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Sphingosine 1-phosphate signaling in the heart and its role in cardioprotection

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Sphingosine 1-phosphate signaling in the heart
and its role in cardioprotection

A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy

in

Biomedical Sciences

by

Christopher Kable Means

Committee in charge:
Professor Joan Heller Brown, Chair
Professor Laurence Brunton
Professor Jerold Chun
Professor Paul Insel
Professor Kirk Knowlton

2007
The dissertation of Christopher Kable Means is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2007
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<th>Full Form</th>
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>AAR</td>
<td>area at risk</td>
</tr>
<tr>
<td>Akt</td>
<td>Akt or protein kinase B</td>
</tr>
<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
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<tr>
<td>cAMP</td>
<td>3’-5’-cyclic adenosine monophosphate</td>
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<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
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<tr>
<td>EPAC</td>
<td>exchange protein activated by cAMP</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<td>guanosine triphosphate</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia reperfusion</td>
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<td>IP$_3$</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
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<td>left ventricle</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>MβCD</td>
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</tr>
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</tr>
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<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine 1-phosphate</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SEW</td>
<td>SEW2871</td>
</tr>
<tr>
<td>SPC</td>
<td>sphingosylphosphorylcholine</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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While working at Aurora Biosciences I had the opportunity to work with and learn from many outstanding scientists such as Dr. Paul Negulescu, Dr. Luxin Feng, and Linda Francis. My experiences working in lab with these individuals prompted me
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receptor localization confers selectivity for G\textsubscript{i} mediated signaling pathways” by Means CK and Brown JH is in preparation for submission.
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Major Field: Biomedical Sciences

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   Professor Joan Heller Brown


Increased susceptibility to myocardial ischemia/reperfusion injury in mice lacking both S1P2 and S1P3 receptors. Experimental Biology. April 11-15, 2003. San Diego, California.


Sphingosine 1-phosphate receptors provide protection against in vivo myocardial ischemia-reperfusion through regulation of Akt. Experimental Biology April 2-6, 2005. San Diego.

Means C.K., Chun J., Brown J.H.

TALKS

ABSTRACT OF THE DISSERTATION

Sphingosine 1-phosphate signaling in the heart
and its role in cardioprotection

by

Christopher Kable Means
Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2007
Professor Joan Heller Brown, Chair

The lysophospholipid sphingosine 1-phosphate (S1P) has been known for many years to induce cellular changes such as proliferation, survival, migration, and contraction. With the discovery of a family of G-protein coupled receptors that use S1P as their ligand, the fundamental mechanism by which S1P signals cellular responses has been clarified.
S1P has been shown to protect cardiomyocytes from death induced by stresses, including hypoxia, and addition of S1P to isolated perfused hearts limits damage induced by ischemia reperfusion. It is not known, however, whether S1P actually serves as a physiological regulator of cardiomyocyte survival in vivo. The studies presented here used S1P receptor knockout mice to ask whether activation of S1P receptors protects the heart from in vivo ischemia reperfusion injury. Following brief arterial occlusion and reperfusion, myocardial infarction develops in WT mice, and to the same extent in mice lacking either S1P2 or S1P3 receptors. In contrast, in S1P2,3 receptor double knockout hearts, infarct size was significantly increased. Ischemia reperfusion leads to activation of MAP kinases and this was not altered in the S1P2,3 receptor double knockout heart. Akt activation, however, was significantly decreased in the S1P2,3 receptor double knockout, but not in either of the single S1P2 or S1P3 receptor knockouts, correlating loss of Akt activation with enlarged infarct size. Thus S1P released during in vivo ischemia reperfusion protects the heart through combined effects on S1P2 and S1P3 receptors and Akt activation.

We subsequently analyzed S1P-mediated signaling pathways in isolated adult mouse ventricular myocytes. S1P activates Akt and MAP kinases downstream of S1P2 and S1P3 receptors, and these responses do not occur when only the S1P1 receptor remains or when it is selectively activated. However the S1P1 receptor is present in S1P2,3 receptor knockout mice and can inhibit isoproterenol-stimulated cAMP accumulation. Subsequent cell fractionation studies showed that the S1P1 receptor is localized to caveolae which are also enriched for adenylyl cyclase. Thus we propose that localization of the S1P1 receptor in caveolae enables it to inhibit adenylyl cyclase
while precluding it from activating Akt or MAP kinases, although all of these responses are regulated through coupling to G_{i}. Conversely, localization of S1P$_2$ and S1P$_3$ receptors outside of caveolae prevent these receptors from accessing adenylyl cyclase while allowing them to access the upstream activators of Akt and MAP kinases. Thus localization of S1P receptors is a critical factor in conferring their selectivity for downstream signaling pathways.
I

Introduction to sphingosine 1-phosphate and sphingosine 1-phosphate receptor mediated signaling

A. History of lysophospholipids and cell signaling

Lysophospholipids, such as lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), lysophosphatidylcholine (LPC), and sphingosylphosphorylcholine (SPC) are a minor lipid species similar in structure to the more abundant phospholipids phosphatidylserine, sphingomyelin, and phosphatidylcholine which comprise mammalian cell membranes. The lysophospholipids were originally thought to be involved in de novo synthesis of phospholipids although they were also known to exhibit signaling properties similar to those of growth factors. The mysterious signaling actions of sphingolipids were the reason that in 1884 J.L.W. Thudichum, the “Father of Neurochemistry,” named the sphingolipid backbone after the mythical Greek sphinx (44). The mechanistic basis for these signaling properties remained a mystery for many years until the discovery of the G-protein coupled receptors (GPCR) which transduce signals mediated by these lysophospholipids.

Sphingosine 1-phosphate has been studied for many years due to its ability to elicit cellular responses such as mobilization of intracellular calcium (19), cell proliferation (112), survival, and muscle contraction. It was thought that S1P was acting as an intracellular second messenger to elicit these responses although several findings such as the pertussis toxin (PTX) sensitivity of some S1P mediated responses ultimately suggested that it was not acting as an intracellular second messenger, but
instead through a receptor-mediated pathway and most likely a G-protein coupled receptor pathway. At the time this pathway was unknown, however, and it remained such until the mid 1990’s when several groups discovered a new family of GPCR’s for which S1P is the high affinity ligand (6; 37).

Experiments from over 40 years ago had shown that LPA could cause smooth muscle contraction and platelet aggregation (69). Several experiments with LPA offered clues into the possibility that it was acting through a GPCR. The actions of LPA such as proliferation, contraction, stress fiber formation and neurite retraction are cell type specific, GTP-dependent (84; 96), subject to desensitization, and blocked by suramin (39; 97), an antagonist of receptor-guanine nucleotide binding protein interactions and the S1P3 receptor in particular (5; 30). In addition the actions of exogenous LPA were not reproduced by microinjected LPA, suggesting that LPA functioned extracellularly at the membrane rather than as a second messenger (14; 15; 39; 40). Finally, several LPA-mediated signaling pathways, such as activation of phospholipase C (PLC) and inhibition of adenylyl cyclase, were shown to be inhibited by pertussis toxin, further suggesting a role for LPA signaling through a G1/o-mediated pathway.

B. Discovery of lysophospholipid receptors

In 1992 a putative LPA receptor was identified in the brain based on the ability of this receptor to bind to a labeled LPA analog (98). It was not until several years later, however, that the first LPA receptor was cloned from a neocortical neuroblast cell line. This receptor was named ventricular zone gene-1 (vzg-1) as the gene showed
an *in situ* expression pattern enriched in cortical neurogenic regions (26). After the cloning of this gene, ligand binding studies and functional assays confirmed that LPA was in fact the ligand for vzg-1. Vzg-1 encoded a seven transmembrane receptor as revealed by hydrophobicity data, and cells overexpressing this gene showed LPA-dependent cell rounding and inhibition of cAMP accumulation that was pertussis toxin sensitive. Receptor expression also increased binding of labeled LPA to the plasma membrane (18). Subsequently gene homology data and degenerate PCR were used to discover two additional receptors for which LPA is the ligand. These receptors were named Edg-4 and Edg-7.

Using homology data from vzg-1, three additional orphan GPCRs were discovered. The first of these genes was cloned in 1990 as an immediate-early gene induced during differentiation of human endothelial cells treated with phorbol 12-myristate 13-acetate (PMA). Thus this gene was named endothelial differentiation gene 1 (Edg-1)(29). Two groups deorphanized this receptor in 1998 by determining that the ligand for Edg-1 is in fact S1P (53; 113).

Subsequently S1P was found to be the ligand for several additional orphan GPCR’s. Edg-5 was discovered in the rat cardiovascular system in 1993 by degenerate PCR as an orphan GPCR with homology to Edg-1 and was given the name AGR16 (79). One year later, another group used a low stringency screening approach with a D2 dopamine reporter probe to discover the same orphan GPCR in the rat nervous system and they gave it the name H218 (61). This receptor was shown to have 58% sequence homology with Edg-1 (111) and in 1999 it was demonstrated by several
groups that S1P was in fact the high affinity ligand for Edg-5 ($K_d = 20-27\text{nM}$)(3; 21; 47; 104; 107; 111).

The third orphan receptor in this family, Edg-3, was isolated by degenerate PCR from a human genomic DNA library (108). This receptor shows 60% amino acid similarity to Edg-1 and it was subsequently shown that S1P is the high affinity ligand for this receptor ($K_d = 23-26 \text{nM}$)(47; 104).

Shortly thereafter, two additional members of this family were identified. Edg-8/nrg-1 (nerve growth factor regulated gene 1) was identified as an orphan GPCR in a screen for genes involved in differentiation of rat pheochromocytoma cells (20) and was determined to be a high affinity S1P receptor ($K_d = 2-10\text{nM}$) and low affinity SPC receptor (33; 34; 62). The final member of this family, Edg-6 was cloned in 1998 as an orphan GPCR in differentiated dendritic cells (23). It was found to be a high affinity S1P receptor ($K_d = 13-63 \text{nM}$) and a low affinity SPC receptor, (100; 109) although it shares the least amount of homology with other Edg receptors and it is possible that it could have a preferred higher affinity ligand other than S1P, such as phytosphingosine 1-phosphate (11).

The lysosphospholipid receptors cloned at this point had been found in several different species. These receptors had been used to show that S1P induced calcium dependent changes in cell morphology, induction of P-cadherin mRNA, increased MAPK activity, decreased cAMP accumulation, and Edg-1 receptor internalization in HEK293 cells or COS-7 cells transfected with human Edg-1(53; 113). S1P also stimulated serum-response element (SRE) activation and alterations in calcium flux (2) in cells transfected with rat H218 or human Edg-3. However, a significant advance
to the field was made with the cloning, in 1999, of the murine homologs of the three most common S1P receptors (111).

The murine genomic clones of the S1P receptors were isolated from a mouse genomic library (using sequences from human or rat Edg-1, H218/AGR16, and Edg-3) and subsequently renamed LpB1, LpB2, and LpB3 respectively. This nomenclature was used to signify that these genes were lysophospholipid receptors (Lp) class “B” for which S1P is the ligand as opposed to lysophospholipid class “A” receptors for which LPA is the ligand. In addition to the importance of finding these receptors in the mouse, this group also made a significant discovery by showing that these murine S1P receptors conferred S1P-specific responses by directly interacting with G-proteins (111). When LpB receptors were expressed in a cell line (RH7777) that does not express any endogenous S1P or LPA receptors, S1P, but not LPA treatment resulted in dose-dependent G-protein activation as detected by the use of GTP\(_\gamma\)S binding assays. Thus it was confirmed that LpB receptors are in fact GPCRs for S1P (111).

In addition to the discovery that these S1P receptors signal through G-proteins, the sequences of these receptors from the mouse offered numerous new opportunities which proved to be critical in the emergent field of S1P signaling. It was now possible to compare sequences within a single species, as well as to analyze expression patterns and even chromosomal location. Of additional importance was the ability to knockout these genes in a species such as the mouse which has well defined genetics. Furthermore, functional studies comparing the efficacy and ligand specificity of these receptors are most accurate when the clones are obtained from the same species. Numerous molecular, biochemical, cellular and genetic techniques have subsequently
been employed to determine the function of these receptors and to explain the cellular responses that had for many years been associated with LPA and S1P treatment.

C. Lysophospholipid Receptor Nomenclature

Due to the discovery of the lysophospholipid receptors by different groups in different species and cell types, the nomenclature remained very confusing. Until 2002 different names were still associated with the same gene and there was no consensus nomenclature for the field. In 2002, these receptors were renamed according to the International Union of Pharmacology (IUPHAR) nomenclature system, which dictates that the receptors be named according to the natural agonist with highest potency followed by subscript numerals for each additional member in this family. Thus the Edg-1 receptor was renamed S1P1, H218/AGR16 was renamed S1P2, and Edg-3 was renamed S1P3. Other lysophospholipid receptors were also renamed as Edg-6 is now known as S1P4, Edg-8 is now S1P5. For LPA receptors, Edg-2 is now known as LPA1, Edg-4 is now known as LPA2, and Edg-7 is now known as LPA3. The revised nomenclature is summarized in Table I.1 (12).

D. Characterization of Sphingosine 1-phosphate receptors

There are currently five receptors for which S1P is the high affinity ligand. It is now clear that activation of these receptors by S1P is the mechanism by which S1P is able to elicit the majority of its biological responses. All of these receptors are GPCRs which have the ability to couple to various G-proteins in order to activate specific signaling pathways. While differential G-protein coupling can explain
Table I.1: Lysophospholipid receptor nomenclature

<table>
<thead>
<tr>
<th>Agonist</th>
<th>IUPHAR Nomenclature</th>
<th>Edg Name</th>
<th>Previous Names</th>
</tr>
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<tbody>
<tr>
<td>LPA</td>
<td>LPA₁</td>
<td>Edg-2</td>
<td>Lpₐ₁, vzg-1, rec1.3</td>
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<tr>
<td>LPA</td>
<td>LPA₂</td>
<td>Edg-4</td>
<td>Lpₐ₂</td>
</tr>
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<td>LPA₃</td>
<td>Edg-7</td>
<td>Lpₐ₃</td>
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<tr>
<td>S1P</td>
<td>S1P₁</td>
<td>Edg-1</td>
<td>Lpₐ₁</td>
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<td>S1P</td>
<td>S1P₂</td>
<td>Edg-5</td>
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<td>S1P₅</td>
<td>Edg-8</td>
<td>Lpₐ₅, nrg-1</td>
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</table>
signaling pathways associated with the members of this receptor family, expression patterns are also important in regulating signaling pathways. Expression of S1P₁, S1P₂, and S1P₃ receptors is ubiquitous, while expression of S1P₄ and S1P₅ receptors is more limited. The characterization of the S1P receptors is detailed below.

