. A difference was considered statistically significant at $P < 0.05$.

RESULTS

S1P receptor expression. RT-PCR analysis was used to determine the pattern of expression of S1P receptors in adult mouse cardiomyocytes and in mouse heart. Transcripts of the $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$ receptors were detected in both isolated cardiomyocytes and the whole adult heart (Fig. 1). $S1P_4$ receptors were not detected in either cardiac preparation, although $S1P_4$ receptor transcripts were detected in other tissues using the same primers (data not shown).

$S1P_2$ and $S1P_3$ receptors mediate protection from I/R injury. Adult $S1P_2^{-/-}$, $S1P_3^{-/-}$, and $S1P_{2,3}^{-/-}$ mice are phenotypically normal, although defects in S1P-mediated cellular signaling have been demonstrated in MEF cells isolated from these animals (15, 17). To determine whether S1P receptors play a role in the response to I/R injury in vivo, we compared WT and S1P receptor-null mice after in vivo I/R by using a previously established model (44). Cardiomyocyte cell death in hearts exposed to 1 h of coronary occlusion followed by 2 h of reperfusion was assessed by using 2,3,5-triphenyltetrazolium chloride staining (described in EXPERIMENTAL PROCEDURES). A representative photomicrograph of a short-axis section from a WT mouse LV is shown in Fig. 2*A*, and the areas quantified to assess ischemic injury are delineated. Evans blue dye-positive areas represent nonischemic tissue, whereas the ischemic area (the AAR) is comprised of the white infarcted necrotic tissue (*1*) plus the red viable salvaged tissue (*2*).

We first compared WT and $S1P_3^{-/-}$ mice. The severity of the ischemic insult was not different in the two groups based on the similar values for AAR expressed relative to total LV mass (Fig. 2*B*). The infarct size, reflective of the amount of nonviable myocardium, was also not significantly different between $S1P_3^{-/-}$ and WT mice, whether expressed relative to AAR or total LV mass (Fig. 2*B*). We subsequently compared WT and $S1P_2^{-/-}$ mice. The severity of the insult was not significantly different between these two groups, as seen by the AAR relative to LV. As observed for $S1P_3^{-/-}$ mice, the size of the infarct relative to either AAR or LV was not significantly different between $S1P_2^{-/-}$ and WT mice (Fig. 3). These data indicate that the loss of either the $S1P_2$ or $S1P_3$ receptor alone does not alter the in vivo response to I/R injury.

The $S1P_2$ and $S1P_3$ receptors could serve redundant functions by coupling to common downstream pathways. Accordingly, we further tested the involvement of S1P receptors in ischemic injury by examining the response to myocardial I/R injury in $S1P_{2,3}^{-/-}$ mice. As shown in Fig. 4, the areas at risk were not different in WT and $S1P_{2,3}^{-/-}$ mice. Importantly, however, infarct size (expressed as a percentage of AAR) was increased by >50% in $S1P_{2,3}^{-/-}$ compared with WT mice (Fig. 4). Infarct size expressed relative to LV mass was also significantly elevated. Thus combined activation of $S1P_2$ and $S1P_3$ receptors provides a protective signal during in vivo I/R that is lost in $S1P_{2,3}^{-/-}$ mice.

