ANALYSIS OF REGULATORS OF G PROTEIN SIGNALING (RGS) 5 REGULATION AND LYSOPHOSPHATIDIC ACID (LPA) SIGNALING IN MUSCLE CELLS

by

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I dedicate this thesis and my accomplishments to my mother, Mrs. Maryse Lespès

for her unconditional love and support!

ABSTRACT

G protein-coupled Receptor (GPCR) signalling pathways are essential for all aspects of cell and organ physiology and the involved proteins work together to transmit signals across the plasma membrane. This process is highly regulated by a number of proteins including RGS (Regulators of G protein Signalling) proteins, which serve as GTPase-activating proteins for the Gα subunit of heterotrimeric G proteins. Previous work in our lab has identified RGS5 as being expressed in the heart as well as being up regulated in the atria but not the ventricules of transgenic (TG4) mice that have a 195-fold cardiac increase in β_2AR levels. To further characterize RGS5 expression, an RGS5 specific anti-serum was generated. Western Blot analysis of a panel of rat tissues demonstrated that basal expression of RGS5 protein was confined to the heart and skeletal muscle tissues, as well as the respective cell lines HL-1 and C2C12. Although GPCRs and RGSs are actively being studied in the heart, little is known regarding the role of GPCRs and RGSs in skeletal muscle. Moreover, several conditions such as atrophy, which is characterized by apoptosis of the muscle fibers, are associated with GPCRs in the skeletal muscle. As a prelude to investigating the role of RGS5 in the regulation of GPCR signaling, we identified a number of GPCRs that are able to signal via ERK1/2 in C2C12 skeletal muscle cells. The signalling and responses induced by one GPCR agonist, Lysophosphatidic Acid (LPA), a potent inducer of survival and apoptosis, were analyzed in C2C12 skeletal muscle cells.

RESUME

Les voies de signalisation cellulaire engendrées par les Récepteurs Couplés aux Protéines G (RCPG) sont vitaux à la bonne physiologie des cellules et des organes, et les protéines qui y sont impliquées travaillent ensemble et de manière coordonnée à transmettre les messages à travers la membrane cellulaire. Ce processus est grandement régulé par bon nombre de protéines incluant les Régulateurs de Signalisation des protéines G (RSG) qui servent à activer l'activité GTPasique de la sous-unité Gα des protéines G heterotrimérique. Des études précédentes dans notre laboratoire ont identifié RSG5 comme étant exprimé dans le coeur et comme étant surexprimé dans les oreillettes mais pas dans les ventricules de souris transgéniques (TG4) ayant 195 fois plus d'expression cardiaque en récepteurs adrénergiques β_2AR. Afin d'approfondir notre étude sur la charactérisation de RSG5, un anticorps spécifique à RSG5 a été fabriqué. L'analyse Western faite sur un ensemble de tissues de rat démontra que l'expression basale de RSG5 se limitait aux tissues cardiaques et ceux du muscle squelettique, ainsi qu'aux cellules en culture respectives, HL-1 et C2C12. Malgré le fait que les RCPG et les RSG sont continuellement analysés dans le coeur, le rôle joué par les RCPG et les RSG dans le muscle squelettique est pratiquement inconnu. De plus, de nombreuses conditions comme l'atrophie, characterisée par l'apoptose des fibres musculaires, sont associées avec les RCPG des muscles squelettiques. En prévision à l'investigation du role de RSG5 dans la régularisation de la signalisation des RCPG, nous avons identifié plusieurs RCPG capable d'induire des signaux à travers l'activation de la kinase ERK1/2 dans les

cellules musculaires et squelettiques C2C12. La signalisation et les réponses suscitées par une agoniste des RCPG, l'Acide lysophosphatidique, une importante médiatrice de survie et d'apoptose, furent analysées dans les cellules C2C12.

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Contributions of Authors

Manuscript #1: Figures 1 and 2 were entirely generated by previous members of the lab. The manuscript was written by my supervisor, Michael T. Greenwood. The data in figures 3, 4 and 5 are produced by me, as well as draft preparation in respect to all figures. Jurgen Heubach and Ursula Ravens provided the TG4 mice extracts.

Manuscript #2: Figure 1A was generated by Zhao Yang. Analysis of genomic DNA cleavage was performed by Chamel Khoury. All the data in other figures were generated and organized by myself. I have also contributed to the preparation of the manuscript, especially regarding preparation of figures as required for publications and writing the manuscript.