1. S1P₁ Receptor

The S1P₁ receptor was the first identified member of the S1P receptor family. Biochemical and signaling data demonstrated that this receptor couples exclusively to the Gᵢ heterotrimeric G-proteins. Prior to the identification of S1P as the ligand for this receptor, a GST fusion protein of the third intracellular loop of S1P₁ was shown to immunoprecipitate with Gᵢ₁ and Gᵢ₃ in HEK cells as well as to associate with Gᵢ in human umbilical vein endothelial cells lysates (51). Subsequently S1P was found to be the ligand for this receptor (Kᵦ = 8nM) and further studies confirmed that the S1P₁ receptor signals through the Gᵢ protein (53; 113). S1P₁ receptor overexpression in a variety of cells, including HEK, CHO, RH7777, and Sf9, has been shown to result in the canonical Gᵢ-mediated response, inhibition of adenylyl cyclase activity (35; 47; 76; 101; 113). In addition S1P₁ overexpression results in pertussis toxin sensitive activation of ERK (53; 56) and calcium mobilization in some cell types (76; 77). Furthermore the S1P₁ receptor has been shown to activate phospholipase C (PLC) and the small GTPase Rac (46; 92). Finally in mouse embryonic fibroblast cells from S1P₂,₃ receptor double knockout mice, the S1P-mediated inhibition of forskolin-stimulated cAMP accumulation was not different from this response in WT cells. As
the only remaining S1P receptor in these S1P<sub>2,3</sub> receptor knockout cells is the S1P<sub>1</sub> receptor, one can conclude that S1P<sub>1</sub> receptor activation is the predominant mechanism for S1P-mediated inhibition of adenylyl cyclase.

While these data obtained from signaling downstream of the S1P<sub>1</sub> receptor are valuable and suggest signaling through G<sub>i</sub>, biochemical assays provided important confirmation that the S1P<sub>1</sub> receptor directly activates G<sub>i</sub> proteins. In the first approach a chimeric G<sub>ai</sub> protein was constructed in which the 4 carboxy terminal amino acids of G<sub>ai</sub>, which determine receptor specificity, were replaced by those residues from G<sub>aq</sub>. Expression of the S1P<sub>1</sub> receptor with native G<sub>aq</sub> in these cells did not lead to increases in intracellular calcium, however when the chimeric G<sub>ai/q</sub> was expressed with the S1P<sub>1</sub> receptor in Jurkat cells, S1P stimulation resulted in an increase in intracellular calcium. These data demonstrated that G<sub>i</sub>, but not G<sub>q</sub>, can interact with the S1P<sub>1</sub> receptor (3). In addition GTP<sub>γ</sub>S binding assays have demonstrated that the S1P<sub>1</sub> receptor promotes exchange of GDP for [<sup>35</sup>S]GTP<sub>γ</sub>S on G<sub>i</sub> proteins, but not G<sub>s</sub>, G<sub>q</sub>, G<sub>12</sub>, or G<sub>13</sub> (107). Thus several approaches have confirmed that the S1P<sub>1</sub> receptor interacts exclusively with G<sub>i</sub> proteins.

*In vivo*, this receptor has been shown to be expressed in many tissues including spleen, brain, heart, lung, liver, kidney, and skeletal muscle. Global knockout of the S1P<sub>1</sub> receptor was found to be embryonic lethal between E12.5 and E14.5 due to vascular abnormalities as a result of defective migration of vascular smooth muscle cells around blood vessels (58). Subsequently a conditional knockout of the S1P<sub>1</sub> receptor on endothelial cells was generated and showed the same phenotype as the
global S1P1 knockout, indicating that vessel maturation is regulated indirectly by S1P1 receptors on endothelial cells, rather than directly by S1P1 receptors on the smooth muscle cells (1). It is now well accepted that the S1P1 receptor is critical for maturation of the vasculature (36; 111).

2. S1P2 Receptor

The S1P2 receptor is also a GPCR, for which S1P is the high affinity ligand \(K_d = 20-27\text{nM}\), but unlike the S1P1 receptor, this receptor has the ability to signal through several different G-proteins. In binding assays the S1P2 receptor showed the ability to promote the exchange of GDP for \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) on \(G_i\), \(G_q\), \(G_{12}\), and \(G_{13}\), but not \(G_s\) in reconstituted Sf9 membranes (107). Functional data are in line with these results as the S1P2 receptor is able to activate many signaling pathways that are typically mediated downstream of these G-proteins. Overexpression of the S1P2 receptor can mediate activation of Rho (78), Rho-dependent stress fiber formation (21), and C3-sensitive activation of a serum response element that responds solely to serum response factor, suggesting that the S1P2 receptor couples to \(G_{12}\). Interestingly, this pathway has also been shown to inhibit Rac activation and thus offsets the pro-migratory effect of Rac activation elicited through activation of the S1P1 receptor. Similar to what is seen with heterologous expression of the S1P2 receptor, the endogenous S1P2 receptor has been shown to be the predominant S1P receptor involved in S1P-mediated activation of Rho, as Rho activation is severely compromised in S1P2 receptor knockout MEF cells. The S1P2 receptor has also been shown to activate mitogen activated protein kinases (MAPK) in a pertussis toxin
sensitive manner (21; 77), as well as to inhibit adenyl cyclase activity (88; 94; 103), suggesting that it couples to G\textsubscript{i}. However the endogenous S1P\textsubscript{2} receptor does not seem to mediate these responses as S1P\textsubscript{2} receptor knockout MEF cells do not show a decrease in S1P-mediated inhibition of forskolin-stimulated cAMP accumulation (38). Finally the S1P\textsubscript{2} receptor has been shown to increase inositol phosphate and intracellular calcium in a pertussis toxin insensitive manner (47; 48; 93), implying that it couples to phospholipase C through G\textsubscript{q}. However the S1P\textsubscript{2} receptor knockout MEF cells do not show a decrease in S1P stimulate PLC activation (38).

\textit{In vivo}, the S1P\textsubscript{2} receptor is expressed ubiquitously in the lung, heart, thymus, brain, liver, kidney, and spleen in the adult mouse although this expression pattern is likely altered during development (36; 111). S1P\textsubscript{2} receptor knockout mice initially showed no obvious phenotype and were born in the expected mendelian ratio although a slight, yet significant, decrease in litter size was noticed (38). More recently it was reported by two groups that the S1P\textsubscript{2} receptor knockout mice are deaf due to degeneration of vestibular and cochlear hair cells \textit{in vivo} (27; 60). Finally the S1P\textsubscript{2} receptor has also been shown to be critical in hepatic wound healing \textit{in vivo}.

3. S1P\textsubscript{3} Receptor

The S1P\textsubscript{3} receptor, like the S1P\textsubscript{2} receptor, couples to a variety of G-proteins but seems to be the primary G\textsubscript{q}-coupled S1P receptor. In heterologous systems, upon binding S1P, the S1P\textsubscript{3} receptor (K\textsubscript{d} = 23-26nM) triggers pertussis toxin insensitive increases in inositol phosphate and diacylglycerol production and subsequent increases in calcium production and protein kinase C activation (3; 47; 77) indicating coupling
to $G_q$. The endogenous S1P$_3$ receptor is also coupled strongly to $G_q$ as MEF cells from S1P$_3$ receptor knockout mice show a complete loss in S1P-induced PLC activation and calcium mobilization, but adenyly cyclase and Rho activation remain intact (36).

Overexpression of the S1P$_3$ receptor can also result in activation of the serum response element and serum response factor in a C3-sensitive manner (2; 4), suggesting coupling to $G_{12}$. This coupling is also suggested by the observation that S1P$_3$ antisense oligonucleotides block S1P-mediated increases in Rho activation and Rho-dependent stress fibers in endothelial cells (52; 81). The indirect data on G-protein coupling of the S1P$_3$ receptor gained from signaling observations have been confirmed by direct binding studies. In GTP$_\gamma$S binding assays, the S1P$_3$ receptor was shown to promote exchange of GDP for $[^{35}\text{S}]$GTP$_\gamma$S on $G_q$, $G_{12}$, $G_{13}$, and $G_i$, but not $G_s$ (107).

In vivo, S1P$_3$ receptor expression is ubiquitous. The S1P$_3$ receptor is present in spleen, lung, heart, thymus, kidney, testis, brain and skeletal muscle in the adult mouse (36; 111). S1P$_3$ receptor knockout mice are viable, born according to the expected mendelian ratio and lack any obvious phenotype. However deletion of both S1P$_2$ and S1P$_3$ receptors results in drastically smaller litter sizes. While the S1P$_2$ receptor appears to be the main pathway for activation of Rho, and the S1P$_3$ receptor remains the main pathway for phospholipase C activation, S1P$_2$ and S1P$_3$ receptors are expressed in many of the same tissues and can couple to the same subset of G-proteins. Thus it is not surprising that in many cases it appears that these receptors function redundantly, and that upon deletion of both receptors, there is increased penetrance of
the *in vivo* phenotypes seen when only the S1P_2 or S1P_3 receptor is deleted. These include loss of hearing (27; 60), and loss of cardioprotection against *in vivo* ischemia reperfusion (65).

4. **S1P_4 Receptor**

The S1P_4 receptor is another high affinity S1P receptor (K_d = 13-63 nM) but also a low affinity SPC receptor and shares the least homology with other S1P receptors (100; 109). In fact phytosphingosine 1-phosphate is the high affinity ligand for this receptor, showing a 50 fold greater affinity than S1P (K_d = 1.6 nM) (11; 13). S1P_4 has been shown to couple to G_i, G_{12}, G_{13}, and G_q much like many of the S1P receptors. Coupling to G_i is shown by S1P-induced pertussis toxin sensitive MAPK activation (100), G_{12/13} coupling is demonstrated by Rho activation and cytoskeletal rearrangement (24), and G_q coupling is inferred from the pertussis toxin insensitive increases in intracellular calcium and phospholipase C activation (24; 100; 109).

Expression of the S1P_4 receptor is much more limited than the other S1P receptors. In both the human and mouse, S1P_4 receptor expression is limited to lymph node, spleen, lung, and thymus. Not much is known about this receptor, although based on its expression pattern, it is likely to have a role in the immune system, possibly in migration of lymphocytes (24).

5. **S1P_5 Receptor**

The S1P_5 receptor is a high affinity S1P receptor (K_d = 2-10 nM) and a low affinity SPC receptor. This receptor is linked to G_i, G_{12}, and G_{13} but not to G_q or G_s.
Coupling to $G_i$ is demonstrated by pertussis toxin sensitive inhibition of adenylyl cyclase activity, although no S1P-mediated increase in MAPK activation was observed in CHO cells overexpressing the S1P$_5$ receptor, and in fact the opposite was observed as S1P$_5$-mediated inhibition of ERK activation (34; 62). Coupling to $G_{12}$ is suggested by activation of Rho (73), while the inability of this receptor to couple to $G_q$ is seen by no increases in PLC activation or intracellular calcium in RH7777 cells overexpressing the S1P$_5$ receptor (62). Finally, direct activation of G-proteins downstream of this receptor has been confirmed with binding assays which show that the S1P$_5$ receptor promotes exchange of GDP for $[^{35}S]GTP_{\gamma}S$ on $G_i$ and $G_{12}$ proteins but not $G_q$ or $G_s$ (62).

Expression of the S1P$_5$ receptor is restricted to the brain, spleen and skin in rats and mice (34; 36). Not much is known about the role of this receptor in vivo, but as this receptor is expressed in oligodendrocytes it likely plays a role in guiding the migration of oligodendrocyte precursor cells during brain development.

**E. Production of Sphingosine 1-phosphate**

S1P is produced by the enzyme sphingosine kinase when it phosphorylates its substrate, sphingosine, which is located in the plasma membrane. This reaction is reversible by action of the enzyme S1P phosphatase. In addition S1P can be irreversibly degraded by the enzyme S1P lyase into phosphoethanolamine and hexadecanal, and subsequently into the phospholipid phosphatidylethanolamine (50). The enzyme sphingosine kinase, of which there are two isozymes, can be activated by numerous growth factor and G-protein coupled receptors. Activation is associated with
Figure I.1: S1P receptor mediated signaling pathways. Reproduced with permission from Elsevier as it appears in: Anliker B, and Chun J. Cell surface receptors in lysophospholipid signaling. *Seminars in Cell & Developmental Biology* 15: 457-465, 2004
Figure I.2: Sphingolipid metabolism
sphingosine kinase translocation to the plasma membrane where it can phosphorylate sphingosine and produce S1P (90).

Large quantities of S1P are stored in platelets, mast cells, and red blood cells as these cells lack the S1P lyase that degrades S1P (110). Upon stimulation by phorbol ester, thrombin, or even inflammatory mediators, these cell types release large quantities of S1P which circulates in the blood bound to albumin and lipoproteins. Circulating concentrations of S1P can reach low micromolar levels in plasma, so binding to proteins such as albumin and lipoproteins is important for sequestering and transporting S1P. Recently by taking advantage of sphingosine kinase conditional knockout mice, which show no detectable S1P in plasma or lymph, it was determined that red blood cells, and not platelets or thrombocytes, are the source of plasma S1P. Furthermore, it was shown that there are two distinct pools of S1P; the radiation-sensitive hematopoietic source derived from bone marrow and a second radiation-insensitive source present in lymph nodes (82).

F. Sphingosine 1-phosphate receptor-independent signaling

Up until the early 1990’s it was assumed that the signaling actions of S1P were a result of its intracellular actions as a second messenger. It is now apparent that most of these signaling effects can be explained as responses to activation of plasma membrane S1P receptors. Nevertheless, there are still several examples of S1P-mediated receptor-independent signaling, although these are difficult to prove due to what has been termed “inside out” signaling (83; 102).
In “inside out” signaling, S1P is produced intracellularly by sphingosine kinase, where it may bind to unknown intracellular targets and function as a second messenger or alternatively, be exported outside of the cell where it may bind to cell surface S1P receptors in an autocrine/paracrine manner. The “inside out” signaling paradigm is supported by two observations: S1P can be produced intracellularly and S1P can be exported from the cell. The mechanism by which S1P is exported from the cell had remained elusive, as the polar head group of S1P clearly would not allow for this molecule to pass through the hydrophobic membrane barrier. Recently, however, it was shown that S1P is exported from cells by ATP binding cassette (ABC) transporters (68). Most, if not all, cells express sphingosine kinase which can be activated by various agonists such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), phorbol ester, acetylcholine, and even S1P to produce intracellular S1P (89). Consequently, the mechanism by which S1P could act in an autocrine/paracrine mechanism is feasible.

In order to confirm the role of S1P acting as a second messenger, one would need to eliminate the possibility of S1P acting in an “inside out” manner. Several experiments using sphingosine kinase to increase intracellular S1P support the notion that S1P acts as an intracellular second messenger. In one set of experiments it was shown that increasing intracellular S1P by overexpression of sphingosine kinase resulted in proliferation and inhibition of apoptosis. These experiments were carried out in mouse embryonic fibroblasts (MEFs) that normally express S1P1, S1P2, and S1P3 receptors. These same responses were shown to occur in pertussis toxin treated S1P2,3 receptor double knockout MEF cells (80). Based on the findings using cells
null for S1P₂ and S1P₃ receptors, and blocking signaling downstream of the Gᵢ
coupled S1P₁ receptor, the authors concluded that S1P signals intracellularly rather
than via its cell surface receptors.