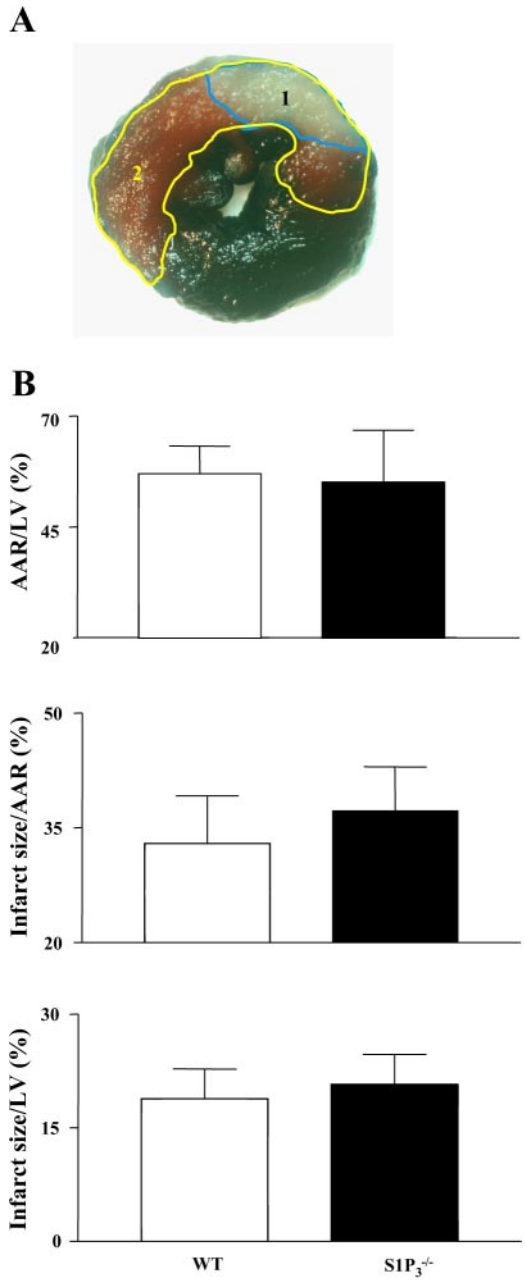

Fig. 2. Comparison of infarct size between wild-type (WT) and $S1P_3$ receptor knockout ($S1P_3^{-/-}$) mice after coronary occlusion followed by reperfusion (ischemia-reperfusion; I/R). *A*: representative photomicrograph of a short axis of the left ventricle (LV) after 1 h of coronary occlusion followed by 2 h of reperfusion. Blue areas, nonischemic tissue; white areas (*1*), infarcted tissue; red areas (*2*), salvaged tissue within the risk area. *B*: myocardial infarct size, area at risk (AAR), and LV size (LV) were calculated from $S1P_3^{-/-}$ mice and their corresponding WT mice ($n = 6$ in each group) as described in EXPERIMENTAL PROCEDURES. Values are means ± SE.

To rule out the possibility that the protective role of S1P receptors is due to S1P receptor-mediated effects on heart rate [through activation of potassium currents (13, 41)], heart rate was monitored by continuous electrocardiographic recording throughout the period of I/R. No differences in heart rate were observed among the groups of mice examined (data not shown), indicating that differences in chronotropic responsiveness do not underlie the altered susceptibility to injury.

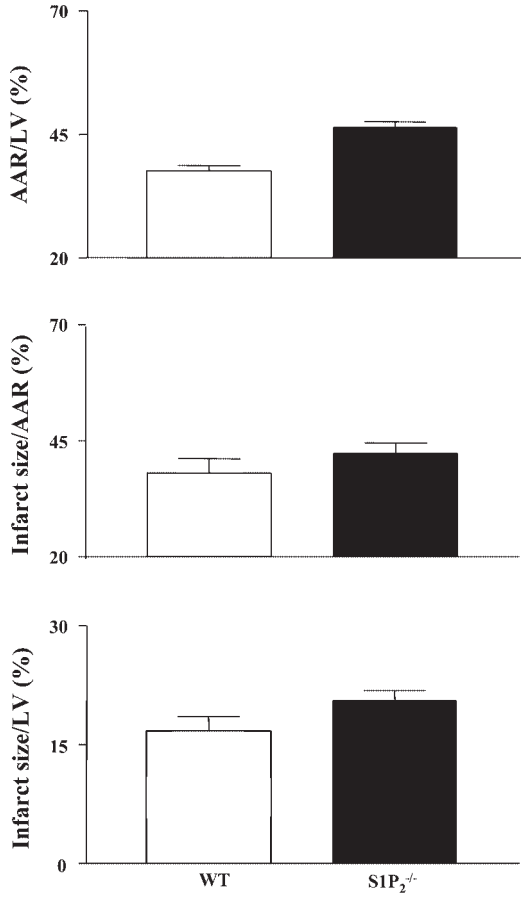

Fig. 3. Comparison of infarct size between WT and $S1P_2$ receptor knockout ($S1P_2^{-/-}$) mice following I/R. Myocardial infarct size, AAR, and LV were calculated from $S1P_2^{-/-}$ mice and their corresponding WT mice ($n = 6$ in each group). Values are means ± SE.

MAP kinase activation pathways are not altered in $S1P_{2,3}^{-/-}$ mice subjected to in vivo I/R. To examine the possible role of MAP kinase activation in the protective effects of S1P receptors, we first characterized the kinetics of activation of various MAP kinases following I/R in WT mice. Phosphorylation of p38, ERK, and JNK MAP kinases was examined by Western blotting with phospho-specific antibodies. Both ischemia and subsequent reperfusion led to increased p38 phosphorylation (Fig. 5*A*), as previously observed in isolated rat and rabbit hearts (4, 29). Phosphorylation of ERK and JNK was not significantly increased during ischemia but increased following reperfusion, with the peak of activation occurring after 15 min of reperfusion (Fig. 5*A*), consistent with previous findings from isolated rat and rabbit heart (4, 36). To determine whether altered activation of these MAP kinases could be responsible for the differential susceptibility to I/R injury, the phosphorylation states of ERK, JNK, and p38 MAP kinases were compared in $S1P_{2,3}^{-/-}$ vs. WT mice subject to ischemia and 15 min of reperfusion. There was no significant difference in the magnitude of reperfusion-induced phosphorylation of any of the MAP kinases in the $S1P_{2,3}^{-/-}$ vs. WT mice (Fig. 5*B*). Thus MAP kinase signaling during in vivo I/R is not compromised in the combined absence of the $S1P_2$ and $S1P_3$ receptors.