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Chapter 1. LITERATURE REVIEW

1.1 G Protein-Coupled Receptors

1.1.1 Introduction

G Protein-Coupled Receptors (GPCRs) represent the largest family of cell surface proteins involved in transmitting signals across the plasma membrane [1]. These integral membrane receptors are able to recognize and transduce sensory signals such as light, odorants and tastes from the extracellular environment, or interact with a wide variety of chemical ligands such as ions, peptides, hormones, nucleotides and lipid molecules within the body and induce a response inside the cell. They owe their name to their well characterized interaction with heterotrimeric G proteins. GPCRs also bind to a plethora of accessory proteins called GPCR interacting proteins (GIPs) that regulate their signaling properties [2]. Their well-conserved structure of seven hydrophobic transmembrane domains, first identified in bacteriorhodopsin, is the hallmark of this family of receptor, with extracellular N- and intracellular C-terminals. GPCRs are encoded by the largest gene family in most animal genomes: over 1% of our genome encode for well over 1000 members with a heptahelical structure, and more than 80% of all hormones signal through these receptors [3]. Needless to say that, due to their diversity and versatility and because they serve to elicit important and various physiological roles defined over time through altered gene expression, they represent the most pursued drug targets in the pharmaceutical industry [4]. With so many members, attempts to classify these receptors have not been that simple. The most popular classification system, that of Kolakowski et al. [5]

attempted to classify GPCRs into six families. However, many newly cloned receptors could not fit into the families previously established. Recently, the classification by Fredriksson et al. [6] provide a better overview of all the mammalian GPCRs grouped into five main families: Glutamate, Rhodopsin, Adhesion, Frizzled/taste2 and Secretin, forming the GRAFS classification system.

1.1.2 GPCR Activation

Although other components have been identified along the years, a simple paradigm of GPCR activation involves a ternary complex made of the agonist, the receptor and the heterotrimeric G-protein [7]. It states that the receptor binds a ligand on the extracellular side, which induces conformational changes that cause the intracellular loops to bind and activate the G-protein. In the basal state, G-proteins form an heterotrimeric structure, made of a GDP-bound α subunit, a β subunit and a γ subunit, the latter of which are tightly bound and function as the $\beta\gamma$ dimer. The inactive G-protein is then recognized by an activated receptor. Interaction of an activated GPCR with a G-protein results in the dissociation of the bound GDP for GTP on the α subunit. Nucleotide exchange leads to the dissociation of the α subunit from the $\beta\gamma$ subunit. Both the GTP-bound α subunit and the $\beta\gamma$ complex are able to interact with and activate effector molecules, which then regulate the production of what are called second messengers. Second messengers such as cAMP, Ca^{2+} and IP3 induce specific biological responses through further regulation of signaling molecules. Also, this classical view of GPCR activation has been questioned lately upon the finding that some GPCRs

have also been shown to signal through non-G protein-mediated events [8]. GPCRs may exist as either homodimers or oligomers [9]. Indeed, GPCR oligomers are proposed to be synthesized as such and represent a functional signaling unit. Hetero-oligomerization is also thought to affect receptor internalization and desensitization [10], processes which occur when GPCRs are excessively exposed to agonist stimulation (section 1.2.2).

1.1.3 Heterotrimeric G Proteins

G-proteins act as molecular switches through their GTP-binding property to regulate a wide range of signaling pathways. Although small monomeric G-proteins such as Ras exist and mediate several biological responses, only the heterotrimeric G-proteins couple GPCR activation to intracellular signaling cascades. To date, more than twenty Gα subunits have been identified and classified in four subfamilies, namely the $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12/13}$ subfamilies. Five β subunits and twelve γ subunits have also been described [7], although main properties of individual G-proteins seem to be primarily determined by the Gα subunit present in the heterotrimeric complex. Most Gα subfamilies are involved in activating effector molecules such as adenylyl cyclase, phospholipase C, RhoGEF and ion channels. However, $G\alpha_i$ prevents cAMP production through inhibition of adenylyl cyclase [7]. The Gα subunit contains the sites for NAD-dependent ADP-ribosylation that can be catalyzed by bacterial toxins [11]. Signaling partners for G-proteins, such as binding partners or molecules that affect the activation/inactivation state of G-proteins have been

