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Lysophospholipid receptor activation of RhoA and lipid signaling pathways

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Abstract

The lysophospholipids sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) signal through G-protein coupled receptors (GPCRs) which couple to multiple G-proteins and their effectors. These GPCRs are quite efficacious in coupling to the $G_{\alpha_{12/13}}$ family of G-proteins, which stimulate guanine nucleotide exchange factors (GEFs) for RhoA. Activated RhoA subsequently regulates downstream enzymes that transduce signals which affect the actin cytoskeleton, gene expression, cell proliferation and cell survival. Remarkably many of the enzymes regulated downstream of RhoA either use phospholipids as substrates (e.g. phospholipase D, phospholipase C-epsilon, PTEN, PI3 kinase) or are regulated by phospholipid products (e.g. protein kinase D, Akt). Thus lysophospholipids signal from outside of the cell and control phospholipid signaling processes within the cell that they target. Here we review evidence suggesting an integrative role for RhoA in responding to lysophospholipids upregulated in the pathophysiological environment, and in transducing this signal to cellular responses through effects on phospholipid regulatory or phospholipid regulated enzymes.

1. Introduction

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are small, bioactive membrane lipid derivatives that play critical roles in many physiological and pathophysiological processes, including development [1], cancer [2], multiple sclerosis [3–5], pain, inflammation, fibrosis [6, 7], atherosclerosis, and myocardial ischemia/reperfusion (I/R) [7–9]. Both S1P and LPA act as extracellular signaling molecules by binding to and activating seven-transmembrane spanning G protein-coupled receptors (GPCRs), which exist as multiple subtypes (S1P_{1–5} [10] and LPA_{1–6} [11]). The cloning and identification of the lysophospholipid receptors opened new avenues for research into the signaling pathways and biological processes elicited by the actions of S1P and LPA. Each of the S1P and LPA receptor subtypes signals through specific G-proteins, $G_{\alpha_{q/11}}$, $G_{\alpha_{12/13}}$, G_{α_i} or their associated $G\beta\gamma$ subunits [10, 12]. The specificity of receptor subtype coupling to the various G proteins depends on their expression levels and cell type; thus, the downstream signals elicited following receptor activation by S1P and LPA are context dependent. Studies using specific agonists and antagonists as well as cloned receptors and knockout mice have, however, provided considerable impetus to uncovering the significance of lysophospholipid signaling through distinct receptor subtypes [6, 7, 11, 13].

The S1P and LPA receptors are amongst the most efficacious of the GPCRs shown to couple to $G_{\alpha_{12/13}}$ and hence to activate guanine nucleotide exchange factors (GEFs) for the low

molecular weight G protein RhoA [14–17]. RhoA functions as a nodal point in transducing extracellular signals to regulate a wide range of cellular responses [18–20]. In this review, we focus on the ability of S1P and LPA to signal through regulation of RhoA activity, and the molecular mechanism by which RhoA controls enzymes involved in, and regulated through, lipid metabolism. We consider the role of this pathway in myocardial I/R injury and briefly discuss its potential role in neuroinflammation.

2. S1P and LPA Regulates RhoA signaling through $G\alpha_{12/13}$

2.1. RhoA activation

RhoA is a member of the Rho family GTPases and has well-established functions in cytoskeletal organization, inducing the formation of actin stress fibers and focal adhesions [20–22]. Constitutive expression of $G\alpha_{12}$ and $G\alpha_{13}$ has been demonstrated to induce stress fiber and focal adhesion formation, as well as tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, all in a Rho dependent manner [23, 24]. In response to GPCR agonists like LPA, RhoA also mediates neurite retraction and inhibiting RhoA function using C3 exoenzyme or dominant negative RhoA leads to neurite outgrowth [25, 26]. Signaling via S1P and LPA to $G\alpha_{12/13}$ and RhoA has also been linked to cell migration and proliferation as well as transcriptional gene regulation [24, 27, 28].

The activity of Rho is regulated by GTPase activating proteins (GAPs), GTP exchange factors (GEFs), and GTP dissociation inhibitors (GDIs). GAPs mediate the inactivation of small G proteins by promoting their intrinsic GTPase activity while GEFs promote the exchange of GDP for GTP and thereby activate the small G protein. Members of the $G\alpha_{12/13}$ family proteins have been shown to interact directly with and thereby activate several RhoGEFs including p115-RhoGEF, PSD-95/Disc-large/ZO-1 homology (PDZ)-RhoGEF, leukemia-associated RhoGEF (LARG), and lymphoid blast crisis (Lbc)-RhoGEF [17, 29]. The p115RhoGEF was also shown to function as a GAP and thereby a signal terminator for $G\alpha_{12/13}$ [30]. All four RhoGEFs are large proteins that are widely expressed in mammals. Dysfunction of the $G\alpha_{12/13}$ RhoGEF pathway can lead to pathophysiological outcomes including cancer, cardiovascular diseases, arterial and pulmonary hypertension [17]. Recent studies demonstrate that $G\alpha_q$ can also regulate RhoGEFs to activate RhoA [15, 31, 32], however this is a less dedicated pathway since $G\alpha_q$ also couples to phospholipase C-beta (PLC β) [33–37].