I/R-induced Akt activation in S1P receptor knockout mice. Similar experiments were then carried out examining Akt activation in response to I/R. Western blotting to detect Akt phosphorylation at Ser^{473} in the catalytic loop revealed that Akt phosphorylation increases during reperfusion following ischemia, consistent with previous studies carried out on isolated, perfused rat hearts (20, 43). In the WT mouse heart the increase in Akt phosphorylation was maximal at 15 min of reperfusion (Fig. 6*A*). Akt phosphorylation was then compared in WT and $S1P_{2,3}^{-/-}$ mouse hearts following I/R. The fivefold increase in phospho-Akt observed in WT mice was markedly attenuated (by ~70%) in the $S1P_{2,3}^{-/-}$ mice (Fig. 6*B*). These data indicate that a significant component of the Akt activation observed during I/R occurs through $S1P_2$ and $S1P_3$ receptor activation and suggest that endogenously released S1P may serve to protect against I/R injury through this pathway.

To further establish a relationship between Akt activation and the protective effect of S1P receptor activation, we tested $S1P_2^{-/-}$ or $S1P_3^{-/-}$ mice, neither of which showed altered infarct size in response to I/R. Mice from both lines were subjected to I/R, and Akt activation was assessed. In contrast to what we observed for the $S1P_{2,3}^{-/-}$ mice, Akt activation by I/R in either $S1P_2^{-/-}$ or $S1P_3^{-/-}$ mice was not significantly different from that of WT mice (Fig. 7).

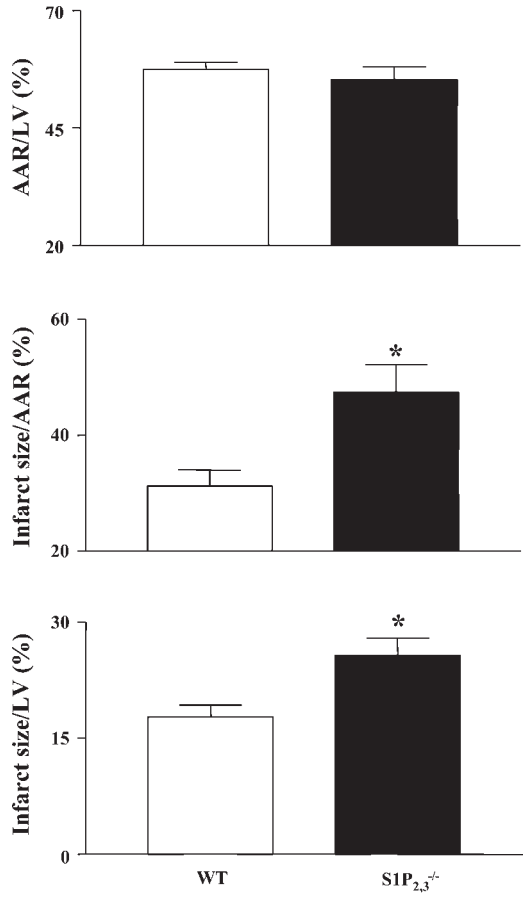

Fig. 4. Comparison of infarct size between WT and $S1P_{2,3}^{-/-}$ receptor double-knockout mice following I/R. Myocardial infarct size, AAR, and LV were calculated from $S1P_{2,3}^{-/-}$ mice and their corresponding WT mice ($n = 11$ in each group). Values are means ± SE. *$P < 0.05$ vs. WT group.