In addition there are also data showing that intracellular S1P is involved in
calcium homeostasis, independent of S1P receptor activation. S1P has been shown to
be involved in regulating calcium levels in yeast and higher plants which do not
express S1P receptors (7; 72). S1P has also been shown to mobilize calcium in
reconstituted membranes that lack S1P receptors, and finally activation or
overexpression of sphingosine kinase has been shown to increase intracellular calcium
in an inositol trisphosphate (IP3)-independent manner (63; 99). This response from
activation or overexpression of sphingosine kinase was subsequently blocked by
sphingosine kinase inhibitors suggesting that S1P, but not sphingosine kinase, has
intracellular targets (66; 67). The idea of S1P acting as a second messenger,
independent of its receptors, is still very controversial as intracellular targets of S1P
have yet to be identified.

G. Sphingosine 1-phosphate in the cardiovascular system

S1P receptors were first discovered in the heart in 1995, a time at which they
were still orphan receptors (10). Subsequently several groups found that the adult
mammalian heart expressed S1P₁, S1P₂, and S1P₃ receptors (28; 64; 65). The S1P₅
receptor has also been detected at the mRNA level in rodent myocytes although this
was not seen in the whole heart. This disparity is most likely due to a very low level of
expression in the myocytes and even lower expression in other cardiac cell types
which diminish the signal when the whole heart is examined (55). Within the heart, expression of S1P1, S1P2, and S1P3 receptors appears to be ubiquitous. However relative expression levels of these receptors may vary widely among the various cardiac cell types, such as myocytes, endothelial cells, or smooth muscle cells (111). In addition to S1P receptors, the myocardium has been shown to express both isozymes of sphingosine kinase and S1P phosphatase (17; 32; 75; 105; 106). Finally, S1P laden blood has ample access to the myocardium. Thus the heart is rich in the components necessary for S1P-mediated signaling.

S1P is known to elicit numerous responses in the heart such as protection, changes in contractility, altered calcium handling, cell migration, angiogenesis, and proliferation of endothelial and smooth muscle cells. S1P increases intracellular calcium concentrations in both neonatal and adult rat cardiomyocytes (57; 71; 85). This latter effect in adult rat cardiomyocytes is mediated predominantly by the S1P1 receptor through an undetermined mechanism which is surprisingly insensitive to pertussis toxin treatment (71). Furthermore S1P1 receptor activation can antagonize β-adrenergic receptor-mediated increases in L-type calcium currents, demonstrating the inhibitory effect of S1P on L-type calcium channels (71). It is unclear whether these changes in intracellular calcium involve S1P-mediated changes at the ryanodine receptor. S1P has also been shown to reduce heart rate, predominantly through the S1P3 receptor (87) as well as to cause negative inotropy in vivo and in the isolated heart (25; 74; 91). Finally chronic S1P treatment of neonatal rat myocytes has resulted in cardiac hypertrophy through an S1P1 receptor-mediated pathway (86).
There is also a wealth of information demonstrating the protective role of S1P in the heart. The earliest studies showed that S1P could protect neonatal rat myocytes from hypoxia while the sphingosine kinase inhibitor, dimethylsphingosine, increased cell death. This increased death from dimethylsphingosine treatment was abolished when cells were treated with the sphingosine kinase activator, GM-1 (45). Recently experiments using sphingosine kinase knockout myocytes have demonstrated that export of intracellular S1P is able to protect myocytes from hypoxia and that this occurs through a receptor-mediated pathway (95). In the intact heart, it was shown that S1P infusion could protect the Langendorff-perfused heart from ischemia reperfusion induced damage, and furthermore it was found that sphingosine kinase is activated during ischemia and this activation is critical for ischemic preconditioning (41-43). Finally *in vivo* we have shown that S1P$_2$ and S1P$_3$ receptor activation is critical in protecting the heart from ischemia reperfusion injury, and propose that S1P is released during inflammation and serves to protect the heart by activating these receptors on myocytes (65). Furthermore, infusion of the high density lipoprotein HDL, which is rich in S1P, has also been shown to protect against ischemia reperfusion injury *in vivo* through an S1P$_3$ receptor-mediated pathway via activation of eNOS on endothelial cells. Thus S1P receptors on multiple cardiac subtypes likely contribute to S1P-mediated cardioprotection.

In the vasculature, S1P treatment causes vasoconstriction *in vivo* and *in vitro* (8; 9), although a concurrent change in blood pressure upon S1P administration is not always observed (9; 16). This response is replicated in endothelial cells, as S1P treatment results in vasoconstriction through an S1P$_1$ receptor-mediated pathway (31)
as well as migration through S1P₁ and S1P₃ receptor-mediated pathways (54; 70). The importance of the S1P₁ receptor in the heart and in endothelial cells may be suggested by the even greater expression levels of this receptor on endothelial cells than in other cardiac cell types, and in fact this receptor is critical for endothelial signaling as the S1P₁ receptor knockout mouse is embryonic lethal due to incomplete vascular maturation. A subsequent conditional endothelial cell S1P₁ receptor knockout has shown that this defect in vascular maturation is due to the absence of S1P₁ receptors on endothelial cells.

S1P also has numerous effects on cardiac fibroblasts. While myocytes are terminally differentiated and non-proliferative, cardiac fibroblasts, which compromise approximately 70% of the cells within the ventricle, are able to divide and migrate in response to S1P. While the S1P₁ receptor is the predominant subtype in cardiac myocytes and endothelial cells, the S1P₃ receptor is the most prevalent S1P receptor subtype in cardiac fibroblasts (49). As a result, these cells respond differently to S1P than other cardiac cell types. Adult cardiac fibroblast migration in response to growth factors is greatly reduced by S1P₃ receptor deletion. This effect is likely cell type-specific as other groups have shown that the S1P₁ receptor is critical for mouse embryonic fibroblast migration in response to growth factors (59) and that this growth factor mediated migration and proliferation is negatively regulated by the S1P₂ receptor (22). Nevertheless, one must pay careful consideration to the effects of S1P on fibroblasts when studying S1P-mediated responses in the heart. In the context of in vivo ischemia, S1P is protective against acute ischemic injury in what is likely due to signaling effects of cells residing in the myocardium, although prolonged S1P
exposure can have deleterious effects by allowing fibroblasts to migrate and proliferate in the heart, permitting increased inflammation, and even causing cell death if the S1P concentrations are too high (45).

Thus S1P has effects on numerous cell types within the myocardium and this S1P signaling is critical for numerous cardiovascular processes. Perturbation of S1P signaling in the heart should be carefully considered as there are numerous cell types within the heart that can respond quite differently to S1P stimulation. This dissertation will further examine the role of cardiac S1P receptors *in vivo* after ischemia reperfusion injury as well as signaling in isolated adult mouse myocytes *in vitro*. 
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Sphingosine 1-phosphate S1P₂ and S1P₃ receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury

A. Abstract

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 injury was examined using mice in which specific S1P receptor subtypes were deleted. Mice lacking either S1P₂ or S1P₃ receptors and subjected to 1 hr coronary occlusion followed by 2 hr of reperfusion developed infarcts equivalent to those of wildtype mice. However, in S1P₂,₃ receptor double knockout mice, infarct size following ischemia-reperfusion was increased by more than 50%. Ischemia-reperfusion leads to activation of ERK, JNK, and p38 MAP kinases however these responses were not diminished in S1P₂,₃ receptor knockout compared to WT mice. In contrast, activation of Akt in response to ischemia-reperfusion was markedly attenuated in S1P₂,₃ receptor knockout mouse hearts. Neither S1P₂ nor S1P₃ receptor deletion alone impaired ischemia-reperfusion induced Akt activation suggesting redundant signaling through these receptors and consistent with the finding that deletion of either receptor alone did not increase ischemia-reperfusion injury. The involvement of cardiomyocytes in S1P₂ and S1P₃ mediated activation of Akt was tested using cells from WT and S1P receptor knockout hearts. Akt was activated by S1P and this was modestly diminished in cardiomyocytes from S1P₂ or S1P₃ 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 ischemia-reperfusion damage in vivo and implicate release of S1P and receptor-mediated Akt activation in this process.

**B. Introduction**

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 (anoxia/reoxygenation) and by cytokines such as TNF\(\alpha\), 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 ischemia-reperfusion (I/R) in the heart (18). While 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 since 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<sub>1</sub> receptor knockout 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<sub>2</sub>, S1P<sub>3</sub>, or S1P<sub>2,3</sub> receptor knockout mice 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<sub>3</sub> receptors alone, Rho activation is regulated by both S1P<sub>2</sub> and S1P<sub>3</sub> receptors, and adenylate cyclase inhibition is regulated by S1P<sub>1</sub> receptors since this response is not lost in MEF cells from S1P<sub>2</sub>, S1P<sub>3</sub>, or S1P<sub>2,3</sub> receptor knockout mice (15;17).

Sphingolipid metabolites such as S1P and ceramide have been suggested to regulate cell survival. While 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 have been established, nor has an \textit{in vivo} protective role for endogenously released S1P been demonstrated.
Accordingly we designed experiments to examine the cell survival pathways regulated by S1P in cardiomyocytes, determine whether S1P receptor activation participated in the response to I/R injury \textit{in vivo}, and 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$_3$ receptor knockout mice but is markedly increased in S1P$_{2,3}$ receptor double knockout 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, while modestly diminished in S1P$_2$ and S1P$_3$ receptor knockout myocytes, is severely compromised in cardiomyocytes and \textit{in vivo} in hearts from S1P$_{2,3}$ receptor knockout 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.

C. Experimental Procedures

1. Animals

Generation and maintenance of S1P$_3$ receptor knockout mice (S1P$_3^{-/-}$), S1P$_2$ receptor knockout mice (S1P$_2^{-/-}$), and S1P$_{2,3}$ receptor double knockout mice (S1P$_{2,3}^{-/-}$) was previously reported (15;17). Animals had free access to water and food. All experiments reported here were performed using 24-28 week-old (25-35 g) mice of either sex. Wildtype littermate animals were used as controls for all experiments with S1P$_2$ or S1P$_3$ receptor knockout mice. For experiments with S1P$_{2,3}$ receptor double
knockout 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 wildtype mice of the same background as controls. All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

2. Reverse Transcription-Polymerase Chain Reaction

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 using Superscript reverse transcriptase (Invitrogen). PCR was performed with an initial denaturation step at 94°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 2 minutes and a final extension step at 72°C for 10 minutes. The following S1P receptor specific primers were used for PCR amplification. S1P1 5’-TCATCGTGCCGGCATACAAACTA-3’ and 5’-GAGTGAGCTTGTAGGTTG-3’. S1P2 5’-ATGGGCAGCTTGTACTCGGAG-3’ and 5’-CAGCCAGCAGACGATAAAGAC-3’. S1P3 5’-CTTGGTCTCTGCAGCTTTCACTC-3’ and 5’-TGCTGATGCAGAAGGCAATGTA-3’. S1P4 5’-TGAACATCAGCTGAGCAGCAG-3’ and 5’-GACGCTTGTAGGATTGAC-3’. Non reverse transcribed RNA was also amplified by PCR to check for contaminating genomic DNA.
3. Isolation of adult mouse cardiomyocytes

Cardiomyocytes were isolated from the hearts of 3 month old WT or S1P receptor knockout mice according to the method adapted from (46) by the Alliance for Cell Signaling (http://www.afcs.org). Briefly, animals were anesthetized with pentobarbital, and hearts were removed, cannulated and subjected to retrograde aortic perfusion at 37°C, at a rate of 3ml/min. Hearts were perfused for 4 minutes in Ca²⁺ free buffer, followed by 8-10 minutes of perfusion with 0.25mg/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 1mM and cells were plated on laminin coated dishes in MEM HBSS medium containing 5% serum. After one hour 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.

4. 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 (Ser473), 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).
5. Animal Model

Occlusion and reperfusion of the coronary artery was 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/minute using a rodent respirator (Model 683, Harvard). 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 an aid of a stereoscope (Nikon, Japan). PE-10 tubing (1-2 mm in length) was placed along the vessel as a cushion, and 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 hour of occlusion by removing the ligature and PE tubing.

6. Assessment of Area at Risk and Infarct Size

Following 2 hours of reperfusion, the LAD was re-occluded, and 5% Evans blue dye (0.2 ml) was injected into the LV cavity with a 27-gauge needle to define the non-ischemic zone. The heart was excised immediately, rinsed in saline to remove excess dye, and the LV was frozen and cut transversely into 5 slices of equal thickness. These samples were incubated in 1% 2,3,5-triphenyltetrazolium chloride
(TTC)-containing Tris-HCl buffer (pH 7.8) at 37°C for 10 minutes to stain the viable myocardium (brick red), and then fixed in 10% formalin-phosphate buffered saline for 24 hours. Each slice was weighed and photographed from both sides using a microscope equipped with a high-resolution digital camera (COOLPIX 990, Nikon). The area at risk, infarcted tissue, and the total LV area were measured by digital planimetry using computer software, NIH Image.

7. Statistical Analysis

All values are expressed as means ± SEM of n independent experiments. Statistical analysis was performed with unpaired t test for 2 groups and 1-way ANOVA followed by Dunnett’s test for 3 or more groups. A difference was considered statistically significant at P < 0.05.
D. Results

1. 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 S1P₁, S1P₂, S1P₃, and S1P₅ receptors were detected in both isolated cardiomyocytes and the whole adult heart (Figure II.1). S1P₄ receptors were not detected in either cardiac preparation, although S1P₄ receptor transcripts were detected in other tissues using the same primers (data not shown).

2. S1P₂ and S1P₃ receptors mediate protection from ischemia-reperfusion injury

Adult S1P₂, S1P₃, and S1P₂,₃ receptor knockout 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 if S1P receptors play a role in the response to ischemia-reperfusion (I/R) injury in vivo we compared WT and S1P receptor null mice after in vivo I/R, using a previously established model (44). Cardiomyocyte cell death in hearts exposed to one hour of coronary occlusion followed by two hours of reperfusion was assessed using TTC staining (described in Methods). A representative photomicrograph of a short-axis section from a WT mouse left ventricle is shown in Figure II.2A and the areas quantified to assess ischemic injury are delineated. Evans Blue Dye positive areas represent non-ischemic tissue, while the ischemic area (area at risk, AAR) is comprised of the white infarcted necrotic tissue (1) plus the red viable salvaged tissue (2).
We first compared WT and S1P3 receptor knockout 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 left ventricular (LV) mass (Figure II.2B). The infarct size, reflective of the amount of nonviable myocardium, was also not significantly different between the S1P3<sup>−/−</sup> and WT mice, whether expressed relative to AAR or total LV mass (Figure II.2B). We subsequently compared WT and S1P2 receptor knockout 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 the S1P3 receptor knockout mice, the size of the infarct relative to either AAR or LV was not significantly different between S1P2<sup>−/−</sup> and WT mice (Figure II.3). These data indicate that the loss of either the S1P2 or S1P3 receptor alone does not alter the *in vivo* response to I/R injury.