identified. These so-called accessory proteins regulate the strength, efficacy and specificity of signals that emanate from the G-proteins. For example, the exchange of a GDP for a GTP on the Gα subunit normally requires the action of a Guanine Nucleotide exchange Factor (GEF) which stimulates the exchange of GDP for GTP [12]. GPCRs themselves are considered GEFs for heterotrimeric G-proteins. Other non-receptor GEFs such as the newly discovered Activators of G-protein Signaling (AGSs) have been identified [13, 14] and are known regulators of the activation state of G-protein. Another group of accessory protein is the Guanine nucleotide Dissociation Inhibitors (GDIs), such as the GoLoco motif-containing proteins, which inhibit exchange of GDP for GTP and have also been shown to prevent re-association of the Gα subunit with the βγ complex [14]. Finally, the last group of accessory proteins for G-proteins involves a set of effector proteins called GAPs (GTPase Activating Proteins) which serve to activate and/or accelerate the GTPase activity of the Gα subunit [15]. One common feature of Gα-proteins is that their rate of GTP hydrolysis is relatively slow and needs to be enhanced. This is especially obvious in the retina where rhodopsin receptor signaling must be quickly inactivated to allow vision to occur [16]. Interaction with GAPs provides the acceleration needed when GTP hydrolysis needs to occur in order for G-protein signaling to stop. One such type of GAP proteins are the Regulators of G-protein Signaling (RGSs) whose functions will be described later (section 1.2.3).

1.1.4 GPCR-associated intracellular signaling pathways

Regulation of the Mitogen-Activated Protein Kinase (MAPK) cascade is a well studied intracellular signaling pathway activated by G-protein Coupled Receptors [17]. The mammalian MAPK cascade consists of a conserved phosphorelay system made of three protein kinase reactions which serve to transduce signals from the extracellular environment to the cytoplasm and nucleus [18, 19]. This system is made of three major components: first a MAPK kinase kinase which phosphorylates and activates the MAPK kinase, which in turn phosphorylates and activates the MAPK. All these kinases are dual specificity kinases and the magnitude and duration of their activation is tightly regulated by phosphatases [20]. To date, five MAPK families have been identified and are ERK1/2, JNK1/2/3, p38α/β/γ/δ, ERK5 (Big MAPK 1) and ERK7.

ERK1/2 is mostly cytoplasmic and translocates to the nucleus upon stimulation where it regulates the activity of nuclear proteins such as transcription factors to mediate proliferation [21]. ERK1/2 has also been shown to control nucleotide synthesis and enhance protein translation [22]. JNK [23] and p38 [24] MAPKs are usually referred to as the stress-activated kinases since they regulate stress-induced apoptotic events. MAPK regulation of biological systems is also mediated by scaffolding proteins which can bind and organize multiple signaling molecules in these cascades as a complex, localize the complex in specific area inside the cell and protect the active kinases from dephosphorylation by phosphatases [25]. A well studied example of a scaffolding protein-dependent MAPK activation pathway involves cytosolic proteins called arrestins [26]. The mechanistic details regarding the scaffolding properties of arrestin will be

discussed in this literature review (section 1.2.2). GPCR-mediated ERK1/2 activation occurs in some cases through transactivation of receptor tyrosine kinases to mediate mitogenic responses [27, 28]. In rat hepatic C9 cells, angiotensin II-induced ERK1/2 activation occurs via transactivation of the EGF receptor in PKCδ and Src/Pyk2 complex-dependent manner [29]. In contrast, β-arrestin-mediated ERK1/2 activation does not appear to regulate any transcriptional event nor induce a mitogenic response. This is explained by the scaffolding property of β-arrestins which is characterized by the cytosolic retention of ERK1/2 [30]. This event has important physiological consequences and may explain why some GPCRs simply do not stimulate proliferation or induce DNA synthesis in some cells. This is seen during activation of wild type PAR2 receptor, which induces ERK1/2 activation in absence of mitogenic responses, whereas a mutant PAR2 that is unable to bind β-arrestins induces the accumulation of a predominant pool of ERK1/2 and subsequent mitogenic responses [31]. Alternatively, β-arrestin-mediated GPCR activation of ERK1/2 include responses such as cell motility, chemotaxis and apoptosis. Other intracellular signaling pathways activated by GPCRs include the PI3K/Akt and Rho-dependent type of pathways to mediate survival and cytoskeleton-related events, respectively [32, 33].

1.2 Termination of GPCR-mediated signaling

1.2.1 Introduction

Signal termination occurs either at the level of the receptor, a process called desensitization (section 1.2.2) because it involves the uncoupling of a given GPCR with a G-protein or at the level of the heterotrimeric G-protein via hydrolysis GTP by the Gα subunit. This step is tightly regulated by GAP activity-containing proteins such as RGSs (section 1.2.3). GDP-bound Gα subunit is then able to re-associate with the βγ complex and this represents the mechanism of G-protein inactivation.