2.2. RhoA effectors

Once activated by GTP binding, RhoA recruits various downstream effectors that relay signals to induce changes in cell shape and movement, proliferation and cell cycle progression, cell survival and gene expression [20, 27, 38]. Many Rho effectors have been discovered using affinity chromatography, yeast two-hybrid and mutational analysis [39, 40]. The most common mechanism for effector activation by GTP-RhoA is the disruption of intramolecular autoinhibition, which exposes the functional domain of the effector protein [20, 40]. RhoA effectors include the extensively characterized Rho associated protein kinase (ROCK), a serine/threonine kinase termed protein kinase N (PKN or PRK) and the mammalian diaphanous (mDia) [40–42]. ROCK is a serine/threonine kinase that is activated

by binding of GTP-RhoA to its C-terminal coiled-coil domain. Activated ROCK subsequently phosphorylates a number of substrates, including LIM kinase and myosin light chain phosphatase, and there by modulates actin-myosin cytoskeletal dynamics and contraction [43]. PKN is another serine/threonine kinase that is a direct target of RhoA [39, 44, 45], which has been shown to be activated by LPA in Swiss 3T3 fibroblasts in a Rho-dependent manner [46, 47]. PKN is involved in cytoskeletal regulation, cell adhesion, and cell cycle [48]. A recent report demonstrated that PKN also mediates cardiac myocyte survival in response to oxidative stress or I/R injury [49]. In addition to the canonical effectors mentioned above, RhoA directly or indirectly regulates a number of phospholipid regulatory and regulated enzymes implicated in biological responses. These lipid regulated/regulatory enzymes are discussed in more detail below.

3. Activation of phospholipases by RhoA

3.1. Phospholipase D

The phosphatidylcholine (PC)-selective phospholipase D (PLD) was recognized as a regulated player in cell signaling events beginning in the late 80s. Amongst the myriad responses that have been subsequently attributed to PLD are cytoskeletal reorganization, membrane trafficking, signal transduction, transformation, proliferation, and cell survival [50–54]. Activated PLD hydrolyzes PC to produce the bioactive lipid phosphatidic acid (PA) and free choline. PA can be further metabolized to generate diacylglycerol (DAG), a second messenger that activates protein kinase C (PKC) and subsequently protein kinase D (PKD). PA can, in addition, be converted to LPA through the actions of secretory type II phospholipase A2 (PLA2) [52, 53, 55]. Two mammalian PC-selective PLD isozymes, PLD1 and PLD2, have been identified. These isozymes share 53% sequence homology and are subject to different regulatory mechanisms. Abundant evidence indicates that RhoA binds to and activates PLD1, but not PLD2 [51, 56, 57]. PLD1 has very low constitutive activity and possesses an inactive conformation until bound to its activators. Interestingly, there are multiple and redundant mechanisms by which RhoA regulates PLD1. First, RhoA can activate PLD1 through direct interaction with its C-terminus [56]. In addition, it can act indirectly through its effector PKN, which also interacts with and activates PLD [58]. Another indirect mode of activation is that RhoA regulates phosphatidylinositol (PI) 4-phosphate 5-kinase PIP5K, increasing the synthesis of PI-4, 5-bisphosphate (PIP2), which serve as a critical co-factor for PLD [53, 59]. S1P and LPA have been shown to activate PLD in various cell types [60–64]. PLD activation by S1P regulates interleukin-8 secretion in human bronchial epithelial cells and therefore contributes to S1P mediated inflammatory response [60, 61]. In human PC-3 prostate cancer cells, LPA was found to activate PLD and induce cell proliferation [63]. Whether RhoA is involved in S1P or LPA mediated PLD activation and downstream effects through mechanisms described above has not been considered.