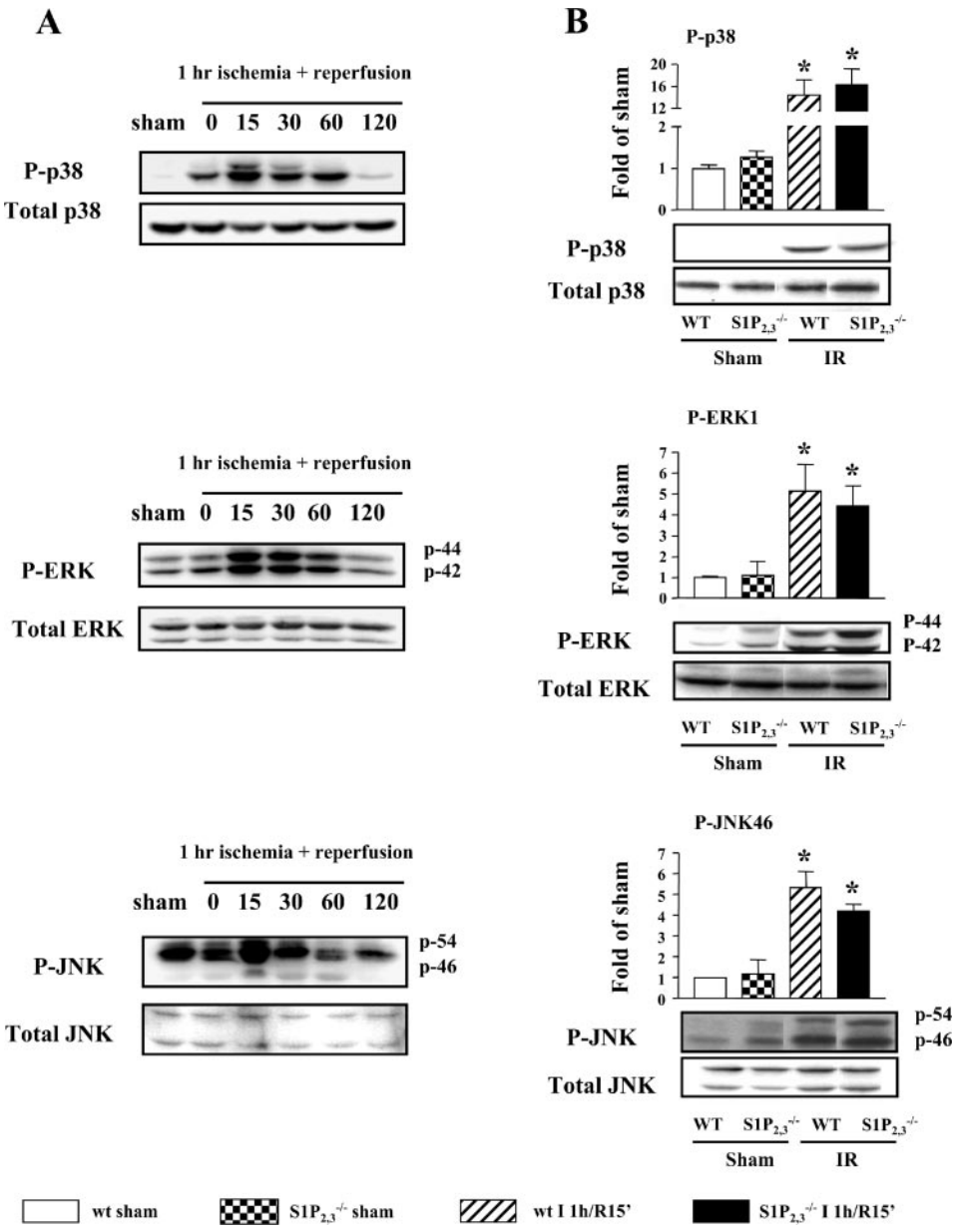

Fig. 5. Phosphorylation of MAP kinases during in vivo I/R. WT and $S1P_{2,3}^{-/-}$ hearts were subjected to 1 h ischemia and various times of reperfusion, and extracted proteins were analyzed by Western blotting. *A*: time course of MAP kinase phosphorylation in WT mouse hearts. *B*: comparison of MAP kinase phosphorylation in WT and $S1P_{2,3}^{-/-}$ mouse hearts at 15 min of reperfusion. Western blots were quantitated by densitometry. Values are means ± SE ($n = 4$–5 in each group). *$P <$ 0.05 vs. sham-operated animals (no I/R).

S1P-mediated Akt activation in WT and S1P receptor knockout adult mouse cardiomyocytes. The data above indicate that Akt activation after in vivo I/R correlates with S1P-mediated protection. Although the heart is largely myocytes, other endogenous or invading cells responsive to S1P (e.g., endothelial cells, macrophages) could be present. To demonstrate that the alterations observed in vivo reflect the response of cardiomyocytes to S1P, we isolated cardiomyocytes from WT, $S1P_2^{-/-}$, $S1P_3^{-/-}$, and $S1P_{2,3}^{-/-}$ mice and assessed the ability of S1P to activate Akt. Treatment of WT adult mouse myocytes with 5 μM S1P (Avanti Polar Lipids) significantly increased Akt phosphorylation (Fig. 8). Although the response was less robust than that elicited by I/R, the pattern observed was similar. S1P induced a smaller and not statistically significant increase in Akt activation in cardiomyocytes from $S1P_2^{-/-}$ or $S1P_3^{-/-}$ mice, whereas deletion of both $S1P_2$ and $S1P_3$ receptors resulted in a complete loss of S1P-mediated Akt activation.

DISCUSSION

Sphingosine is released from, and S1P is formed in, isolated rabbit hearts subject to hypoxia and acidosis (5). The addition of S1P to neonatal rat ventricular myocytes has been demonstrated to confer cardioprotection against hypoxia (22), and S1P also protects against global I/R damage in isolated mouse hearts (19). A role for S1P in conferring ischemic preconditioning in the isolated heart has also been suggested (24). Activation of sphingosine kinase, the upstream kinase responsible for producing S1P, has more recently been suggested to protect the isolated perfused heart from I/R damage (18). Our findings provide the first in vivo evidence that G protein-coupled $S1P_2$ and $S1P_3$ receptors are stimulated during I/R and promote cardiomyocyte survival.