The S1P2 and S1P3 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 S1P2,3 receptor double knockout mice. As shown in Figure II.4, the areas at risk were not different in WT and S1P2,3<sup>−/−</sup> mice. Importantly, however, infarct size (expressed as a percentage of AAR) was increased by more than 50% in S1P2,3<sup>−/−</sup> compared to WT mice (Figure II.4). Infarct size expressed relative to LV mass was also significantly elevated. Thus combined activation of S1P2 and S1P3 receptors provides a protective signal during *in vivo* I/R that is lost in S1P2,3 receptor null mice.

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
heart rate was monitored by continuous electrocardiographic recording throughout the period of I/R. No differences in heart rate were observed amongst the groups of mice examined (data not shown), indicating that differences in chronotropic responsiveness do not underlie the altered susceptibility to injury.

3. MAP kinase activation pathways are not altered in S1P$_{2,3}^{-/-}$ mice subjected to in vivo ischemia-reperfusion

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 (Figure II.5A) as previously observed in isolated rat and rabbit hearts (4;29). Phosphorylation of ERK and JNK were not significantly increased during ischemia, but increased following reperfusion with the peak of activation occurring after 15 min of reperfusion (Figure II.5A), 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$_{2,3}^{-/-}$ versus 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$_{2,3}$ receptor double knockout versus WT mice (Figure
II.5B). Thus MAP kinase signaling during in vivo I/R is not compromised in the combined absence of the S1P$_2$ and S1P$_3$ receptors.

4. Ischemia-reperfusion 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 Ser473 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 (Figure II.6A). Akt phosphorylation was then compared in WT and S1P$_{2,3}$ receptor knockout mouse hearts following I/R. The 5 fold increase in phospho-Akt observed in WT mice was markedly attenuated (by ~70%) in the S1P$_{2,3}^{-/-}$ mice (Figure II.6B). 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$_3$ receptor knockout 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 mice lacking either S1P$_2$ or S1P$_3$ receptors alone was not significantly different from that of WT mice (Figure II.7).
5. 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. While 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, S1P2, S1P3, and S1P2,3 receptor knockout 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. (Figure II.8). While 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 S1P2 or S1P3 receptor knockout mice, while deletion of both S1P2 and S1P3 receptors resulted in a complete loss of S1P mediated Akt activation.

E. Discussion

Sphingosine is released from, and S1P 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 S1P2 and S1P3 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 in the S1P3 receptor knockout versus WT mice. Based on our previously published studies there is nearly complete loss of S1P-mediated phosphoinositide hydrolysis in MEF cells from S1P3 receptor knockout mice (15) and we also observe complete loss of S1P stimulated phosphoinositide hydrolysis in myocytes isolated from S1P3 receptor knockout myocytes (data not shown). Thus if elevated S1P elicits phospholipase C activation and generation of its downstream second messengers through S1P3 receptors in the ischemic myocardium, these responses do not appear to be required for S1P mediated protection.

MAP kinase pathways have also been implicated in control of cell survival in the myocardium. In vivo, all three MAP kinase pathways (ERK, JNK, p38) are activated by reperfusion following ischemia (Figure II.5)(7). However, neither ERK, JNK, nor p38 activation by I/R are impaired in the S1P2,3−/− mice. This finding suggests that the major pathways leading to MAP kinase activation in I/R are not initiated through stimulation of the S1P2 or S1P3 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 S1P2,3−/− 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 S1P2,3−/− mice. Smaller and insignificant decreases are affected by loss of either S1P2 or S1P3 receptors alone. Thus stimulation of both S1P2 and S1P3 receptors appears to contribute to activation of Akt \textit{in vivo}. The redundant or overlapping functions of these receptors in coupling S1P actions to phosphorylation of Akt is also seen in our \textit{in vitro} studies on isolated cardiomyocytes. Cardiomyocytes lacking either the S1P2 or S1P3 receptor demonstrate a partial loss in Akt phosphorylation, whereas there is complete loss of S1P-mediated phosphorylation of Akt in S1P2,3 receptor double knockout cardiomyocytes. That either SIP2 or SIP3 receptors can mediate Akt activation and concomitant cardioprotection further explains why I/R damage is not aggravated in mice lacking only S1P2 or only S1P3 receptors.

A surprising aspect of our studies is that the S1P1 receptor, still present in the S1P2,3 receptor knockout mice, does not confer greater protection against I/R injury. Akt activation by I/R is markedly diminished in the S1P2,3 receptor knockout mice despite the presence of the S1P1 receptor, shown in other systems to couple to Akt activation (3;25;33). The S1P1 receptor in adult mouse cardiomyocytes also couples poorly to this pathway as no Akt activation is observed in cells from S1P2,3 receptor knockout mice. The reason that the S1P2 and S1P3 receptors, but not the S1P1 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 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 S1P3 receptor (34). More recently it has been reported that administration of exogenous S1P is able to protect the heart from I/R injury via this S1P3 receptor mediated pathway (42). Interestingly our data indicate that in cardiomyocytes, Akt activation occurs predominantly through the S1P2 receptor and to a lesser extent through the S1P3 receptor. In vivo, we find that both the S1P2 and S1P3 receptors contribute to I/R induced Akt activation and an increase in ischemic damage is not seen unless both S1P2 and S1P3 receptors are deleted. Since 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 (eg. 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 S1P3 receptor activation of eNOS, via Akt, in endothelial cells, as well as S1P2 and S1P3 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 S1P2 or
S1P₃ receptors could therefore be novel therapeutic modalities for limiting the extent of cardiomyocyte loss associated with acute I/R injury in the heart.

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Figure II.1: Expression of S1P receptor mRNA in adult mouse cardiomyocytes and whole mouse heart. Reverse transcriptase (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 (+RT) and non-reverse transcribed (-RT) cDNA using receptor subtype specific primers. Sizes of PCR products are 270 base pairs (S1P1), 415 base pairs (S1P2), 617 base pairs (S1P3), and 305 base pairs (S1P5).
Figure II.2. Comparison of infarct size between wild-type and S1P3 receptor knockout mice after coronary occlusion followed by reperfusion (ischemia-reperfusion). (A) Representative photomicrograph of a short-axis of the left ventricle (LV) after 1 hour of coronary occlusion followed by 2 hours of reperfusion. Blue areas, non-ischemic 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 S1P3−/− mice and their corresponding wild-type mice (n=6 in each group) as described under Methods. Values are means ± SEM.
Figure II.3: Comparison of infarct size between wild-type and S1P₂⁻/⁻ mice following ischemia-reperfusion. Myocardial infarct size, area at risk (AAR) and LV size (LV) were calculated from S1P₂⁻/⁻ mice and their corresponding wild-type mice (n=6 in each group). Values are means ± SEM.
Figure II.4: Comparison of infarct size between wild-type and S1P$_{2,3}^{-/-}$ mice following ischemia-reperfusion. Myocardial infarct size, area at risk (AAR) and LV size (LV) were calculated from S1P$_{2,3}^{-/-}$ mice and their corresponding wild-type mice (n=11 in each group). Values are means ± SEM. *P<0.05 vs. wild-type group.
Figure II.5: Phosphorylation of MAP kinases during ischemia-reperfusion in wild-type and S1P\textsubscript{2,3}\textsuperscript{-/-} mouse hearts. Wild-type and S1P\textsubscript{2,3}\textsuperscript{-/-} hearts were subjected to 1 hour 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\textsubscript{2,3}\textsuperscript{-/-} mouse hearts at 15 minutes of reperfusion. Western blots were quantitated by densitometry. Values are means ± SEM (n=4-5 in each group). *P<0.05 vs. sham.
Figure II.6: Phosphorylation of Akt during ischemia-reperfusion in wild-type 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 at 15 minutes of reperfusion. Western blots were quantitated by densitometry. Values are means ± SEM (n=4-5 in each group). *P<0.05 vs. sham.
Figure II.7: Phosphorylation of Akt during ischemia-reperfusion in wild-type, S1P2^−/−, and S1P3^−/− mouse hearts. Wild-type, S1P2^−/−, and S1P3^−/− hearts were subjected to 1 hour ischemia, 15 minutes reperfusion, and extracted proteins were analyzed by Western blotting. A comparison of Akt phosphorylation in these hearts after 1 hour ischemia and 15 minutes reperfusion. Western blots were quantitated by densitometry. Values are means ± SEM (n=4 in each group). There was no significant difference (p>0.05) between Akt activation by IR in WT, S1P3^−/− and S1P2^−/− hearts.
Figure II.8: S1P-mediated phosphorylation of Akt in wild-type, S1P3−/−, S1P2−/−, and S1P2,3−/− adult mouse cardiomyocytes. Cardiomyocytes were stimulated with S1P (5 µM) for 5 minutes and then assayed for phosphorylation of Akt by Western blotting. Phosphorylation was quantitated by densitometry and normalized to vehicle controls of each genotype. Values are means ± SEM (n≥5 in each group). *P<0.05 vs. vehicle.
G. References


S1P receptor localization confers selectivity for G_{i} mediated signaling pathways

A. Abstract

Adult mouse ventricular myocytes (AMVMs) express S1P_{1}, S1P_{2}, and S1P_{3} receptors. S1P activates Akt and ERK in AMVMs through a pertussis toxin sensitive (G_{i/o}-mediated) pathway. In S1P_{3} receptor knockout myocytes Akt and ERK activation by S1P are reduced by 35% and 25% respectively. In S1P_{2} receptor knockout myocytes Akt and ERK activation by S1P are each reduced by approximately 60%. With combined S1P_{2,3} receptor knockout, activation of Akt is abolished and ERK activation is reduced by nearly 90%. Thus the S1P_{1} receptor which is still present in S1P_{2,3} receptor double knockout myocytes is unable to mediate Akt or ERK activation in response to S1P. S1P treatment leads to pertussis toxin sensitive inhibition of isoproterenol-stimulated cAMP accumulation in WT myocytes. This response was unchanged in S1P_{2,3} receptor double knockout myocytes demonstrating that the S1P_{1} receptor in these cells is functional and couples to G_{i}. SEW2871, an S1P_{1} receptor selective agonist, also decreased isoproterenol-stimulated cAMP accumulation but this agonist failed to activate either ERK or Akt. To explore the possibility that localization of the S1P_{1} receptor and its effectors is important in mediating this signaling specificity, the effect of the caveolae disrupting drug, methyl-β-cyclodextrin (MβCD) was examined. MβCD treatment did not affect S1P mediated activation of ERK or Akt but fully abolished S1P and SEW2871 mediated inhibition of cAMP accumulation. The S1P_{1} receptor was concentrated in the caveolar fraction and this
localization was disrupted by MβCD treatment. Taken together, the data indicate that localization of S1P1 receptors to caveolae is required for the ability of this receptor to inhibit adenylyl cyclase, but at the same time compromises the coupling of this receptor to Akt and ERK pathways.

B. Introduction

The lysophospholipid, sphingosine 1-phosphate (S1P), regulates numerous cellular responses including survival, proliferation, migration, angiogenesis, and actin cytoskeletal rearrangements (6; 18; 42). These responses are mediated through binding of S1P to its cognate cell surface G-protein coupled receptors. There are currently 5 receptors for which S1P is the high affinity ligand. Upon binding S1P, these receptors activate a variety of G-proteins of the Gi, Gq, and G12,13 families of heterotrimeric G-proteins to generate second messengers that elicit a range of cellular responses. S1P1, S1P2, and S1P3 receptors are expressed in numerous tissues and have been studied extensively, whereas expression of S1P4, and S1P5 receptors is limited to a few select tissues and their function is less clearly understood.

The S1P1 receptor has been shown to signal exclusively through the heterotrimeric G-protein, Gi, based on biochemical and signaling data. Binding assays with [35S]GTPγS have shown that in Sf9 and HEK cells expressing the S1P1 receptor, S1P promotes the exchange of GDP for GTP only on Gi proteins, but not on Gs, Gq, G12, or G13 (51). In addition S1P1 receptors expressed on HEK, Sf9, and COS cells have been shown to activate ERK (27; 32; 53) and inhibit forskolin-stimulated cAMP accumulation (32; 50; 53). The fact that all of these S1P1 receptor mediated responses
were completely abolished by pertussis toxin (PTX) treatment further corroborates the role of G\textsubscript{i} signaling downstream of the S1P\textsubscript{1} receptor.

While the S1P\textsubscript{1} receptor couples exclusively to G\textsubscript{i} proteins, the S1P\textsubscript{2} and S1P\textsubscript{3} receptors are more promiscuous in coupling to the G\textsubscript{i}, G\textsubscript{q}, and G\textsubscript{12/13} families of heterotrimeric G-proteins. Coupling of S1P\textsubscript{2} and S1P\textsubscript{3} receptors to these G-proteins has been confirmed by GTP\textsubscript{γ}S binding assays and analysis of the signaling pathways downstream of these receptors links them to G\textsubscript{i}, G\textsubscript{q}, and G\textsubscript{12/13} proteins (51). S1P\textsubscript{2} receptor overexpression has been shown to activate ERK in a PTX sensitive manner (32), as well as to increase inositol phosphate hydrolysis (25) and activate Rho (33), suggesting coupling of the receptor to G\textsubscript{q} and G\textsubscript{12} as well as G\textsubscript{i}. Furthermore, endogenous levels of the S1P\textsubscript{2} receptor have been shown to be important for the majority of S1P-mediated Rho activation in MEF cells as these cells isolated from S1P\textsubscript{2} receptor knockout mice show greatly diminished S1P-mediated Rho activation (19).

In a similarly promiscuous nature, overexpression of S1P\textsubscript{3} receptors has been shown to increase intracellular calcium, inositol phosphate formation (25; 32), and Rho activation (33) while inhibiting adenylyl cyclase (32), once again suggesting coupling to G\textsubscript{q}, G\textsubscript{12}, and G\textsubscript{i}. Furthermore, in MEF cells from the S1P\textsubscript{3} receptor knockout mice, we have shown that endogenous levels of the S1P\textsubscript{3} receptor are required for the majority of S1P-mediated inositol phosphate hydrolysis and a minor fraction of the S1P-mediated activation of Rho.

While heterologous expression systems have revealed much about the biochemistry of the S1P receptors and which pathways these receptors can signal
through, it is less clear which pathways are activated by the endogenous receptors. The lack of subtype specific agonists and antagonists, as well as poor receptor antibodies, has made it difficult to study the function of each endogenous receptor subtype. Thus the use of mice in which individual S1P receptors have been deleted provides a powerful tool for investigating the signaling downstream of discrete S1P receptor subtypes (17; 20).