PLD signaling has been implicated in myocardial protection and there is considerable evidence that PLD is activated and regulated by oxidative stress, a cellular response associated with various cardiac pathologies including coronary heart disease [65]. It has been reported that PLD activation by oxidation involves activities of protein tyrosine kinases

and phosphatases [66]. Whether oxidative activation of PLD also occurs through RhoA is not known, but RhoA has a redox-sensitive motif and can be directly activated by reactive oxygen species (ROS) [67, 68]. We recently determined that RhoA is rapidly and robustly activated by hydrogen peroxide and by I/R injury [69]. The possibility that RhoA and PLD work together to mediate cardiac protection is supported by the observation that disrupting the direct interaction of RhoA and PLD prevents adenosine-induced cardioprotection from I/R injury [70]. PLD is also activated by ischemic preconditioning and contributes to preconditioning mediated cardioprotection, a paradigm in which brief periods of I/R, applied before the sustained I/R event, protect against the subsequent sustained I/R insult [71, 72]. Of particular interest, SIP released by ischemic preconditioning or exogenously applied to the heart can serve as a powerful pharmacological preconditioning stimulus to protect the heart against I/R injury [73, 74]. This cardioprotective pathway will be discussed in detail later in section 5.1 in this review.

3.2. Phospholipase C-epsilon

Rho family GTPases have been shown to regulate phospholipase C (PLC) signaling, and earlier work suggested that this effect was not due to direct PLC activation, but rather through the regulation of PLC substrate PIP2 availability [75, 76]. Rho family proteins stimulate the activity of PIP5K, a phospholipid kinase that catalyzes the phosphorylation of the membrane phospholipid phosphatidylinositol 4-phosphate to generate PIP2 [59, 77, 78]. More recent studies revealed that RhoA actually has direct effect on a family member of PLC, Phospholipase C-epsilon (PLC ϵ), through physical interaction with its catalytic domain [79–82]. PLC ϵ is a recent addition to the PLC family (see [83–85] for reviews). Like other PLC enzymes, PLC ϵ hydrolyzes PIP2 to generate two important second messengers, DAG and inositol 1,4,5-triphosphate (IP₃). Generation of DAG is required for the activation of all of the conventional and novel PKC family members as well as other C1 domain containing, DAG sensitive enzymes. IP₃ controls Ca²⁺ release from intracellular IP₃ sensitive Ca²⁺ stores. These second messengers can be generated through any of the more commonly studied PLC isoforms including PLC β and PLC γ . Unlike PLC β , which is regulated by binding to the G α_q subunit, and PLC γ , which is regulated by tyrosine phosphorylation, PLC ϵ is unique in being regulated by small GTPases of the Ras family including Ras, Rap1 and RhoA [80, 83, 85–87]. Ras binds to the Ras-associating (RA) domain, and RhoA to a site in the catalytic domain of PLC ϵ leading to its activation [80, 83, 85, 86, 88]. PLC ϵ is also unique in containing an extended N terminus which includes a CDC25 domain not found in other PLC family members. This domain allows it to function as a GEF for Rap1. The activated Rap1 formed in this manner could bind to the C terminal RA domain, of PLC ϵ , forming a positive feedback loop which leads to sustained PLC signaling [84].

The observation that heterologous expression of G $\alpha_{12/13}$ increases PLC ϵ activity lead to the subsequent demonstration that PLC ϵ is activated through Rho [79, 82, 85, 89]. Co-transfection of RhoA in COS-7 cells along with PLC ϵ , significantly increased inositol phosphate production whereas co-transfection with other small G proteins like Rac1 and Cdc42 did not [82]. A region in a linker within the XY catalytic motif of PLC ϵ , not present in other PLCs, was identified as the region with which RhoA directly interacts to regulate

PLC ϵ [82]. LPA and S1P regulate PLC ϵ via G $\alpha_{12/13}$ mediated activation of RhoA. This was shown by co-expressing PLC ϵ and the RGS domain of p115RhoGEF, which binds and activates the GTPase activity of G $\alpha_{12/13}$. When co-expressed, LPA and S1P induced PLC ϵ activation was found to be significantly reduced [90]. Consistent with this data, the Harden group found that in COS-7 cells, LPA induced inositol phosphate production via PLC ϵ was dependent on functional Rho [79].

Though LPA receptors can also couple to G α_q and hence to PLC β , there are differences in the kinetics of inositol phosphate (IP) production depending on whether PLC β or PLC ϵ are activated. In Rat-1 fibroblasts, knockdown of PLC β affected only short-term phosphoinositide hydrolysis whereas PLC ϵ knockdown affected sustained IP generation [79, 90]. Our group was the first to use PLC ϵ knockout cells to demonstrate that PLC ϵ is the major phospholipase C isoform activated by S1P, LPA, or thrombin and is responsible for the long term IP production in primary astrocytes. In contrast IP production in response to carbachol, which acts on the G α_q coupled muscarinic receptor, was not decreased in astrocytes from PLC ϵ KO mice [91].

Two PLC ϵ knockout mouse models have been generated: one lacks a portion of its catalytic core and EF hand, making it catalytically inactive, and the other, generated by deletion of exon 6 of PLC ϵ , shows complete loss of PLC ϵ protein [84]. PLC ϵ knockout mice exhibit increased susceptibility to hypertrophy and cardiac enlargement in response to adrenergic stress [92]. PLC ϵ knockout mice have also been shown to have diminished generation of proinflammatory mediators in models of cancer, in particular skin cancer [93, 94].