1.2.2 GPCR desensitization

Desensitization is a process by which rapid attenuation of receptor responsiveness to a given agonist occurs and it results from a set of mechanisms. First, receptor phosphorylation induces the uncoupling of the receptor from the G-protein; the phosphorylated receptor is then internalized into intracellular compartments; finally, downregulation of the total cellular amounts of receptor proteins, possibly through lysosomal degradation. Two types of receptor desensitization have been described based on the activation state of the GPCR [34, 35]. Homologous desensitization involves phosphorylation of agonist-occupied receptors by a family of proteins called G-protein-coupled Receptor Kinases (GRKs) [36]. Indeed, GRKs have been shown to phosphorylate serine and threonine residues within the third intracellular loop and carboxyl-terminal tail domains of many GPCRs. To date, seven mammalian GRKs have been

identified, namely GRK1 (rhodopsin kinase), GRK2 or βARK1 (β-adrenergic receptor kinase-1), GRK3 or βARK2 (β-adrenergic receptor kinase-2), GRK4, GRK5, GRK6 and GRK7 (cone opsin kinase) [35]. With the exception of GRK1 and GRK4 whose expression are almost exclusively confined to the retina and testes respectively, and GRK2 and GRK6 who are most abundantly expressed in skeletal muscle [37], GRKs are ubiquitously expressed. Functions of GRKs are highly regulated, mainly through subcellular localization, alterations of their kinase activity or at the expression levels [38, 39]. In addition, phosphorylation of GPCRs by GRKs serves to recruit and promote the binding of arrestin proteins [35, 40], which prevents further receptor-mediated G-protein binding and activation, hence desensitization. The arrestin protein family contains only four members, namely arrestin 1 (rod arrestin), arrestin 2 (β-arrestin 1), arrestin 3 (β-arrestin 1) and arrestin 4 (cone arrestin) [40]. Arrestin 1 and 4 are expressed exclusively in retinal rods and cones, respectively where they regulate the rhodopsin and color opsin receptors and are thus commonly referred to as the visual arrestins. Arrestin 2 and 3 display a marked preference for binding βARK-phosphorylated β2-adrenergic receptors (β2AR) *in vitro* [41, 42], hence the name β-arrestin. A general paradigm regarding the function of β-arrestins in GPCR desensitization is their ability to block coupling of the activated receptor to the heterotrimeric G-protein required for signaling. This is true, at least in the case of the β2-adrenergic receptors, for which it has been originally reported [43, 44]. Recently, the arrestins have emerged as key players that also mediate GPCR internalization and recycling, signaling, and even degradation [26, 40]. Indeed, β-

arrestins are known endocytic adaptors that couple receptors to several components of the endocytic machinery. Physical interactions of β-arrestins with clathrin [45], the adaptor protein AP-2 [46], N-ethylmaleimide-sensitive fusion protein (NSF) [47] and ADP-ribosylation factor 6 (ARF6) [48] which are elements involved in internalization and/or vesicular trafficking have all been demonstrated. Following clathrin-coated pits-mediated internalization of GPCRs, some β-arrestin-associated mechanisms dictate the fate of the receptor. Receptor recycling to the cell surface has been shown to be β-arrestin-dependent. Class A receptors such as the β2AR, which bind only transiently to β-arrestin are likely to recycle rapidly, whereas class B receptors (angiotensin II receptor) bind more tightly to β-arrestin, internalize together with it and recycle much slower [49]. Once internalized, the ligand-bound receptor can still activate intracellular cytoplasmic signaling pathways, a process largely mediated by the scaffolding properties of β-arrestins. These proteins have been found to serve as adaptor molecules and specific binding between the β-arrestin isoforms and MAPK cascade proteins, non-receptor tyrosine kinases and other proteins such as IκBα have been demonstrated, thereby allowing the GPCR to signal in a G-protein-independent manner [26]. In addition, the finding that β-arrestin was able to bind MDM2, an E3 ubiquitin ligase, suggest that ubiquitination plays a role in sorting the receptor to the lysosome for subsequent degradation [50]. For example, Marchese *et al.* have reported that ubiquitination was required for the targeting of the CXC chemokine receptor 4 (CXCR4) to lysosomes for degradation and prior to this event, CXCR4 internalization is β-arrestin-dependent [51]. In summary,

the multifunctional nature of arrestin proteins allows them to act as both negative and positive regulators of GPCR signaling.