We have previously shown that S1P₁, S1P₂, and S1P₃ receptors are present in the heart and that activation of S1P₂, and S1P₃ receptors contributes to protection from ischemia reperfusion injury in vivo (29). Others have also shown a role for S1P in protecting myocytes and the heart from hypoxia and ischemia (4; 21; 22), as well as in regulation of heart rate (38). Thus S1P signaling plays an important role in the heart and understanding the signaling pathways activated by the individual receptor subtypes on myocytes is critical to understanding the regulatory role played by these receptors in controlling cardiovascular responses.

C. Materials and methods

1. Reagents

Sphingosine 1-phosphate was obtained from Avanti polar lipids. SEW2871 and pertussis toxin were obtained from Calbiochem. Methyl-β-cyclodextrin, isoproterenol, and carbachol were purchased from Sigma. Phospho Akt, phospho ERK, phospho JNK, total Akt, total ERK, and total JNK antibodies were purchased from Cell Signaling Technologies and the caveolin-3 and S1P₁ receptor antibodies were purchased from Abcam. The cAMP enzyme immunoassay was purchased from
GE Healthcare. QPCR primers and reagents were purchased from Applied Biosystems.

2. Animals

Generation and maintenance of S1P3 receptor knockout mice (S1P3<sup>-/-</sup>), S1P2 receptor knockout mice (S1P2<sup>-/-</sup>), and S1P2,3 receptor double knockout mice (S1P2,3<sup>-/-</sup>) was previously reported (17; 19; 20). Animals had free access to water and food. All experiments reported here were performed using 8-16 week-old male mice. Wildtype littermate animals were used as controls for all experiments with S1P2 or S1P3 receptor knockout mice. For experiments with S1P2,3 receptor double knockout 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 wildtype mice of the same background as controls. All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

3. Quantitative PCR

Total RNA was isolated from WT and S1P receptor knockout myocytes using the RNeasy kit and converted to cDNA as previously reported (29). Resulting myocyte cDNA was used for quantitative PCR analysis with Taqman gene specific primers for S1P1, S1P2, and S1P3 receptors and glyceraldehyde 3-phosphate dehydrogenase using an ABI7500 system. Values for comparison of a single gene across genotypes were determined using cycle threshold (Ct) data fitted to a standard curve. For comparison
of multiple transcripts in a single sample, equal amplification efficiency of primers was confirmed and then the $2^{-\Delta\Delta Ct}$ method was applied to the Ct values (28). Data represents triplicates for each primer set (in single genotype, multiple gene studies) or triplicates for each genotype (in multiple genotype, single gene studies).

4. Immunoblot analysis

For western blotting studies, cells were stimulated with 5µM S1P or 5µM SEW2871 and then lysates were prepared as described previously (29). For studies with Methyl-β-cyclodextrin (MβCD), cells were treated with 1mM MβCD for 1 hour prior to stimulation. Equal amounts of total protein were loaded as determined by a Bradford assay.

5. cAMP enzyme immunoassay

Myocytes were treated with isobutylmethylxanthine (500µM) for 10 minutes, followed by 5-15 minutes with either S1P (1µM), SEW2871 (1µM), or carbachol (30µM), and then stimulated with 1mM isoproterenol for 10 minutes. Cells were lysed and assay was performed according to kit protocol. Results were obtained by fitting data to a standard curve and then normalizing to total protein per sample. For Methyl-β-cyclodextrin studies, cells were treated with 1mM MβCD for 1 hour prior to the start of the assay.

6. Isolation of adult mouse cardiomyocytes
Cardiomyocytes were isolated from the hearts of 2-4 month old WT or S1P receptor knockout mice according to the method adapted from (13; 52) by the Alliance for Cell Signaling (http://www.afcs.org). Briefly, animals were anesthetized with pentobarbital, and hearts were removed, cannulated and subjected to retrograde aortic perfusion at 37°C, at a rate of 3ml/min. Hearts were perfused for 4 minutes in Ca\(^{2+}\) free buffer, followed by 8-10 minutes of perfusion with 0.25mg/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 1mM and cells were plated on laminin coated dishes in MEM HBSS medium containing 5% serum. After one hour, cells were washed and serum free medium was added back. Cells remained in serum free medium overnight (31) and cellular responses were measured the next day.

7. Preparation of caveolar fractions

Caveolar fractions were isolated from cardiomyocytes using an alkaline, detergent-free procedure (40). Cardiomyocytes from a 10cm dish were scraped into 1ml carbonate buffer (150mM sodium carbonate pH 11, 1mM EDTA), lysed by passing through a 23 gauge needle 10 times, sonicated, mixed with 1ml 80% sucrose in MBS (25mM MES, pH 6.5, 150mM NaCl, 2mM EDTA) and loaded into the bottom of a 12 ml ultracentrifuge tube. Next 6 ml of 35% sucrose was layered on top of the lysate, and finally 4 ml of 5% sucrose was layered on top. Tubes were spun at
4°C for 3 hr at 39,000 rpm in a SW41 swinging bucket rotor. Ten fractions, each 1.2 ml in volume were removed. Equal volumes of each fraction were analyzed by western blotting.

D. Results

1. Pertussis toxin sensitivity of S1P mediated Akt and MAP Kinase activation

Adult mouse ventricular myocytes (AMVMs) were pretreated with 100 ng/ml pertussis toxin (PTX) overnight and then stimulated with 5 μM S1P for 5 minutes. Activation of ERK, JNK, and Akt was assessed by western blotting with phospho specific antibodies. S1P stimulation resulted in a 5 fold increase in ERK phosphorylation, a 4 fold increase in JNK phosphorylation, and a 1.6 fold increase in Akt phosphorylation relative to vehicle control (Figure III.1). After pertussis toxin treatment, S1P-mediated activation of ERK was reduced by 70%, activation of JNK was reduced by 90%, and activation of Akt was reduced by 77%, indicating that a significant component of S1P-mediated Akt and MAP kinase activation is mediated by a G_i-coupled S1P receptor (Figure III.1).

2. S1P mediated activation of ERK, JNK, and Akt in WT and S1P receptor knockout myocytes

WT or S1P receptor knockout myocytes were stimulated with S1P (5μM) for 5 minutes and activation of ERK, JNK, and Akt was assessed by western blotting. ERK activation, relative to WT cells, was reduced by 25% in S1P_3 receptor knockout myocytes, by 60% in S1P_2 receptor knockout myocytes, and by 88% in S1P_2,3 receptor
double knockout myocytes. JNK activation was reduced by 25% in S1P3 receptor knockout myocytes and by 75% in both S1P2 and S1P2,3 receptor double knockout myocytes. Finally, Akt activation was reduced by 35% in S1P3 receptor knockout myocytes, by 65% in S1P2 receptor knockout myocytes and fully inhibited in S1P2,3 receptor knockout myocytes (Figure III.2). Thus nearly all S1P-mediated Akt and MAP kinase activation is abolished in S1P2,3 receptor double knockout myocytes, indicating that the S1P1 receptor, which is still present in the S1P2,3 receptor double knockout myocytes, is not a major mediator of these S1P-promoted responses. This is particularly surprising since the S1P1 receptor has been shown to couple exclusively to Gi, which mediates these responses (Figure III.1).

3. Relative expression of the S1P1 receptor mRNA in WT and S1P receptor knockout myocytes

To determine whether S1P1 receptor expression was altered in the S1P2,3 receptor double knockout myocytes, levels of S1P1, S1P2, and S1P3 receptor mRNA were assessed by quantitative PCR (QPCR). Based on mRNA levels the S1P1 receptor is the predominant type of receptor in WT myocytes, with the S1P2 and S1P3 receptor mRNA being expressed at much lower levels (Figure III.3A). S1P1 receptor mRNA expression is 12 fold higher than that of the S1P2 receptor, and the S1P3 receptor mRNA is expressed at over 2 fold the level of the S1P2 receptor. Levels of S1P1 receptor mRNA expression were not different in WT, S1P2, S1P3, or S1P2,3 receptor double knockout myocytes (Figure III.3B). Thus it is unlikely that loss of S1P1
receptor expression accounts for the markedly attenuated Akt and ERK responses in S1P$_{2,3}$ receptor double knockout myocytes.

**4. S1P mediated inhibition (via G$_i$ coupling) of isoproterenol-stimulated cAMP accumulation in WT and S1P$_{2,3}$ receptor double knockout myocytes.**

The question of whether the S1P$_1$ receptor is active in the myocyte cell membrane was then addressed. The canonical response regulated through the alpha subunit of G$_i$ is inhibition of adenylyl cyclase activity. The ability of S1P to inhibit isoproterenol-stimulated cAMP accumulation was assessed using an enzyme immunoassay. Isoproterenol (1uM) stimulation for 10 minutes led to a 6 fold increase in cAMP accumulation (data not shown). Addition of S1P (1uM) or carbachol (30uM) five minutes prior to isoproterenol stimulation resulted respectively in 35% and 65% reductions in cAMP accumulation. This response was identical in WT and S1P$_{2,3}$ receptor double knockout myocytes, indicating that the inhibition of adenylyl cyclase activity, in contrast to the activation of Akt or ERK, is mediated exclusively through the S1P$_1$ receptor (Figure III.4). The involvement of G$_i$ in this response was verified as PTX treatment completely blocked S1P-mediated inhibition of isoproterenol-stimulated cAMP accumulation (Figure III.4). Thus S1P$_1$ receptors are intact and fully coupled to G$_i$ in S1P$_{2,3}$ receptor double knockout myocytes.

**5. SEW2871 and S1P mediated inhibition of isoproterenol-stimulated cAMP accumulation in WT myocytes**
The ability of the S1P₁ receptor to inhibit isoproterenol-stimulated cAMP accumulation in the S1P₂,₃ receptor double knockout myocytes was confirmed by using the S1P₁ receptor specific agonist SEW2871. Stimulation with 1μM SEW2871 resulted in a 30% decrease in isoproterenol-stimulated cAMP accumulation, an effect comparable to that seen when S1P was added in parallel experiments (Figure III.5). This finding provides additional evidence that the S1P₁ receptor mediates adenylyl cyclase inhibition in cardiomyocytes.

6. Inability of SEW2871 to activate ERK or Akt

The effect of SEW2871 on Akt and ERK activation was then examined. WT myocytes were stimulated with SEW2871 (5μM) for 15 minutes and ERK and Akt activation were assessed by western blotting. Neither ERK nor Akt phosphorylation was increased by SEW2871 stimulation (Figure III.6), although this agonist elicited Gᵢ- mediated inhibition of cAMP accumulation at this concentration and timepoint (as shown in Fig 5). As a positive control for ERK and Akt activation, these cells were stimulated with S1P for 15 minutes; significant increases in the activation of both ERK and Akt were observed. Additional times of SEW 2871 treatment were also tested (5-30 min) and failed to show ERK or Akt activation (data not shown).

7. Methyl-β-cyclodextrin treatment blocks S1P- and SEW2871-mediated inhibition of isoproterenol-stimulated cAMP accumulation

It is not clear why the cardiomyocyte S1P₁ receptor, which clearly couples to Gᵢ, cannot signal through this G-protein to activate ERK or Akt. We hypothesized the
existence of compartments, specifically that S1P₁ receptors might be localized to caveolae. To test this hypothesis, WT myocytes were treated with 1mM methyl-β-cyclodextrin (MβCD) for 1 hour to disrupt caveolae. Cells were then stimulated with S1P (1µM), SEW2871 (1µM), or carbachol (30µM) for 15 minutes, followed by addition of isoproterenol using the same protocol as in the experiments shown in figure III.4 and III.5. Isoproterenol elicited a greater than 10 fold increase in cAMP accumulation and carbachol retained its ability to decrease isoproterenol stimulated cAMP accumulation by 60% (Figure III.7). In contrast, neither S1P nor SEW2871 were able to inhibit cAMP accumulation following MβCD treatment (Figure III.7).

8. Methyl-β-cyclodextrin treatment does not block S1P mediated activation of ERK or Akt

Since treatment with MβCD clearly affected S1P-mediated inhibition of adenylyl cyclase, we examined the effect of this treatment on the ability of S1P to activate Akt and ERK. WT myocytes were treated with 1mM MβCD for 1 hour prior to 5 minutes of S1P stimulation and then activation of ERK and Akt was measured by western blotting. MβCD increased basal ERK activation. This effect is consistent with previous findings showing enhanced basal ERK activation in MβCD- or caveolin siRNA-treated cells (11) or in caveolin knockout myocytes (7). Importantly, however, MβCD treatment did not block the ability of S1P to further activate ERK. Additionally, MβCD treatment lowered basal Akt activation but the extent of the response, in terms of the effect of S1P on Akt was unchanged (Figure III.8). The lack
of effect of MβCD on S1P-mediated Akt and ERK activation contrasts sharply with its disruptive effect on S1P-mediated inhibition of cAMP formation.

9. **S1P₁ receptor localizes to caveolar fractions**

Caveolar fractions were isolated from WT myocytes and the presence of the S1P₁ receptor was tested by western blotting. A band of ~47kd, corresponding to the expected size of the S1P₁ receptor, was detected in fractions 4 and 5. This band was not seen when the S1P₁ receptor antibody was preincubated with a blocking peptide for the S1P₁ receptor. Caveolin-3 was also enriched in these same fractions, thus confirming the presence of the S1P₁ receptor in caveolae. In addition, treatment of myocytes with 1mM MβCD for 1 hour prior to caveolar fractionation resulted in a redistribution of caveolin-3 and the S1P₁ receptor from their characteristic locations in fractions 4 and 5 into a more even distribution in fractions 4-10.

E. **Discussion**

Sphingosine 1-phosphate signaling pathways have been under intense investigation in numerous systems since the cloning of the first S1P receptor subtypes nearly ten years ago. However due to a deficiency in pharmacological tools, much of the research investigating the role of these receptors has relied upon biochemical or heterologous expression techniques to analyze S1P receptor signaling. We recently published data showing that activation of endogenous S1P₂ and S1P₃ receptors provides protection against ischemic injury in the heart and showed that this response was mediated at least in part by S1P receptors on cardiomyocytes (29). There is
additional evidence in the cardiovascular system showing that activation of S1P receptors on myocytes or endothelial cells can protect the heart from ischemic damage (48). In the current study we aimed to determine which signaling pathways occur downstream of endogenous S1P receptor subtypes using a differentiated system of adult mouse ventricular myocytes. We find that the endogenous S1P receptor selectivity for signaling pathways is considerably different from what is seen in heterologous expression systems and we show that as a result of compartmentation, the S1P1 receptor is much less promiscuous than was previously proposed and in fact only signals to a limited subset of Gi-mediated pathways.

The data presented here reveal a surprising finding about the S1P1 receptor. It is well accepted that this receptor couples exclusively to the Gi family of heterotrimeric G-proteins (26; 51). In addition, it is well documented that MAP kinase and Akt activation occur via activation of Gi-coupled GPCR’s (2; 24; 35; 44) and the use of pertussis toxin in the present study has confirmed a role for Gi-mediated responses in activation of these pathways in myocytes (5; 14). What our studies demonstrate, using myocytes from S1P receptor knockout mice, is that the major Gi coupled S1P receptor subtype, S1P1, cannot mediate activation of ERK or Akt in cardiomyocytes.