4. Activation of lipid regulated enzymes

4.1. Protein kinase D

PKD is a serine/threonine protein kinase regulated by DAG and PKC. Accordingly, PKD serves as a downstream mediator of the actions of growth factors and GPCR agonists that activate the PLC family of enzymes [95]. There are three PKD isoforms (PKD1, PKD2, and PKD3) all of which contain two C1 domains that mediate DAG and phorbol ester binding, an autoinhibitory PH module, and a carboxy-terminal kinase segment. PKD activation occurs secondary to accumulation of DAG, which recruits PKD through its C1 domains. DAG also activates PKC, which phosphorylates PKD at two conserved serines in the PKD activation loop, promoting its autophosphorylation at a distinct site. Both phosphorylation events are required for full activation of PKD [95, 96].

The Rozengurt group has extensively examined the mechanism of GPCR activation of PKD, focusing on the actions of LPA. They demonstrated that LPA treatment leads to PKD activation through G $\alpha_{12/13}$ and Rho [97, 98]. PKD activation mediated by RhoA has also been shown to occur in response to loss of cell-cell adhesions in HeLa cells, and to result in NF κ B activation [99]. The molecular mechanism for RhoA mediated PKD activation has not been fully elucidated despite the fact that a link between these signals has been known for over a decade. Theoretically RhoA mediated stimulation of either PLD or PLC ϵ , could increase DAG generation and PKC activation and thereby lead to recruitment and phosphorylation of PKD.

PKD is primarily cytosolic but can be localized to different cellular compartments in response to receptor activation [97, 100]. Moreover, studies using a Fluorescence Resonance Energy Transfer (FRET) reporter to reveal the dynamics and intracellular localization of PKD activation demonstrate that PKD activation is sustained in particular cellular compartments where DAG and PKC are abundant, e.g. the Golgi [101, 102]. The cellular localization of PKD is likely to be of fundamental importance for specifying its substrates and accordingly which of its diverse biological effects (cell proliferation and differentiation, Golgi vesicle fission and transport, survival) are elicited. For example PKD regulates cell motility via phosphorylation of SSH1L, cortactin, E-cadherin, SNAIL, and RIN1 and has thus been speculated to be important for cancer metastasis [95]. Several extensive reviews concerning PKD regulation and functions have been published [95–97]. It is noteworthy that PLC ϵ has been reported to be localized to the Golgi [103], and accordingly sustained activation of PLC ϵ and generation of DAG could be responsible for PKD activation in that cellular compartment.

In the cardiovascular system, PKD activation has been implicated in the development of pressure overload induced hypertrophy, through activation of a nuclear target, the class II histone deacetylases (HDACs) [104–106]. Phosphorylation of HDAC5 by PKD leads to its nuclear export and subsequent increase in myocyte enhancer factor 2 (MEF2) transcriptional activation of a specific set of hypertrophic gene expression [107]. The Olson laboratory reported that cardiac specific PKD1 gene deletion prevented cardiac hypertrophy in response to pressure overload or chronic adrenergic and angiotensin II signaling *in vivo* [105].

Our laboratory recently demonstrated that PKD is required for the cardioprotective effect of RhoA activation in a cardiac specific inducible RhoA transgenic mouse model [69]. The hypothesis that PKD might be a novel target for cardioprotection against oxidative stress, with potential targets at the mitochondria, is consistent with recent reports by the Storz group demonstrating that PKD promote survival in response to oxidative stress. Their studies concluded that PKD is activated at the mitochondria in response to increased ROS, and that this leads to NF κ B mediated MnSOD expression, which contributes to mitochondrial ROS detoxification [108–112]. Recently, our lab used a neonatal rat cardiomyocyte model to show that PKD activation by H₂O₂ and RhoA protects against oxidative stress induced cell death [69]. Notably, S1P is highly protective against I/R injury, and we hypothesize that it achieves this protection in part through activation of PKD.

4.2. AKT

The serine/threonine protein kinase Akt (also known as protein kinase B) is another lipid regulated signal transducer which is activated through various cell surface receptors including receptor tyrosine kinases and GPCRs for agonists such as S1P and LPA. Aberrant Akt activity underlies the pathophysiological properties of a variety of complex diseases thus dysregulation of this protein kinase is recognized to be at the core of human pathophysiology and disease [113, 114].