The data presented here also provide insight into the signaling pathways by which S1P can affect cardioprotection in vivo. As demonstrated here, the extent of I/R damage did not differ

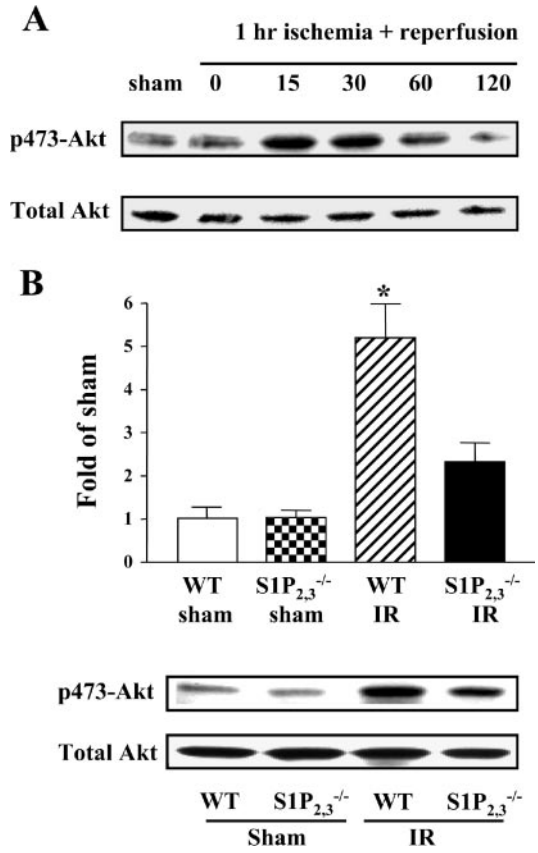

Fig. 6. Phosphorylation of Akt during in vivo I/R in WT and $S1P_{2,3}^{-/-}$ mouse hearts. *A*: time course of Akt phosphorylation in WT mouse hearts. *B*: comparison of Akt phosphorylation in WT and $S1P_{2,3}^{-/-}$ mouse hearts after 1 h ischemia and 15 min of reperfusion. Western blots were quantitated by densitometry. Values are means ± SE ($n = 4$–5 in each group). *$P < 0.05$ vs. sham-operated animals (no I/R).

in the $S1P_3^{-/-}$ vs. WT mice. According to our previously published studies (15), there is nearly complete loss of S1P-mediated phosphoinositide hydrolysis in MEF cells from $S1P_3^{-/-}$ mice, and we also observed complete loss of S1P-stimulated phosphoinositide hydrolysis in myocytes isolated from $S1P_3^{-/-}$ hearts (data not shown). Thus if elevated S1P elicits PLC activation and generation of its downstream second messengers through $S1P_3$ receptors in the ischemic myocardium, these responses do not appear to be required for S1P-mediated protection.

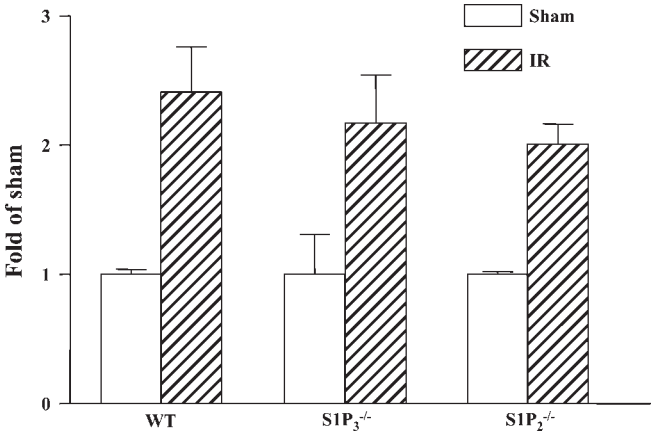

Fig. 7. Phosphorylation of Akt during I/R in WT, $S1P_2^{-/-}$, and $S1P_3^{-/-}$ mouse hearts. WT, $S1P_2^{-/-}$, and $S1P_3^{-/-}$ hearts were subjected to 1 h ischemia and 15 min reperfusion, and extracted proteins were analyzed by Western blotting. Western blots were quantitated by densitometry. Values are means ± SE ($n = 4$ in each group). There was no significant difference ($P > 0.05$) between Akt activation by I/R in WT, $S1P_2^{-/-}$, and $S1P_3^{-/-}$ hearts.

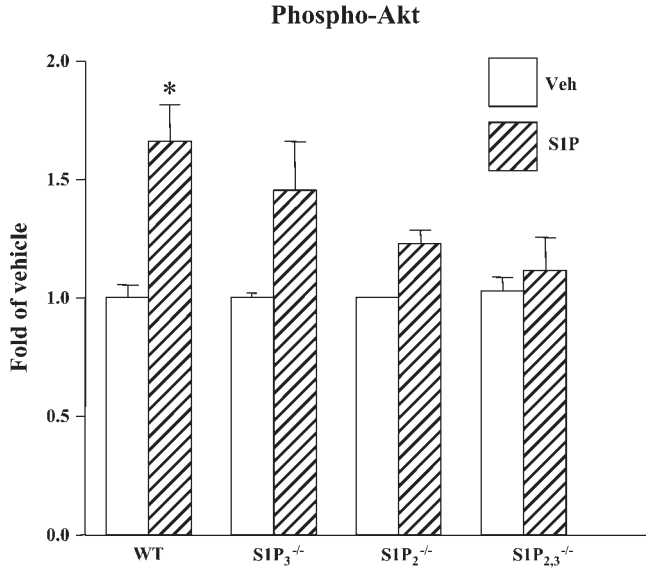

Fig. 8. S1P-mediated phosphorylation of Akt in WT, $S1P_2^{-/-}$, $S1P_3^{-/-}$, and $S1P_{2,3}^{-/-}$ adult mouse cardiomyocytes. Cardiomyocytes were stimulated with S1P (5 μM) for 5 min and then were assayed for phosphorylation of Akt by Western blotting. Phosphorylation was quantitated by densitometry and normalized to vehicle controls of each genotype. Values are means ± SE ($n \geq 5$ in each group). *$P < 0.05$ vs. vehicle.