A possible explanation for absence of S1P mediated ERK or Akt activation in the S1P2,3 receptor knockout myocytes may reside in the loss of interactions between S1P receptors and other growth factor receptors such as PDGF or EGF receptors (46). However there is no reason to think that the ability of the S1P1 receptor to engage these receptors would be altered by deletion of either S1P2 or S1P3 receptors. It is also
possible, although not yet demonstrated, that multiple S1P receptor subtypes may be necessary for mediating certain responses, though the data from SEW2871-treated WT myocytes argues against this idea. When WT myocytes, which do not have altered S1P receptor expression, are stimulated with the S1P1 agonist, SEW2871, no activation of ERK or Akt is seen, indicating that the absence of S1P2 or S1P3 receptors is not the reason why S1P1 is unable to couple to these responses. Thus data from both genetic deletion of S1P receptors and pharmacological tools indicate that the S1P1 receptor in cardiomyocytes is unable to activate Akt or MAP kinases.

In order to look for activity downstream of the S1P1 receptor we measured the classical Gι-mediated response, inhibition of adenylyl cyclase activity (1; 47). S1P has been documented to inhibit cAMP accumulation in cells overexpressing the S1P1 receptor (25; 53), and studies on S1P receptor knockout MEF cells demonstrate that S1P is still able to inhibit forskolin-stimulated cAMP accumulation in S1P2,3 receptor double knockout cells (19). Our data confirm that the S1P1 receptor is capable of inhibiting isoproterenol-stimulated cAMP accumulation through activation of Gι, even though it cannot couple to Akt or ERK activation.

Membrane organization has been proposed as a mechanism of regulating cell signaling by localizing receptors, G-proteins and effectors (30) and more recently the S1P1 receptor was found to be localized in caveolae (16). Caveolae, or caveolin containing invaginations of the plasma membrane have been well studied as membrane domains heavily enriched in signaling components (37; 39) and thus we sought to determine if localization of S1P receptors might determine their coupling to downstream signaling pathways. The pharmacological agent MβCD has been shown
to disrupt caveolae by depleting cellular cholesterol (10) and this has been documented in myocytes (23; 36). Since the S1P$_1$ receptor inhibits cAMP accumulation and caveolae are known to be enriched in adenylyl cyclase (3; 12; 15), we asked whether the coupling between this receptor and effector requires caveolar compartmentation. In myocytes treated with MβCD both S1P- and SEW2871-mediated inhibition of isoproterenol stimulated cAMP accumulation were completely abolished. Remarkably, S1P was still able to activate ERK and Akt after MβCD treatment, indicating that S1P-mediated activation of ERK and Akt does not require or occur in caveolae.

The experiments with MβCD provide indirect evidence for S1P$_1$ receptor localization and signaling in caveolae. To directly determine whether the S1P$_1$ receptor was localized to caveolae we used a detergent free alkaline lysis method to isolate caveolae (40) from myocytes (34). Fractions were immunoblotted to examine localization of the S1P$_1$ receptor and caveolin-3, the muscle specific isoform of caveolin (41). Similar to what was observed with endothelial cells overexpressing the S1P$_1$ receptor (16), we observe that the endogenous S1P$_1$ receptor is localized to caveolae in myocytes. Furthermore MβCD treatment was shown not only to disrupt caveolin-3 but also S1P$_1$ receptor localization to the 5% and 35% sucrose gradient interface where caveolae are typically found. Thus we have confirmed that S1P$_1$ receptors are localized to caveolae and confirmed that signaling through adenylyl cyclase occurs in this compartment.

Our data using cells from KO mice indicate that ERK and Akt are activated almost exclusively through S1P$_2$ and S1P$_3$ receptors (Figure III.2). The lack of effect
of MβCD on these responses indicates that they are not confined to the caveolar compartment, although this cannot be directly determined due to lack of adequate antibodies. What remains to be determined is why activation of S1P₁ receptors and Gᵢ in caveolae does not lead to activation of ERK or Akt, and why activation of S1P₂ and S1P₃ receptors and Gᵢ outside of caveolae does not contribute to inhibition of adenylyl cyclase located within caveolae. One possible explanation is that different Gᵢ subunits are involved in these responses. Inhibition of the major isoforms of cyclase in the heart (ACV/VI) is known to be mediated by Gᵢ subunits (45), while activation of MAP kinases and Akt is thought to occur through Gᵦ subunits (8; 9; 43; 44; 49). While the Gᵦ subunits may be mobile, the membrane linked Gᵦ subunits downstream of the caveolar S1P₁ receptor may be unable to access upstream effectors of ERK or Akt activation such as PI3 kinase. Conversely activated Gᵦ subunits, downstream of S1P₂ and S1P₃ receptors, may be unable to reach ACV/VI confined within caveolae. Interestingly even after treatment of cells with MβCD to disrupt caveolae and redistribute the S1P₁ receptor, SEW2871 stimulation does not result in activation of ERK or Akt. Thus non-caveolar S1P receptor mediated signaling pathways must also be organized such that redistribution the S1P₁ receptor out of caveolae is not sufficient to activate these pathways.

Since the discovery of the first S1P receptor nearly ten years ago, much research has been done using heterologous expression systems to determine which G-proteins interact with S1P receptor subtypes and which pathways are activated downstream of individual S1P receptors. While the foundation for S1P signaling has
been laid, this present research, focused on endogenous S1P receptor subtypes in differentiated adult cells, provides new insights into the specificity of these receptors in regulating signaling pathways. Our data show that S1P receptor signaling is more specialized than was once thought. Moving forward, understanding which S1P receptors are involved in certain common cellular responses and signaling pathways will be critical to determining whether pharmacological modulation of these receptors is appropriate and which receptors might be targeted to obtain a beneficial response.

F. Acknowledgements

This chapter is material in preparation for submission to the Journal of Biological Chemistry. Christopher K. Means and Joan Heller Brown “S1P receptor localization confers selectivity for G_i mediated signaling pathways.” The dissertation author was the primary investigator in the development and execution of the study, and is the primary author of this manuscript. This research was supported by a predoctoral fellowship from the American Heart Association (CKM).
Figure III.1: Pertussis toxin sensitivity of S1P mediated activation of ERK, JNK, and Akt in WT adult mouse myocytes. WT myocytes were stimulated with 5µM S1P for 5 minutes and then assayed for phosphorylation of ERK, JNK, and Akt by western blotting. Representative blots are shown. Phosphorylation was quantitated by densitometry and normalized to vehicle. Values are means ± SEM (n>5 for each group). *P<0.05 vs. vehicle.
Figure III.2: S1P mediated activation of ERK, JNK, and Akt in WT, S1P\textsubscript{3}\textsuperscript{-/-}, S1P\textsubscript{2}\textsuperscript{-/-}, and S1P\textsubscript{2,3}\textsuperscript{-/-} adult mouse myocytes. Myocytes were stimulated with 5µM S1P for 5 minutes and then assayed for phosphorylation of ERK, JNK, and Akt by western blotting. Phosphorylation was quantitated by densitometry and normalized to vehicle of each genotype. Values are means ± SEM (n>5 for each group). *P<0.05 vs. vehicle, †P<0.05 vs. WT S1P treatment.
Figure III.3: Quantitative PCR analysis of S1P receptor mRNA expression in WT and S1P receptor knockout myocytes. A. Relative expression of S1P₁, S1P₂, and S1P₃ receptor mRNA in WT mouse myocytes. Results were calculated using $2^{\Delta \Delta Ct}$ method. n>4 for each probe set. B. Expression levels of S1P₁ receptor mRNA in WT, S1P₂⁻/⁻, S1P₃⁻/⁻, and S1P₂,₃⁻/⁻ mouse myocytes. Results were calculated using the standard curve method. n>4 for each genotype.
Figure III.4: S1P mediated inhibition (via G$_i$) of isoproterenol stimulated cAMP accumulation in WT and S1P$_{2,3}^{-/-}$ myocytes. WT and S1P$_{2,3}^{-/-}$ myocytes were treated with IBMX for ten minutes followed by S1P (1µM), carbachol (30µM), DMSO (control for SEW2871) or media (control for S1P) treatment for 5 minutes and then stimulated with isoproterenol (1µM) for 10 minutes. For pertussis toxin, cells were treated overnight with pertussis toxin and the remainder of the assay was performed as above. Cells were lysed and the amount of cAMP was determined by ELISA. Amount of cAMP in vehicle treated sample was subtracted from all other samples and then amount of cAMP in isoproterenol only treated sample was normalized to 100%. All other treatments are expressed as a percent of isoproterenol (Ctrl) stimulated cAMP accumulation. Values are means ± SEM (n>6 for each group). *P<0.05 vs. control.
Figure III.5: S1P and SEW2871 mediated inhibition of isoproterenol stimulated cAMP accumulation in WT myocytes. WT myocytes were treated with IBMX for 10 minutes, followed by 15 minute stimulation with S1P (1µM), SEW2871 (1µM), media (control for S1P), or DMSO (control for SEW2871, 1:2000), and then stimulated with isoproterenol (1µM) for 10 minutes. Cells were lysed and the amount of cAMP was determined by ELISA. Amount of cAMP in vehicle treated sample was subtracted from all other samples and then amount of cAMP in samples only treated with isoproterenol were used to normalize the other results. All other treatments are expressed as a percent of isoproterenol (Ctrl)-stimulated cAMP accumulation. Values are means ± SEM (n>6 for each group). *P<0.05 vs. control.
Figure III.6: S1P and SEW2871 mediate Akt and ERK activation in WT myocytes. WT myocytes were stimulated with S1P (5µM), SEW2871 (5µM), vehicle (media control for S1P), or DMSO (control for SEW2871, 1:2000) for 15 minutes and then assayed for phosphorylation of Akt and ERK by western blotting. Representative blots are shown. Phosphorylation was quantitated by densitometry and normalized to vehicle. Values are means ± SEM (n>4 for each group). **P<0.05 vs. vehicle.
Figure III.7: Methyl-β-cyclodextrin treatment blocks S1P and SEW2871 mediated inhibition of isoproterenol-stimulated cAMP accumulation. WT myocytes were treated with methyl-β-cyclodextrin (1mM) for 1 hour followed by 500µM IBMX treatment for 10 minutes, and then S1P (1µM), SEW2871 (1µM), media (control for S1P), or DMSO (control for SEW2871, 1:2000) for 15 minutes. Finally cells were stimulated with isoproterenol (1µM) for 10 minutes. Cells were lysed and amount of cAMP was determined by ELISA. Amount of cAMP in vehicle treated sample was subtracted from all other samples and then amount of cAMP in the isoproterenol only treated sample was normalized to 100%. All other treatments are expressed as a percent of isoproterenol (Ctrl)-stimulated cAMP accumulation. Values are means ± SEM (n>6 for each group). *P<0.05 vs. control.
Figure III.8: Methyl-β-cyclodextrin treatment does not block S1P mediated activation of ERK or Akt. WT myocytes were treated with methyl-β-cyclodextrin (1mM) for 1 hour prior to stimulation with S1P (5µM) for 5 minutes and then assayed for phosphorylation of ERK and Akt by western blotting. Representative blots are shown. Phosphorylation was quantitated by densitometry and normalized to vehicle. Values are means ± SEM (n>4 for each group). *P<0.05 vs. vehicle.
Figure III.9: Localization of the S1P$_1$ receptor in caveolar fractions. WT myocytes were lysed in detergent free bicarbonate buffer and prepared by centrifuging on a discontinuous 40%/35%/5% sucrose gradient. Equal volumes of each fraction were analyzed by western blotting. For methyl-β-cyclodextrin experiments, cells were treated with methyl-β-cyclodextrin (1mM) for 1 hour prior to fractionation. Fraction 1 corresponds to the top fraction while fraction 10 corresponds to the bottom fraction.
G. References


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IV

Implications/Future directions

Over the last decade, tremendous strides have been made in understanding S1P signaling. Initial gains were made using molecular and biochemical techniques to understand the receptors. However, due to a lack of pharmacological agonists/antagonists and receptor antibodies, there is still a deficiency in understanding how these receptors function. More recently S1P related research has tried to circumvent the lack of useful pharmacological reagents by using S1P receptor knockout mice which allow one to better define the function of these endogenous receptors in vitro and in vivo.

In our earlier studies we demonstrated a role for activation of S1P$_2$ and S1P$_3$ receptors in protecting the heart against in vivo ischemia reperfusion injury. While the increased infarct size in the S1P$_{2,3}$ receptor double knockout heart is concomitant with decreased Akt activation, we cannot conclusively conclude that loss of Akt activation is the downstream cause of the increased damage. Akt is a well known mediator of cardioprotection, but the mechanisms underlying this protection are not well understood. Although we did not test this, we postulate that eNOS activation may be the next step in this Akt mediated protection, as Akt is known to phosphorylate eNOS and eNOS knockout mice show increased ischemia reperfusion damage. Other pathways which may be involved in S1P-mediated cardioprotection include MAP kinase and Rho activation. We concluded that MAP kinase activation is not involved in this response as neither ERK, JNK, nor p38 activation was changed, relative to WT
mice, in S1P$_{2,3}$ receptor double knockout mice which have an increased infarct size. However we have not examined Rho activation and what role it may play in S1P-mediated cardioprotection from ischemia reperfusion injury. Understanding this mechanism of S1P-mediated cardioprotection will be critical in being able to determine whether pharmacological intervention is appropriate in protecting or preventing ischemic injury in the heart.

Our infarct studies were very acute and further studies should be carried out to more completely determine the effect of S1P receptor loss on long term responses to ischemia. Thus our initial results may reflect S1P and Akt induced short term changes in the heart, but one also needs to consider the longer term effects of S1P as inflammatory mediators and other cell types migrate into the heart. While S1P protects the terminally differentiated myocytes, it causes proliferation and migration of other cell types such as fibroblasts. Consequently, while S1P may be protective in the short term, prolonged or sustained ischemia induced S1P signaling in the heart may result in deleterious effects similar to what is seen in a fibrotic heart. These concerns could be addressed with longer term infarct studies looking at infarct size, functional recovery, fibrosis, or the inflammatory response in the S1P receptor knockout mice.