S1P and LPA can induce robust Akt activation [6, 115–117]. Commonly activation of Akt in response to GPCR agonist is mediated through a G α_i signaling pathway, secondary to release of $\beta\gamma$ subunits from G α_i . Indeed our laboratory and others have demonstrated that in

adult mouse ventricular myocytes, S1P mediated Akt activation is $G\alpha_i$ -dependent, since this response is fully blocked by pretreatment with pertussis toxin [117–119]. However another mechanism for activating AKT is through RhoA, which we showed to activate Akt in neonatal rat cardiomyocytes by signaling through FAK and phosphoinositide-3 kinase (PI3K) [120].

Akt is best known for its role in regulating cell survival. In the context of myocardial protection, there is abundant evidence supporting a crucial role of Akt activation. A variety of cardioprotective agents including S1P, as well as adenosine, and bradykinin, converge on Akt activation [116, 118, 121, 122], which then leads to inhibition of pro-apoptotic proteins and initiation of protective signaling cascades [114, 123]. More recently, Akt activation has been implicated in promoting cardiac stem cell survival [124]. *In vivo* studies using knockout mice further supported the role of Akt in myocardial protection as Akt1 knockout mice show increased apoptosis and decreased survival after cardiomyopathic injury in addition to reduced organ size [125]. Conversely, mice with genetic deletion of PHLPP1, a novel protein phosphatase that dephosphorylates and inactivates Akt, showed increased Akt phosphorylation and decreased injury following myocardial I/R [126]. There is also evidence indicating that Akt is activated in response to brain injury and contributes to neuronal survival *in vivo* in a traumatic brain injury (TBI) mouse model [127] and a subarachnoid hemorrhage rat model [128].

4.3. PTEN

First identified as a tumor repressor that was mutated in cancer cells, PTEN (phosphatase and tensin homologue deleted on chromosome 10) plays an important role in the regulation of cell migration and invasion [129]. It has also been recognized to function as an essential modulator of cell survival [130]. The effect of PTEN on cell survival can be explained in part through its role in Akt signaling; PTEN dephosphorylates PIP3 and therefore antagonizes the PIP3 generation and Akt activation that occur in response to PI3K stimulation [130]. Evidence that S1P and RhoA regulate PTEN was first reported in several studies in 2005 [131, 132]. Studies from the Sanchez laboratory showed that S1P induces an antimigratory effect, which was lost in PTEN^{loxP/loxP} mouse embryonic fibroblasts (MEFs) and restored upon PTEN expression. They also demonstrated that the antimigratory effect of S1P was mediated through the S1P₂ receptor subtype and a RhoA-dependent pathway [131]. Paradoxically in human glioma cells, S1P₂ receptor mediated migration was reported to be Rho- but not PTEN-dependent, [133]. A study conducted by the Wu laboratory demonstrated that RhoA regulates the intracellular localization and stimulates the phosphatase activity of PTEN through its downstream effector ROCK. They found that ROCK interacts with and phosphorylates PTEN, and further identified key residues on PTEN that are required for this regulation [132]. Notably, in the studies cited above, Akt activation, used as one of the readouts for PTEN activity, was decreased by S1P treatment and RhoA expression, consistent with up regulation of PTEN activity by RhoA. However, as indicated earlier multiple studies have demonstrated an activation of Akt by S1P and LPA stimulation in other cell types including cardiomyocytes [6, 115–117].

5. Lysophospholipid and RhoA mediated signaling in myocardial I/R injury and neuroinflammation

5.1. Protection against myocardial I/R injury

The lysophospholipids, in particular S1P, have been shown to contribute to protection against myocardial I/R injury. S1P is present at micromolar concentrations in serum where it is bound to serum albumin and high density lipoproteins (HDL) [134, 135]. Interestingly, some of the cardioprotective effects of HDL have been shown to be mediated by S1P [134, 135]. Of the five S1P receptors, S1P₁₋₃ are expressed in the cardiovascular system with S1P₁ being the dominant subtype in cardiomyocytes and vascular endothelial cells, and S1P₃ predominating in fibroblasts [9]. S1P has well established roles in mediating ischemic pre- and post-conditioning, two powerful experimental maneuvers that protect the heart from I/R injury [73, 136, 137]. I/R injury occurs after acute myocardial infarction and is associated with cardiomyocyte cell death and myocardial dysfunction. S1P is released by cardiomyocytes in response to preconditioning in neonatal rat cardiomyocytes. When S1P is exogenously provided to the isolated perfused heart before or after ischemia, cardiac contractility is preserved and infarct development in response to I/R is markedly attenuated. Pharmacological pre- and postconditioning with the S1P precursor sphingosine also protects the heart against I/R injury [74, 137].