MAP kinase pathways have also been implicated in control of cell survival in the myocardium. In vivo, all three MAP kinase pathways (ERK, JNK, and p38) are activated by reperfusion following ischemia (Fig. 5) (7). However, neither ERK, JNK, nor p38 activation by I/R is impaired in the $S1P_{2,3}^{-/-}$ mice. This finding suggests that the major pathways leading to MAP kinase activation in I/R are not initiated through stimulation of the $S1P_2$ or $S1P_3$ receptors. Thus activation of another receptor likely contributes to the activation of MAP kinases in in vivo I/R. In addition, the observation that infarct size is significantly increased in $S1P_{2,3}^{-/-}$ mice, even in the face of unaltered MAP kinase activation, indicates that activation of MAP kinases is not sufficient to support cardiomyocyte survival.

The phosphorylation of Akt that accompanies I/R is, in contrast, markedly attenuated in the $S1P_{2,3}^{-/-}$ mice. Smaller and insignificant decreases are affected by loss of either $S1P_2$ or $S1P_3$ receptors alone. Thus stimulation of both $S1P_2$ and $S1P_3$ receptors appears to contribute to activation of Akt in vivo. The redundant or overlapping functions of these receptors in coupling S1P actions to phosphorylation of Akt is also seen in our in vitro studies on isolated cardiomyocytes. Cardiomyocytes lacking either the $S1P_2$ or $S1P_3$ receptor demonstrate a partial loss in Akt phosphorylation, whereas there is complete loss of S1P-mediated phosphorylation of Akt in $S1P_{2,3}^{-/-}$ cardiomyocytes. That either $S1P_2$ or $S1P_3$ receptors can mediate Akt activation and concomitant cardioprotection further explains why I/R damage is not aggravated in mice lacking only $S1P_2$ or only $S1P_3$ receptors.

A surprising aspect of our studies is that the $S1P_1$ receptor, still present in the $S1P_{2,3}^{-/-}$ mice, does not confer greater protection against I/R injury. Akt activation by I/R is markedly diminished in the $S1P_{2,3}^{-/-}$ mice despite the presence of the $S1P_1$ receptor, shown in other systems to couple to Akt activation (3, 25, 33). The $S1P_1$ receptor in adult mouse cardiomyocytes also couples poorly to this pathway, because no Akt activation is observed in cells from $S1P_{2,3}^{-/-}$ mice. The reason that the $S1P_2$ and $S1P_3$ receptors, but not the $S1P_1$ receptor, regulate Akt activation in cardiomyocytes is under study.

Akt is a well-established mediator of cardioprotection in I/R injury both in vitro and in vivo, as demonstrated by transfection, gene delivery, and transgenic approaches (10, 30–32). Mechanisms of Akt-mediated cardioprotection are under intense investigation. Akt has been shown to increase endothelial NO synthase (eNOS) phosphorylation, and a role for NO in protection against ischemic damage is suggested by experiments with eNOS knockout mice (11, 21). In endothelial cells, S1P activates eNOS via an Akt-mediated pathway, and this occurs via the $S1P_3$ receptor (34). More recently, it has been reported (42) that administration of exogenous S1P is able to protect the heart from I/R injury via this $S1P_3$ receptor-mediated pathway. Interestingly, our data indicate that in cardiomyocytes, Akt activation occurs predominantly through the $S1P_2$ receptor and to a lesser extent through the $S1P_3$ receptor. In vivo, we find that both the $S1P_2$ and $S1P_3$ receptors contribute to I/R-induced Akt activation and that an increase in ischemic damage is not seen unless both $S1P_2$ and $S1P_3$ receptors are deleted. Because the Akt activation in response to in vivo I/R is greater than the Akt activation seen in isolated cardiomyocytes, it is likely that other cell types (e.g., vascular endothelial cells or fibroblasts) or other activators of Akt are contributing to this response. As a working hypothesis, we suggest that the protective effects of S1P released in response to I/R involve $S1P_3$ receptor activation of eNOS, via Akt, in endothelial cells, as well as $S1P_2$ and $S1P_3$ receptor activation of Akt in cardiomyocytes.

In conclusion, our findings indicate that S1P activation of its cognate G protein-coupled receptor on cardiomyocytes serves as a signal for Akt activation and cardiomyocyte protection during I/R in vivo. Subtype-selective agonists for $S1P_2$ or $S1P_3$ receptors could therefore be novel therapeutic modalities for limiting the extent of cardiomyocyte loss associated with acute I/R injury in the heart.