One novel approach being employed to study the time-dependent role of S1P in cardiac signaling and pathophysiology *in vivo* is the use of an S1P sequestering antibody. While the S1P receptor knockout mice allow for the complete and permanent disruption of a receptor and signaling downstream of that receptor, one cannot subsequently restore these pathways. On the contrary, the sequestering antibody should allow for temporary inactivation of S1P signaling at specific times.
and subsequent full restoration of these pathways upon removal of the antibody. While some are skeptical about the utility of this antibody, future discoveries may yield more pharmacological inhibitors which will allow for more thorough and specific analysis of the role that S1P plays in pathophysiology \textit{in vivo}. Thus while we have chosen to remove the receptor, a different approach would remove the ligand. A final approach to examine the role of S1P signaling in the pathophysiology of ischemia reperfusion \textit{in vivo} would involve subjecting the sphingosine kinase knockout mice to \textit{in vivo} ischemia reperfusion. Mice null for sphingosine kinase 1 and sphingosine kinase 2 show nearly undetectable levels of circulating S1P in their blood and as a result S1P receptors should not be activated during ischemia reperfusion. Consequently, these mice should allow one to determine the role of S1P receptor activation in the pathophysiology of ischemia reperfusion injury. Together, these complementary approaches will yield the greatest amount of information about the role of S1P signaling in protecting the heart from ischemic injury.

Another question to be resolved is what cell types or tissues are involved in the increased damage that we observe in the S1P$_{2,3}$ receptor double knockout mice after \textit{in vivo} ischemia reperfusion. The S1P receptor knockout mice used in this study are global knockouts, thus altered responses could be the result of loss of S1P signaling in any number of tissues, including various cell types within the myocardium. To address this issue, cardiac-specific S1P receptor knockout mice may be of value, or on a more basic level, \textit{in vitro}, a Langendorff system of ischemia reperfusion could be used to demonstrate the local effects within the heart as this system employs an isolated heart which is closed off to other systems.
The Langendorff model has been used for many years to study the isolated perfused heart. While our ischemia reperfusion model is extremely powerful since it is performed \textit{in vivo} and the endogenous S1P is released naturally, the Langendorff model could still be of value in answering several questions. The Langendorff model is advantageous as it studies an isolated heart and as a result does not involve cell types or responses that originate from outside the heart. Additionally pharmacological agents can be added or removed in known quantities and at desired times in the isolated heart. These studies would be challenging to perform \textit{in vivo} as one does not know how much, if any, of the agent reached the heart and one could not rule out secondary effects of the agent acting on other non cardiac systems. Thus the isolated perfused heart could be a useful system to answer appropriate questions.

While we have shown that the S1P$_2$ and S1P$_3$ receptors mediate protection from \textit{in vivo} ischemia reperfusion, the role of the S1P$_1$ receptor in mediating this protection is not known. It is possible that the S1P$_1$ receptor also mediates cardioprotection \textit{in vivo} as this receptor couples to G$_i$ pathways and subsequent activation of ERK and Akt in many cell types. However, without an S1P$_1$ receptor knockout mouse, we are unable to address this question. Alternatively, this issue could be addressed with the Langendorff system perfused with S1P or the S1P$_1$ agonist, SEW2871.

In the \textit{in vivo} ischemia reperfusion protocol, we observe that the ligand for the S1P$_2$ and S1P$_3$ receptors is supplied naturally. However a major question in the field of S1P-mediated cardioprotection involves the source of S1P. It is not known with certainty whether S1P is produced locally within the heart, or whether it enters the
heart, via circulation, during reperfusion. These studies may be addressed once more with the isolated perfused heart model. We already know that following in vivo ischemia reperfusion, the S1P$_{2,3}$ receptor double knockout hearts show an increased infarct size and decreased Akt activation in comparison to WT hearts. In the Langendorff system, the ischemia reperfusion could be done with and without perfusing exogenous S1P. If the infarct sizes are comparable between WT and S1P$_{2,3}$ receptor double knockout hearts in the absence of perfused S1P, then one might conclude that in vivo, S1P is supplied from outside the heart. A larger infarct size in the S1P$_{2,3}$ receptor double knockout heart might indicate the contrary, that S1P is normally released locally from within the heart during ischemia or reperfusion. If S1P is in fact supplied from outside the heart, perfusion with S1P should restore protection to the WT heart while having a much lesser, or even no, effect on the S1P$_{2,3}$ receptor double knockout heart.

Another conclusion that these studies demonstrate is the potential limitations of heterologous systems. In overexpression systems, many of the S1P receptors will couple to common responses such as MAPK and PLC activation or inhibition of cAMP accumulation. However looking at MEF cells or primary myocytes we see that this is not the case. Thus one should be careful to extrapolate data from heterologous expression systems to the endogenous protein. The difference could be a consequence of altered localization, artificial interactions, or changes in stoichiometry elicited via overexpression, in addition to the cell type specificity. Thus in terms of physiological significance, it would seem more valuable to infer signaling from gene knockout or cellular knockdown systems than it is to do so with overexpression systems, provided
one is alert to the shortcomings associated with knockdown such as compensation by other endogenous proteins.

While there are many S1P receptors that can couple to the same subset of G-proteins, it appears that there is less redundancy between receptors than was originally perceived. There are multiple levels of regulation such as receptor expression in various tissues and some selectivity for coupling to certain G-proteins, but we show here that localization of receptors or G-proteins within cellular compartments, in particular caveolae, is also another important regulatory factor. This may not have been realized previously as overexpression can alter or mask localization of endogenous proteins. Antibodies against transmembrane proteins are notoriously difficult to work with, especially for the detection of endogenous proteins, and those commercially available antibodies against the relatively new S1P receptor family are no exception. Thus it has only been possible to examine localization by overexpressing epitope tagged receptors.

Our studies suggest that localization of the endogenous S1P1 receptor within caveolar compartments enhances signaling to inhibit cAMP accumulation while precluding this receptor from coupling to other common Gi mediated pathways. This was not expected from previous data generated by heterologous systems. While we could not show the converse for the S1P2 and S1P3 receptors due to nonspecific receptor antibodies, it is an important finding which illustrates the significance of localization and scaffolding in regulating signaling. With the development of improved antibodies, subcellular localization of S1P2 and S1P3 receptors may be possible. However rather than using fractionation studies to analyze subcellular
localization, microscopy may yield even more important findings to answer why certain S1P receptors prefer to couple to particular downstream second messengers. These questions must be addressed at the single cell level to reveal spatiotemporal regulation of S1P mediated signaling pathways.

These studies here have advanced our understanding of endogenous S1P receptor signaling in a primary differentiated cell type which is more closely related to cells *in vivo* than the immortalized cell lines commonly used for heterologous expression. When combined with complementary approaches such as overexpression systems, study of these cells provides the best approach to understanding cell signaling *in vitro*. Ultimately, conclusions gained from our *in vitro* experiments have contributed to our understanding about how these receptors function in cardiomyocytes *in vivo*.

The future goal of these studies is to translate biological discoveries into medical therapies. In order to do so we need to understand the function of all these receptors at the organ and whole animal level. Our studies are some of the early ones to address the role of S1P receptors in the heart *in vivo*, and we have complemented these by analyzing signaling downstream of endogenous S1P receptors in primary adult mouse cardiomyocytes. However further studies need to be conducted to determine when and where S1P receptors might be modulated in order to obtain a beneficial cardiovascular outcome. Furthermore, one needs to determine which S1P receptor(s) would be the best target for therapeutic intervention. Development of new compounds with specificity and selectivity for particular S1P receptor subtypes will be advantageous for further advancement of this field.
Appendix

A. Transverse aortic constriction induced hypertrophy mediated by the S1P\textsubscript{3} receptor in vivo

1. Introduction

Myocardial hypertrophy is an adaptive response to increased hemodynamic load. In this process the heart enlarges by increasing cardiomyocyte size and contractile protein content which in the short term results in increased cardiac function by reducing wall stress (8). The causes of this increased load can include both mechanical and hormonal stimuli, and if left uncorrected this adaptive hypertrophy will progress into dilated hypertrophy and the heart will fail by decompensation (3).

A number of circulating, locally released factors have been demonstrated to cause cardiac hypertrophy as a result of binding to and activating G\textsubscript{q} coupled G-protein coupled receptors. These factors include angiotensin II, endothelin-I, norepinephrine, phenylephrine, and prostaglandin F\textsubscript{2α} (1; 12; 19; 22-24). Overexpression of G\textsubscript{αq} in cultured neonatal rat ventricular myocytes results in hypertrophic growth, and even more sustained G\textsubscript{αq} activity that is achieved with a constitutively active G\textsubscript{αqQ209L} mutant results in hypertrophy and eventual cell death by apoptosis (2; 3). Similar results are seen \textit{in vivo} as G\textsubscript{q} transgenic mice exhibit hypertrophy and peripartal stress induces a lethal cardiomyopathy in G\textsubscript{αq} transgenic mice (3; 10). These findings were confirmed by another constitutively active G\textsubscript{αq}
transgenic mouse made by a separate group which also showed dilated cardiomyopathy, albeit more delayed in onset, but also a lack of correlation between PLC activity and dilated cardiomyopathy, suggesting the involvement of other signaling pathways in this phenotype (15). These responses are associated with increases in p38 MAP kinase and JNK activity, both of which have been implicated as apoptotic mediators in other systems. While overexpression of G\(_{aq}\) induces a modest increase in p38 and JNK activity, expression of the constitutively active G\(_{aQ209L}\) mutant leads to more significant increases in p38 and JNK activation. Similarly, \(\text{in vivo}\), p38 and JNK activity were significantly increased in the peripartum period in G\(_{aq}\) transgenic mice (3). Involvement of G\(_{aq}\) has been further confirmed as injection of a G\(_{aq}\) neutralizing antibody resulted in inhibition of \(\alpha1\) adrenergic receptor mediated cardiomyocyte hypertrophy (13). Finally a necessary role for G\(_{q}\) in hypertrophy was confirmed by experiments using transgenic mice expressing a minigene peptide inhibitor of G\(_{aq}\) as well as G\(_{q/11}\) knockout mice. G\(_{aq}\) minigene inhibitor transgenic mice and G\(_{q/11}\) knockout mice both resisted transverse aortic constriction-induced hypertrophy, and the G\(_{aq}\) minigene inhibitor transgenic mice were also shown to resist phenylephrine and angiotensin II induced hypertrophy (4; 26).

While agonists such as phenylephrine and endothelin are known mediators of hypertrophy, little is known about what role S1P might play in cardiac hypertrophy. Two studies have shown that S1P causes hypertrophy in neonatal rat ventricular myocytes (16; 20). These studies showed that S1P stimulation resulted in cardiac hypertrophy as measured by enhanced cytoskeletal reorganization and increases in
phenylalanine incorporation, MAPK, Akt, and P70S6K activation, BNP secretion and ANF expression (16). In addition these studies showed that S1P-mediated hypertrophy is mediated in part by both G\textsubscript{i} and Rho pathways as pertussis toxin and C3 toxin partially inhibited the effects of S1P on phenylalanine incorporation and cytoskeletal organization. The hypertrophic effects of S1P were also shown to occur in part downstream of the S1P\textsubscript{1} receptor as blocking antibodies against the extracellular regions of this receptor reduced S1P-mediated phenylalanine incorporation while blocking antibodies against an intracellular region of the S1P\textsubscript{1} receptor showed no effect in inhibiting this response (16). While these studies use custom antibodies designed against the S1P\textsubscript{1} receptor to imply a role for the S1P\textsubscript{1} receptor in mediating hypertrophy, they fail to show that this antibody specifically detects the S1P\textsubscript{1} receptor and does not nonspecifically interact with other S1P receptor subtypes. To date all studies examining the role of S1P in cardiac hypertrophy have been performed \textit{in vitro} and it remains to be determined whether S1P is involved in hypertrophy \textit{in vivo}.

As S1P has been implicated in cardiac hypertrophy \textit{in vitro} and G\textsubscript{q} signaling is a known mediator of hypertrophy \textit{in vitro} and \textit{in vivo}, we wished to determine whether the G\textsubscript{q} activation by S1P receptors is involved in cardiac hypertrophy \textit{in vivo}. Our previous work had shown the S1P\textsubscript{3} receptor is efficiently coupled to G\textsubscript{q} signaling pathways as mouse embryonic fibroblast cells from the S1P\textsubscript{3} receptor knockout mice showed a near complete loss in S1P stimulated inositol phosphate production which was restored by adding back the S1P\textsubscript{3} receptor to these cells (11). Thus we hypothesized that the S1P\textsubscript{3} receptor might also be a strong G\textsubscript{q} coupled receptor in adult mouse cardiac myocytes and that deletion of this receptor would abolish S1P
induced G_q stimulation *in vivo*. Accordingly we examined the effect of S1P_3_ receptor deletion on the hypertrophic response to transverse aortic constriction (TAC).

2. Results/Discussion

*In vivo*, hypertrophy can be artificially induced by transverse aortic constriction (TAC). During this procedure, the mice are anesthesized with ketamine and xylazine and subsequently placed on a ventilator in a supine position. The chest cavity is opened and a suture is placed around the transverse aorta and constricted by approximately 70% in order to generate a pressure gradient. The mice are allowed to recover, and over time this pressure gradient causes pressure overload induced cardiac hypertrophy (17; 18).

S1P_3_ receptor knockout mice and the corresponding age-matched WT mice were subjected to 7 days of transverse aortic constriction (TAC) after which the hearts were removed and the ratio of left ventricle to body weight was determined as a measure of hypertrophy. For sham conditions, surgery was performed as for TAC conditions but the suture was not constricted. These experiments were performed by Yusu Gu of the Seaweed Canyon Cardiovascular Physiology Laboratory at UCSD.

WT and S1P_3_ receptor knockout mice subjected to sham conditions showed left ventricle to body weight (LV/BW) ratios of 0.35% and 0.36% respectively. TAC treatment of WT mice significantly increased the LV/BW ratio to 0.61%. In contrast S1P_3_ receptor knockout mice showed a LV/BW ratio of only 0.50% after TAC. The LV/BW ratio difference between TAC treated S1P_3_ receptor knockout mice and TAC treated WT mice was considered statistically significant with p value of <0.01,
implicating a role for S1P in cardiac hypertrophy. In addition these data indicate that S1P acts via the S1P3 receptor to produce a considerable proportion of the TAC-induced cardiac hypertrophy. The fact that the S1P3 receptor knockout mice still displayed a significant degree of hypertrophy, as assessed by the LV/BW ratio, is likely the result of the actions of other hypertrophic mediators such as angiotensin, VEGF, epinephrine, or norepinephrine acting through their own canonical receptor mediated pathways or S1P acting on other S1P receptor subtypes.

To consider the signaling pathways by which S1P could cause hypertrophy, we isolated myocytes from the S1P3 receptor knockout mice and analyzed S1P-mediated signaling in these cells. As S1P is known to activate Gq-mediated pathways, such as inositol phosphate hydrolysis, and Gq pathways are known to be involved in cardiac hypertrophy both in vitro and in vivo, this appeared to be a plausible mechanism to explain the in vivo results.

S1P-mediated Gq signaling was assessed in WT and S1P3 receptor knockout myocytes by measuring inositol phosphate hydrolysis, as determined by the ratio of S1P-stimulated inositol monophosphate (IP1) normalized to total inositol. We chose to measure the ratio of IP1/total inositol as an endpoint rather than total inositol phosphates (IP’s) as Dr. Elizabeth Woodcock had previously shown that no changes in total IP’s were seen in S1P-stimulated neonatal rat myocytes, but changes in IP1 could be detected due to the much smaller background of IP1.