Which S1P receptor subtype mediates cardioprotection remains uncertain. Our laboratory demonstrated using S1P receptor knockout mice that S1P₂ and S1P₃ receptors play important and partially redundant roles in limiting infarct size in response to endogenous S1P released following I/R *in vivo* [116]. Cardioprotection from exogenously applied S1P appears to be mediated from S1P₃ receptor since intravenously administered S1P failed to reduce infarct size in S1P₃ knockout mice in an *in vivo* I/R model [135]. In the isolated perfused rat heart, Karliner's group demonstrate that blocking S1P signaling through S1P₁ and S1P₃ receptors with the receptor antagonist VPC23019 prevented effects of both pre- and postconditioning on contractile function and infarct size following I/R [73]. Interestingly pre- or post-conditioning with the S1P receptor agonist and FDA approved drug FTY720, which affects S1P₁ and S1P₃ in the heart [138, 139] was shown to improve functional performance but failed to reduce infarct size [140] [141]. The same group demonstrated that SEW2871, a S1P₁ selective agonist, did not improve functional recovery [141], and Gottlieb's laboratory reported that SEW2871 had marginal effect on reducing infarct size in the isolated perfused heart [142]. Taken together these studies suggest that it may be the S1P₃ receptor subtype that primarily confers cardioprotective responses.

The clinical applicability of using S1P or its receptor modulators to diminish I/R damage is limited by the fact that acute administration of S1P, FTY720, or the non-selective S1P agonist (AAL-R) induces sinus bradycardia *in vivo*. There is evidence that this effect may be mediated through S1P₃ receptors since the bradycardia seen with FTY720 was abolished in S1P₃ receptor KO mice [143, 144]. On the other hand, studies in humans showed that BAF312, an S1P₁ and S1P₅ selective agonist without S1P₃ activity induces bradycardia, suggesting a contribution of S1P₁ receptors to S1P induced bradycardia in humans [145]. A possible mechanism for FTY720 induced bradycardia mediated by the S1P₁ receptor, which

couples only to G_{q_i} -dependent pathways, would be through activation of the G protein gated potassium channel $I_{K_{ACH}}$ in atrial myocytes [146]. Although the acute bradycardic effect of S1P receptors in the heart is modest, it can cause exacerbation of arrhythmia induced by I/R. In fact, FTY720 cannot be used as a postconditioning treatment since it increases mortality following global I/R due to induction of fatal ventricular tachyarrhythmias [140]. Similarly the S1P₁ receptor agonist SEW2871 exacerbates reperfusion arrhythmias [142]. Collectively these studies and those cited in the previous paragraph suggest that cardioprotection could be mediated through S1P₃ and arrhythmias through S1P₁ receptors, in which case it might be possible to dissociate these responses therapeutically. It is clearly critical to further dissect the differential roles of each S1P receptor subtype in regulating cardiac functions under physiological and pathophysiological conditions.

There is evidence supporting the notion that both S1P₂ and S1P₃ receptors can couple to RhoA [147–150]. As mentioned earlier in this review, many RhoA effectors, including both its direct and indirect targets, are potential mediators of cell survival and cardioprotection (shown in Figure 1). One such downstream effector regulated in response to RhoA is Akt. There is abundant evidence supporting a cardioprotective role of AKT, which is activated during reperfusion [151] and is associated with ischemic pre- and post-conditioning [152–154]. AKT is activated in response to PIP3 generation through PI3K, and inhibition of PI3K by LY294002 was shown to reverse the decrease in infarct size and increase in functional recovery by preconditioning in the isolated rat heart model [155, 156]. Our laboratory has previously demonstrated that adenoviral infection of neonatal rat cardiomyocytes with a constitutively activated form of RhoA (L63) stimulates the FAK/PI3K/AKT pathway and protects cardiomyocytes from apoptotic insults. The anti-apoptotic effect by RhoA activation is reversed by inhibition of ROCK, FAK or PI3K [120], further supporting a pro-survival role of RhoA and AKT in cardiomyocytes. Furthermore, our laboratory showed that deletion of PHLPP1, an AKT phosphatase that dephosphorylates and inhibits Akt, promotes cardiomyocyte survival and significantly reduces infarct size following I/R [126]. Our studies using adult mouse hearts and isolated adult cardiomyocytes indicate that AKT activation and protection are mediated by the S1P₂ and S1P₃ receptors; AKT activation by S1P or by I/R was markedly reduced and *in vivo* infarct size increased >50% by S1P_{2/3} gene deletion despite the fact that the S1P₁ receptor was still highly expressed [116, 117].

There are several pathways through which AKT activation could provide cardioprotection. First, AKT phosphorylates and activates endothelial nitric oxide synthase, leading to increases in nitric oxide production and ventricular relaxation [157]. In addition AKT phosphorylates and inhibits pro-apoptotic targets such as glycogen synthase kinase-3 [158] and the Bcl2 proteins Bax [159, 160] and Bad [161], leading to reduced apoptotic cell death. Finally AKT translocates to mitochondria and phosphorylates hexokinase-II which regulates the mitochondrial permeability transition pore [162] and protects the cells from apoptotic and necrotic cell death [123, 163].