ACKNOWLEDGMENTS

Present addresses: C.-Y. Xiao, Beth Israel Deaconess Medical Center, Cardiovascular Research, 330 Brookline Ave., E/SL-146, Boston, MA 02215; I. Ishii, Dept. of Molecular and Cellular Neurobiology, Gunma University Graduate School of Medicine, Showamachi 3-39-22, Maebashi 371-8511, Japan.

GRANTS

This work was supported by National Institutes of Health Grants HL-46345, (to J. H. Brown), HL-28143 (to J. H. Brown), HL-43026 (to J. H. Omens), MH-56199 (to J. Chun), and MH-01723 (to J. Chun) and by an American Heart Association postdoctoral fellowship (to T. Zhang) and an American Heart Association predoctoral fellowship (to C. K. Means).

REFERENCES

1. **Adams JW, Pagel AL, Means CK, Oksenberg D, Armstrong RC, Brown JH.** Cardiomyocyte apoptosis induced by Gαq signaling is mediated by permeability transition pore formation and activation of the mitochondrial death pathway. *Circ Res* 87: 1180–1187, 2000.
2. **Allende ML, Yamashita T, Proia RL.** G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood* 102: 3665–3667, 2003.
3. **Balthasar S, Samulin J, Ahlgren H, Bergelin N, Lundqvist M, Toescu EC, Eggo MC, Tornquist K.** Sphingosine 1-phosphate receptor expression profile and regulation of migration in human thyroid cancer cells. *Biochem J* 398: 547–556, 2006.
4. **Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH.** Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart: p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia and reperfusion. *Circ Res* 79: 162–173, 1996.
5. **Cavalli AL, Ligutti JA, Gellings NM, Castro E, Page MT, Klepper RE, Palade PT, McNutt WT, Sabbadini RA.** The role of TNFα and sphingolipid signaling in cardiac hypoxia: evidence the cardiomyocytes release TNFα and sphingosine. *Basic Appl Myol* 12: 167–175, 2002.
6. **Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S, Tigyi G.** International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev* 54: 265–269, 2002.
7. **Clerk A, Fuller SJ, Michael A, Sugden PH.** Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J Biol Chem* 273: 7228–7234, 1998.
8. **Cordis GA, Yoshida T, Das DK.** HPTLC analysis of sphingomyelin, ceramide and sphingosine in ischemic/reperfused rat heart. *J Pharm Biomed Anal* 16: 1189–1193, 1998.
9. **Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S, Spiegel S.** Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* 381: 800–803, 1996.
10. **Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K.** Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 101: 660–667, 2000.
11. **Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC.** Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597–601, 1999.
12. **Hernandez OM, Discher DJ, Bishopric NH, Webster K.** Rapid activation of neutral sphingomyelinase by hypoxia-reoxygenation of cardiac myocytes. *Circ Res* 86: 198–204, 2000.
13. **Himmel HM, Meyer zu Heringdorf D, Graf E, Dobrev D, Kortner A, Schuler S, Jakobs KH, Ravens U.** Evidence for Edg-3 receptor-mediated activation of I(K.ACh) by sphingosine-1-phosphate in human atrial cardiomyocytes. *Mol Pharmacol* 58: 449–454, 2000.
14. **Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ.** Lysophospholipids–receptor revelations. *Science* 294: 1875–1878, 2001.
15. **Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J.** Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* 276: 33697–33704, 2001.
16. **Ishii I, Fukushima N, Ye X, Chun J.** Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem* 73: 321–354, 2004.
17. **Ishii I, Ye X, Friedman B, Kawamura S, Contos JJA, Kingsbury MA, Yang AH, Zhang G, Brown JH, Chun J.** Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate receptors, $S1P_2/LP_{B2}$/EDG-5 and $S1P_3/LP_{B3}$/EDG-3. *J Biol Chem* 277: 25152–25159, 2002.
18. **Jin ZQ, Goetzl EJ, Karliner JS.** Sphingosine kinase activation mediates ischemic preconditioning in murine heart. *Circulation* 110: 1980–1989, 2004.
19. **Jin ZQ, Zhou HZ, Zhu P, Honbo N, Mochly-Rosen D, Messing RO, Goetzl EJ, Karliner JS, Gray MO.** Cardioprotection mediated by sphingosine-1-phosphate and ganglioside GM-1 in wild-type and PKC epsilon knockout mouse hearts. *Am J Physiol Heart Circ Physiol* 282: H1970–H1977, 2002.
20. **Jonassen AK, Sack MN, Mjos OD, Yellon DM.** Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 89: 1191–1198, 2001.