Myocytes were loaded overnight with 12uCi/ml of [3H] inositol and stimulated the following day with 1µM S1P for 30 minutes in the presence of 10mM LiCl. In WT myocytes S1P stimulation lead to a 30% increase in IP1 accumulation. Endothelin was
a more efficacious agonist, yielding a 2.5 fold increase in inositol phosphate formation. However in S1P3 receptor knockout myocytes, S1P was unable to stimulate IP1 accumulation while endothelin still yielded a 2.5 fold increase. Thus Gq signaling is still present in S1P3 receptor knockout myocytes as indicated by the response to endothelin, however S1P mediated Gq signaling appears to be abolished in S1P3 receptor knockout myocytes.

In other studies described in chapter 3, we show that the S1P3 receptor is also capable of activating Akt and ERK, two known mediators of hypertrophy. Several groups have shown that overexpression of an activated Akt in the heart results in cardiac hypertrophy (6; 25) and expression of a myristoylated or phosphomimetic Akt in the heart results in even greater hypertrophy (14; 21). The necessity of Akt in cardiac hypertrophy was tested with Akt knockout mice which show blunted exercise-induced hypertrophy but normal growth in response to TAC, demonstrating that Akt can function as a positive regulator of physiological hypertrophy and negative regulator of pathological hypertrophy (7). A role for ERK in hypertrophy is demonstrated from studies showing that transgenic mice expressing cardiac specific MEK exhibit cardiac hypertrophy (5), while transgenic mice expressing a dominant negative RAF1, an upstream activator of MAP kinases, show decreased ERK activation and attenuated cardiac hypertrophy in response to TAC (9).

However, in Figure III.2 we show that the S1P3 receptor only contributes to a minor portion of Akt and ERK activation as S1P3 receptor knockout myocytes show decreases of 35% and 25% in S1P stimulated Akt and ERK activation respectively.
Thus it is also possible that the decreased Akt and ERK activation are also involved in
the decreased cardiac hypertrophy seen in TAC treated S1P3 receptor knockout hearts.

While S1P mediated $G_q$ signaling is abolished in S1P3 receptor knockout
myocytes in vitro, and this is correlated with the decreased hypertrophy in vivo after
TAC, several questions still remain. Could this small phosphatidylinositol (PI) signal
actually contribute to the in vivo hypertrophy response. It seems unlikely that the 30%
increase in S1P-mediated inositol phosphate production could be responsible for such
a significant increase in hypertrophy given that there are numerous other non-S1P
mediated hypertrophic pathways. This conundrum could possibly be explained by
localization. Much as $G_i$ mediated signaling downstream of the S1P1 receptor may be
explained by subcellular localization, it is possible that the S1P3 receptor is localized
near certain effectors such as PLC and while S1P only stimulates a small increase in
activity it may be a much greater signal if it could be measured locally. These pools
may allow this signal to be focused and concentrated on certain pathways involved in
hypertrophy, thus allowing for the S1P3-mediated hypertrophy seen in vivo. It is also
possible that amplification of this rather modest PI response could allow for the
significant changes in TAC induced hypertrophy. Finally while the S1P3 receptor
plays only a minor role in activation of Akt and ERK, we cannot disregard the
possible role that these known hypertrophic pathways, in conjunction with the PI
response, may play in regulating cardiac hypertrophy in vivo.

Future studies subjecting the other S1P receptor knockout mice to TAC may
yield further information about the mechanism of S1P-induced hypertrophy in vivo.
Our data show that the S1P3 receptor is the primary receptor involved in S1P-mediated
PLC activity. Thus S1P2 receptor knockout myocytes should have intact S1P-induced PI responses and if PLC activation is involved in hypertrophy, these mice should not show reduced TAC induced cardiac hypertrophy in comparison to WT mice. However the S1P2 receptor contributes a much larger amount of the S1P-mediated Akt and ERK activation and if these molecules are involved in the TAC-induced hypertrophy, the S1P2 receptor knockout mice would show decreased hypertrophy relative to TAC treated WT hearts. Finally S1P2,3 receptor knockout mice may be subjected to TAC as the remaining cardiac S1P1 receptor in these mice appears to only contribute to inhibition of cAMP accumulation with an insignificant or lack of involvement in Akt or ERK activation. Thus these mice should show a decrease in TAC induced hypertrophy relative to WT mice, and if ERK and Akt are involved in S1P mediated hypertrophy, these S1P2,3 receptor knockout mice should show an even greater reduction in TAC-induced hypertrophy than the S1P3 receptor knockout mice.

3. Acknowledgements

The dissertation author was the primary investigator in the development and execution of this study. I would like to thank Yusu Gu for performing the transverse aortic constriction surgery.
Figure V.1: Transverse aortic constriction (TAC) induced cardiac hypertrophy is diminished in S1P3 receptor knockout hearts. WT and S1P3 receptor knockout mice were subjected to 7 days of transverse aortic constriction after which hearts were removed and hypertrophy was assessed by measuring the left ventricle to body weight ratio (LV/BW). For sham conditions surgery was performed identically although the vessel was not constricted. n=12 per group.
Figure V.2: S1P mediated inositol phosphate hydrolysis is abolished in S1P3 receptor knockout myocytes. WT and S1P3 receptor knockout myocytes were loaded overnight with 3H inositol and then stimulated with 1 µM S1P for 30 minutes prior to harvesting lipids and measuring the ratio of inositol phosphate (IP1) to total inositol. n>12 per group, *p<0.05 vs. veh
4. References


B. Regulation of cardiomyocyte contractility by S1P receptors

1. Introduction

S1P is known to elicit numerous responses on cardiomyocytes including increases in intracellular calcium, changes in heart rate, and even altered contractility (15; 18). These responses to S1P are thought to be mediated by its interaction with cell surface G-protein coupled receptors that induce changes in second messengers and various ion channels. However little is known about how S1P regulates myocyte contractility and it is unclear which S1P receptors are involved in regulation of contractility. Thus we chose to examine the effects of S1P on contractility in myocytes isolated from WT and S1P receptor knockout mice.

S1P is involved in activation of a number of pathways that are involved in regulation of cardiac contractility. In atria, S1P activates G-protein activated potassium channels via a pertussis toxin sensitive pathway resulting in an inward rectifying potassium current (\(I_{K.Ach}\)) (2; 9; 12). These potassium channels are known to be critical for the control of resting membrane potential, heart rate, and duration of action potentials (3; 19). Furthermore, in human atrial myocytes, it is thought that the receptor mediating this channel is the S1P\(_3\) receptor (5). Finally S1P administration in vitro has also been shown to slow spontaneous pacing of rabbit sinoatrial cells by activation of \(I_{K.Ach}\) (4) while in vivo infusion of S1P or FTY720, a molecule sharing structural similarity with S1P that is also an agonist at all S1P receptors except S1P\(_2\), results in a decrease in heart rate (7; 17) and even bradycardia via S1P\(_3\) receptor activation (16).
In ventricular myocytes, S1P depresses the excitability of action potentials by reversibly decreasing sodium currents ($I_{Na}$) rather than by altering the inward rectifier potassium current (10). This response is not blocked by pertussis toxin, suggesting that it may be the result of S1P signaling through $G_q$ and not $G_i$ proteins, or even S1P directly interacting with the sodium channel rather than interacting with an S1P receptor (10), as has been shown with other extracellularly applied lipids (1; 6). Additionally in neonatal rat ventricular myocytes S1P treatment induces calcium overload in a pertussis toxin insensitive manner via the S1P$_1$ receptor. However this is a controversial finding as the S1P$_1$ receptor is known to couple exclusively to $G_i$ and the authors of this study propose that the S1P$_1$ mediated calcium overload may occur through $G_q$ signaling (11).

The ability of S1P to activate $G_i$ proteins and subsequently inhibit cAMP accumulation also allows S1P to regulate myocyte contractility. Numerous calcium handling proteins involved in myocyte contractility are regulated by PKA and through its $G_i$ signaling, S1P is able to antagonize the actions of PKA. Typically, PKA is activated downstream of β-adrenergic receptors, and this serves to phosphorylate the ryanodine receptor (RyR) and phospholamban. Phosphorylation of the ryanodine receptor causes dissociation of FKBP and thus results in the ryanodine receptor becoming more sensitive to calcium-induced calcium release. Phosphorylation of phospholamban results in increased activity of the sarcoplasmic reticulum calcium ATPase (SERCA) allowing for cardiac relaxation as calcium reenters the sarcoplasmic reticulum (14). More recently it was even shown that β-adrenergic signaling can effect cardiac contractility in a PKA-independent mechanism by activating a mechanism
involving the Rap guanine nucleotide exchange factor, EPAC, and its downstream effector PLCε (13; 20).

2. Results/Discussion

Adult mouse ventricular myocytes were suspended in Tyrodes solution and plated on glass coverslips. Cells were paced at 0.5 Hz and contractility was measured using a video based edge detection system to measure sarcomere length. After equilibration, cells were infused with 1µM S1P and sarcomere length was measured approximately 10 minutes after S1P infusion. For pertussis toxin (PTX) experiments, cells were treated with 1.5µg/ml PTX for 3 hours at 37°C prior to measuring contractility.

WT cells showed a marked negative inotropic response to S1P, as indicated by a 50% reduction in the electrically-induced change in sarcomere length. After equilibration, the sarcomere length of WT myocytes during contraction was reduced by approximately 50%. Before S1P infusion, the sarcomere length of WT myocytes changed from 1.8µm in relaxed myocytes to 1.65 µm in contracted myocytes. After S1P infusion, the sarcomere length of the relaxed WT myocytes was still 1.8 µm, but in contracted myocytes, the sarcomere length only shortened to approximately 1.74 µm (Figure V.3). Thus, following S1P infusion, WT myocytes show a decreased strength of contraction.

As decreases in cardiac inotropy are known to be mediated in part by Gi signaling, we treated cells with pertussis toxin prior to S1P stimulation. After pertussis toxin treatment, myocytes exhibited sarcomere lengths of 1.7µm in relaxed myocytes
and 1.55\(\mu\)m in contracted myocytes. Upon S1P infusion to these pertussis toxin treated cells, the sarcomere length changes were not significantly changed by S1P (Figure V.4). Thus most of the S1P mediated negative inotropy is mediated by G\(_i\) signaling pathways, presumably through inhibition of adenylyl cyclase and PKA.

Finally, as S1P decreases cardiac inotropy through G\(_i\) signaling pathways, we examined the effects of S1P on contractility in S1P\(_{2,3}\) receptor double knockout myocytes. As shown in chapter 3, S1P\(_{2,3}\) receptor double knockout myocytes still express the S1P\(_1\) receptor, which is thought to couple exclusively to G\(_i\) signaling pathways. We have shown that nearly all of the effects of S1P on inhibition of cAMP accumulation are mediated by the S1P\(_1\) receptor coupling to G\(_i\) and inhibition of adenylyl cyclase activity. Thus we hypothesized that if S1P was decreasing inotropy by inhibiting adenylyl cyclase and subsequent PKA activation, the effects of S1P on contractility should be intact in S1P\(_{2,3}\) receptor double knockout myocytes.

When S1P\(_{2,3}\) receptor double knockout myocytes are treated with S1P, the negative inotropic effect seen in WT myocytes is still intact. Prior to S1P infusion, we observe sarcomere lengths of 1.7\(\mu\)m in relaxed cells and 1.5\(\mu\)m in contracted cells. Following S1P infusion, the sarcomere length of relaxed cells is still 1.7\(\mu\)m and contracted cells show a sarcomere length of 1.6\(\mu\)m (Figure V.5). Thus we still observe S1P mediated negative inotropy in the S1P\(_{2,3}\) receptor double knockout myocytes.

The above experiments demonstrate that S1P causes negative inotropy in myocytes primarily through the G\(_i\) coupled S1P\(_1\) receptor. This is consistent with the accepted notion that inhibition PKA activity by decreasing cAMP accumulation would
decrease β-adrenergic signaling and cardiac contractility. Our data using genetic and pharmacological approaches demonstrate that inhibition of adenylyl cyclase activity and cAMP accumulation is mediated primarily by the S1P₁ receptor, thus this is likely the pathway by which S1P decreases cardiac contractility.

This observation that not all of the S1P mediated negative intropy is blocked by pertussis toxin treatment could imply a possible role for non Gi proteins in mediating this response. However, incomplete inhibition of Gi proteins by the short pertussis toxin treatment time may be the reason that not all of the negative inotropic effects of S1P are blocked by pertussis toxin. This length and concentration of pertussis toxin treatment has previously been shown to potentiate the contractile response of rat cardiomyocytes to β2-adrenergic stimulation but not β1-adrenergic stimulation (8; 21). While the S1P₁ receptor is thought to couple exclusively to Gi proteins, the S1P₂ and S1P₃ receptors are known to couple to Gq signaling pathways, and others have noted that S1P₃ receptor activation may be involved in regulating heart rate and myocyte excitation (16). Thus analysis of S1P mediated contractility in S1P₂ or S1P₃ receptor knockouts may be of interest. If S1P is able to elicit a negative inotropic effect in either the S1P₂ or S1P₃ receptor knockout myocytes, then other non Gi mediated pathways are likely involved.

In addition, while S1P decreases contractility in electrically stimulated cells we would like to look at whether S1P has the same effect on cells stimulated with a β agonist such as isoproterenol. If S1P is inhibiting contraction by decreasing cAMP accumulation and PKA activity, it should antagonize the increased contractility observed with isoproterenol stimulation. Finally, the involvement of the S1P₁ receptor
in mediating negative inotropy can be further confirmed by measuring sarcomere
length in WT myocytes perfused with the S1P1 receptor agonist, SEW2871.

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measuring cardiac contractility.
Figure V.3: S1P induced negative inotropy in WT adult mouse myocytes. A: Sarcomere length in WT myocytes prior to S1P infusion. B: Sarcomere length in WT myocytes after S1P infusion.
Figure V.4: S1P induced negative inotropy is blocked by pertussis toxin. A: Sarcomere length in WT myocytes treated with 150ng/ul pertussis toxin for 3 hours. B: Sarcomere length in WT myocytes treated with 150ng/ul pertussis toxin for 3 hours after S1P infusion.
Figure V.5: S1P induced negative inotropy is not altered in S1P$_{2,3}$ receptor double knockout myocytes. A: Sarcomere length in S1P$_{2,3}$ receptor double knockout myocytes before S1P infusion. B: Sarcomere length in S1P$_{2,3}$ receptor double knockout myocytes after S1P infusion.
Figure V.6: Quantitation of S1P induced changes in sarcomere length. WT or S1P2,3 receptor double knockout myocytes were treated with pertussis toxin and/or S1P and changes in sarcomere length were measured. Data is normalized to percent of sarcomere length change in vehicle treated WT myocytes. ***p<.001 vs. WT veh, *p<.05 vs. WT veh
4. References