By comparison with AKT, the role of PLD as a cardioprotective mediator has received only modest attention. In 1996 the Downey group reported that PLD played a role in ischemic preconditioning [71]. PLD activity was doubled in the isolated rabbit heart following preconditioning, and activation of PLD using sodium oleate before a 30-minute coronary

occlusion reduced infarct size by 50% [71]. These findings were supported by data from the Das group, in which PLD was shown to be increased following ischemic preconditioning in the isolated rat heart, and associated with generation of DAG and PA and with translocation and activation of PKC. Inhibition of PLD with a PLD-specific antibody reversed preconditioning protection during I/R [72]. The Liang group later revealed a connection between RhoA signaling and the cardioprotective effect of PLD by demonstrating that RhoA inhibition with C3 exoenzyme or dominant negative RhoA (T19N) inhibited adenosine induced PLD activation and cardioprotection in chick embryo cardiomyocytes [164]. Later more direct evidence of RhoA and PLD involvement in cardioprotection was provided by the same group who showed that a direct RhoA-PLD interaction was required for adenosine induced cardioprotection against ischemia in chick embryo cardiomyocytes [70]. They hypothesized that adenosine protects through a mechanism involving PLD activation and DAG generation, which activates PKC and stimulates the mitoK_{ATP} channels to induce cardioprotection [70].

Our recently published studies using an inducible transgenic RhoA mouse model demonstrated that RhoA activation in adult cardiomyocytes does not affect basal cardiac morphology or function [69]. However, infarct size following I/R injury is reduced by 60–70% and functional performance is significantly improved [69]. The novel PKC isoforms PKC ϵ and PKC δ are both activated in the RhoA transgenic and more interestingly PKD is robustly activated. We demonstrated that pharmacological inhibition of PKD with CID755673 [165] reversed the protection in the RhoA transgenic heart. Our studies also revealed that RhoA and PKD are stress responsive proteins, which are activated in response to oxidative stress including I/R or H₂O₂ treatment [69]. The work cited above supports the hypothesis that release of S1P in response to ischemic stress contributes to RhoA and PKD activation and that this mechanism contributes to S1P mediated cardioprotection.

5.2. Neuroinflammation

Inflammation underlies many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Microglia, the macrophages of the brain become activated upon pathogen invasion or tissue damage, and respond in order to initiate repair. Astrocytes, which were once considered to simply serve as structural elements in the brain, also become activated during CNS injury and disease in a process known as astrogliosis [166]. Astrogliosis and microgliosis can be detrimental for neuronal survival and regeneration, in part due to activation of signaling cascades (like the NF κ B pathway) that exacerbate inflammation via cytokine production and generation of neurotoxic levels of ROS

Signaling via S1P and LPA has been linked to neuroinflammation. For example, injection of S1P and LPA into the striatum of mice was found to cause astrogliosis characterized by an increase in GFAP expression and astrocyte proliferation [167]. S1P was also found to increase proinflammatory cytokines including TNF- α and IL-1 β in microglia [168]. The importance of S1P signaling in neuroinflammation is also evidenced by the efficacy of FTY720 (which targets all S1P receptor subtypes except S1P₂), in the treatment of multiple sclerosis. FTY720 initially acts as an agonist at S1P receptors but acts as a functional

antagonist at later times due to its ability to cause S1P₁ receptor internalization [169]. FTY720 appears to function primarily through S1P₁ receptor blockade in lymph node T cells, preventing lymphocyte egress and imparting its immunosuppressive effect [170–172]. Moreover, it has direct effects in the CNS [173]. Strikingly the Chun group, using S1P₁ conditional null mutants in different CNS lineages, showed that the S1P₁ receptors on astrocytes are responsible for the efficacy of FTY720 in the treatment of Experimental Autoimmune Encephalomyelitis (EAE), which is used as a model of MS [174]. The precise molecular mechanism by which FTY720 elicits protective signals remains to be fully elucidated. Activation of S1P₁/Gq_i signaling by FTY720 could result in ERK1/2 and AKT activation and subsequent survival signaling [175–177]. Activation of S1P₃ and S1P₅ receptors by FTY720 could elicit RhoA activation and protection by mechanisms discussed earlier in this review. Evidence that FTY720 acting through these receptor subtypes activates RhoA comes from experiments in which oligodendrocyte precursor cells were shown to undergo neurite retraction which was blocked by a Rho kinase inhibitor [175].