21. **Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourd'Heuil D, Huang PL, Lefer DJ.** Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 276: H1567–H1573, 1999.
22. **Karliner JS, Honbo N, Summers K, Gray MO, Goetzl EJ.** The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. *J Mol Cell Cardiol* 33: 1713–1717, 2001.
23. **Kurrelmeyer KM, Michael LH, Baumgarten G, Taffet GE, Peschon JJ, Sivasubramanian N, Entman ML, Mann DL.** Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. *Proc Natl Acad Sci USA* 97: 5456–5461, 2000.
24. **Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN.** Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. *J Mol Cell Cardiol* 34: 509–518, 2002.
25. **Lee JF, Zeng Q, Ozaki H, Wang L, Hand AR, Hla T, Wang E, Lee MJ.** Dual roles of tight junction associated protein, zonula occludens-1, in sphingosine-1-phosphate mediated endothelial chemotaxis and barrier integrity. *J Biol Chem* 281: 29190–29200, 2006.
26. **Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, Hla T.** Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* 279: 1552–1555, 1998.
27. **Liliom K, Sun G, Bunemann M, Virag T, Nusser N, Baker DL, Wang DA, Fabian MJ, Brandts B, Bender K, Eickel A, Malik KU, Miller DD, Desiderio DM, Tigyi G, Pott L.** Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors. *Biochem J* 355: 189–197, 2001.
28. **Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL.** Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest* 106: 951–961, 2000.
29. **Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue TL.** Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation* 99: 1685–1691, 1999.
30. **Matsui T, Li L, del Monte F, Fukui Y, Franke TF, Hajjar RJ, Rosenzweig A.** Adenoviral gene transfer of activated phosphatidylinositol 3′-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation* 100: 2373–2379, 1999.
31. **Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M, Force TL, Franke TF, Hajjar RJ, Rosenzweig A.** Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 104: 330–335, 2001.
32. **Miao W, Luo Z, Kitsis RN, Walsh K.** Intracoronary, adenovirus-mediated Akt gene transfer in heart limits infarct size following ischemia-reperfusion injury in vivo. *J Mol Cell Cardiol* 32: 2397–2402, 2000.
33. **Morales-Ruiz M, Lee MJ, Zollner S, Gratton JP, Scotland R, Shiojima I, Walsh K, Hla T, Sessa WC.** Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J Biol Chem* 276: 19672–19677, 2001.
34. **Nofer JR, van der GM, Tolle M, Wolinska I, von Wnuck LK, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B.** HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* 113: 569–581, 2004.
35. **O'Connell TD, Ishizaka S, Nakamura A, Swigart PM, Rodrigo MC, Simpson GL, Cotecchia S, Rokosh DG, Grossman W, Foster E, Simpson PC.** The alpha(1A/C)- and alpha(1B)-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse. *J Clin Invest* 111: 1783–1791, 2003.
36. **Omura T, Yoshiyama M, Shimada T, Shimizu N, Kim S, Iwao H, Takeuchi K, Yoshikawa J.** Activation of mitogen-activated protein kinases in in vivo ischemia/reperfused myocardium in rats. *J Mol Cell Cardiol* 31: 1269–1279, 1999.
37. **Payne SG, Milstien S, Spiegel S.** Sphingosine-1-phosphate: dual messenger functions. *FEBS Lett* 531: 54–57, 2002.
38. **Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Welch S, Schaefer E, Walsh K, Rosenzweig A, Torella D, Nurzynska D, Kajstura J, Leri A, Anversa P, Sussman MA.** Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circ Res* 94: 884–891, 2004.
39. **Spiegel S, Milstien S.** Sphingosine 1-phosphate, a key cell signaling molecule. *J Biol Chem* 277: 25851–25854, 2002.
40. **Spiegel S, Milstien S.** Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 4: 397–407, 2003.
41. **Sugiyama A, Aye NN, Yatomi Y, Ozaki Y, Hashimoto K.** Effects of sphingosine 1-phosphate, a naturally occurring biologically active lysophospholipid, on the rat cardiovascular system. *Jpn J Pharmacol* 82: 338–342, 2000.
42. **Theilmeier G, Schmidt C, Herrmann J, Keul P, Schafers M, Herrgott I, Mersmann J, Larmann J, Hermann S, Stypmann J, Schober O, Hildebrand R, Schulz R, Heusch G, Haude M, von Wnuck LK, Herzog C, Schmitz M, Erbel R, Chun J, Levkau B.** High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* 114: 1403–1409, 2006.
43. **Tong H, Chen W, Steenbergen C, Murphy E.** Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. *Circ Res* 87: 309–315, 2000.
44. **Xiao CY, Hara A, Yuhki K, Fujino T, Ma H, Okada Y, Takahata O, Yamada T, Murata T, Narumiya S, Ushikubi F.** Roles of prostaglandin I(2) and thromboxane A(2) in cardiac ischemia-reperfusion injury: a study using mice lacking their respective receptors. *Circulation* 104: 2210–2215, 2001.
45. **Zhang T, Johnson EN, Gu Y, Morissette MR, Sah VP, Gigena MS, Belke DD, Dillmann WH, Rogers TB, Schulman H, Ross J Jr, Brown JH.** The cardiac-specific nuclear δ_B isoform of Ca^{2+}/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. *J Biol Chem* 277: 1261–1267, 2002.
46. **Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, Wang S, Lakatta EG, Cheng H, Xiao RP.** Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. *Am J Physiol Heart Circ Physiol* 279: H429–H436, 2000.