Phospholipid regulatory signaling molecules and associated responses downstream of S1P and LPA receptors and RhoA were detailed in the sections above. Other effects of RhoA not yet discussed result from RhoA mediated gene transcription. The ability of RhoA to lead to transcriptional activation was first reported in 1995 [28]. This work demonstrated RhoA-dependent regulation of serum response factor (SRF) target genes in response to LPA and serum, as indicated by the ability of C3 exoenzyme to functionally inactivate RhoA and prevent this response. RhoA has also been demonstrated to regulate NFκB, a key transcription factor involved in immune and inflammatory responses. The Lacal group demonstrated that RhoA activates NFκB through phosphorylation and degradation of IκBα [178]. Their studies also demonstrated that LPA induces RhoA and NFκB in prostate cancer cells, and that inhibition of RhoA with C3 exoenzyme blocked LPA induced NFκB activation [179]. Thus lysophospholipids activation of GPCRs that signal to RhoA can participate in transcriptional responses that underlie inflammation.

PLCε and PKD discussed above in terms of their regulation by lysophospholipids and RhoA have been reported to be involved in inflammatory responses and to regulate NFκB. The Kataoka group demonstrated increased inflammation and cyclooxygenase-2 (COX-2) expression in an intestinal tumorigenesis model and showed that this was significantly attenuated in PLCε KO mice [93]. PLCε has also been reported to cooperate with NFκB to modulate expression of certain cytokines like MCP-1 [180]. As described above, PKD is a downstream effector of Rho which is activated by LPA [97, 98]. PKD has also been linked to inflammation through NFκB. LPA was found to induce expression of the proinflammatory cytokine IL-8 through PKD and NFκB, and knockdown of PKD abrogated LPA stimulated NFκB activation and IL-8 production in epithelial cells [181]. Work by the same group and others have also showed that GPCR agonists including LPA, as well as oxidative stress, activate NFκB through PKD in various cell types [182]. Our current studies have also linked S1P and LPA to inflammatory gene expression mediated through PKD and NFκB in astrocytes. The ability of lysophospholipids to activate RhoA mediated gene transcription is likely to have implications for neuroinflammation. A hypothetical schema demonstrating pathways by which RhoA and its effectors contribute to S1P and LPA mediated neuroinflammation is shown in Figure 2.

6. Conclusions and Future Directions

The underlying premise of this review is that RhoA is a critical effector of lysophospholipid signaling through S1P and LPA receptors. Which endogenously expressed subtypes of S1P and LPA receptors couple to activation of RhoA in physiologically relevant cell and organ systems is not fully known but is of central importance to future research. The pathophysiological relevance of elucidating lysophospholipid and RhoA mediated pathways is underscored by the fact that S1P and LPA are released or made available at sites of cell injury, where they can serve adaptive or maladaptive functions. Lysophospholipid receptors that couple to RhoA signaling are involved in various pathophysiological conditions, suggesting these receptors as important targets for disease therapy. The review details specific signaling pathways that are engaged through the downstream actions of RhoA, outside of the well recognized effects of RhoA on the actin cytoskeleton. Of particular interest is the fact that RhoA directly activates signaling molecules that regulate or are regulated by intracellular phospholipids. Examples discussed in detail here include the direct activation of PLD and PLC ϵ and the indirect regulation of PKD and the Akt regulating enzymes PI3K and PTEN. Notably, these signaling molecules have either established or emerging importance as therapeutic targets in cardioprotection and neuroinflammation. Therefore understanding the specificity of lysophospholipid receptor coupling to RhoA activation and these lipid signaling pathways may provide additional strategies for the treatment of acute myocardial ischemic injury or of chronic diseases involving neuroinflammation.

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Fig. 1.

S1P and LPA signaling through RhoA pathways in cardiomyocytes. S1P and LPA activate RhoA through $G\alpha_{12/13}$ and RhoGEFs. RhoA then binds to and activates downstream effectors such as PLD1, PLC ϵ and Rho kinase ROCK. Activation of PLD1 and PLC ϵ leads to DAG generation and PKC activation, which subsequently activates PKD. PKD phosphorylates HDAC5 in the nucleus, resulting in MEF2 mediated hypertrophy. PKD also promotes NF κ B mediated MnSOD expression and mitochondrial translocation, which detoxify mitochondrial ROS and protects cells from oxidative stress. RhoA effect on the actin cytoskeleton through ROCK leads to activation of FAK, PI3K and AKT can also protect cardiomyocytes from oxidative stress. ROCK also mediates RhoA activation of PTEN, which leads to inhibition of AKT.

S1P and LPA **Fig. 2.**

S1P and LPA signaling through RhoA in neuroinflammation. S1P and LPA activate RhoA through $G\alpha_{12/13}$ and RhoGEFs. PKD activation results from increases in phospholipase activity leading to DAG generation. This promotes NF κ B mediated inflammatory gene expression, which contributes to astrogliosis and microgliosis.