The second mode of GPCR desensitization occurs via second messenger-dependent protein kinase-mediated phosphorylation of the receptors, such as that induced by cyclic-AMP-dependent protein kinase (PKA) or protein kinase C (PKC) [52]. However, agonist occupancy of the receptor does not seem to be required in order for phosphorylation to occur. As such, unactivated receptors, including receptors for other ligands can be desensitized through heterologous desensitization.

1.2.3 Regulators of G-protein Signaling (RGSs)

Regulators of G-protein Signaling (RGSs) are classically defined as GTPase activating proteins (GAPs) for heterotrimeric G proteins. Instead of activating GTP hydrolysis, they rather accelerate the rate of GTP hydrolysis of the Gα subunit [16]. Consequently, RGSs are important negative regulators of G protein signaling and thus provide another level of regulation in the process of GPCR/G protein signal termination. The first evidences for the existence of the RGSs came after the observation that fast physiologic deactivation rates of G-protein signaling which occur in a cellular context did not correlate with the slow GTP hydrolysis rates seen in *in vitro* reconstituted systems [53]. These findings prompted the search for proteins that could accelerate the rates of GTP hydrolysis and thus explain this timing discrepancy. The discovery of RGS proteins and functions came from studies in the yeast *Saccharomyces cerevisiae* where the yeast Sst2 protein was identified [54]. Sst2p was found to regulate G-protein-

mediated response to mating pheromone. At the same time, another gene (*flba*) was identidied as a negative regulator of G-protein signaling involved in development of the fungus *Aspergillus nidulans* [55]. While other researchers were identifying proteins with similar properties and conserved domains, full evidence that a superfamily of negative regulators of G-protein signaling did exist came after the identification of the *Caernorhabditis elegans* RGS gene EGL-10 [56]. All these proteins were assembled into one family, named Regulators of G-protein Signaling (RGSs). RGS proteins are mostly dissimilar in size and sequence. However, all these proteins contain a conserved ~128 amino acids domain called the RGS domain (RGS box). Today, human RGS protein family comprises 37 highly divergent and multifunctional proteins. Based on amino acid identities within the conserved RGS domain, mammalian RGSs proteins have been classified into eight subfamilies, named from A to H [14]. However, two multiple RGS-box containing proteins D-AKAP2 and RGS22 did not belong to any of the eight subfamilies.

The molecular mechanism by which RGSs act as GAPs is actively being studied. The RGS domain is believed not to make direct contact with the bound GTP, which further confirms the idea that RGSs do not take part in the chemistry of GTP hydrolysis but rather modify the confirmation of the GTP-Gα complex such that GTP hydrolysis can occur more efficiently [16]. GAP activity for many RGS proteins has been demonstrated, especially for the small RGSs of the B subfamily [57, 58], which contain no other functional domain aside from the RGS domain. Also, with respect to the negative regulation that they provide to G-protein signaling, RGS proteins can act as effector antagonists that prevent G-

proteins from binding to their effectors. Indeed, RGS2 was found to block slow muscarinic inhibition by N-type channels in HEK293 cells by functioning as an effector antagonist rather than as a GAP [59]. Furthermore, overexpression of RGS1 in COS cells results in the inhibition of $G_{\alpha 12}$-induced signaling, although RGS1 failed to exhibit GAP activity towards $G_{\alpha 12}$ [60]. Effector antagonist capacities of many other RGSs such as RGS5 have not been assessed.

Aside from the defining RGS-box, many RGS proteins contain a variety of additional motifs which confer other properties than just the GAP activity. Indeed, many RGSs contain a variety of structural domains, allowing them to act as novel effectors or scaffolding proteins of G-protein signaling [14, 61].

Which of the small RGS proteins interact with which Gα subunits and how selectivity for these interactions is determined is still unknown. An important concept in the RGS field is that of receptor/G-protein selectivity and specificity of RGS proteins. Given that there are about 20 Gα subunits and more than 30 RGS proteins, it was predicted that pairing an RGS with a Gα subunit would be fairly possible. We now know from *in vitro* assays, that RGSs are quite promiscuous regarding which Gα subunit they bind [58]. However, recent studies have provided insights into the molecular mechanisms regulating the recruitment of a particular RGS to a given G-protein [62], stating that the presence of the coupled receptor provide the specificity and selectivity needed and seen *in vivo*. It has been suggested that domains outside the RGS-box may confer selectivity towards a given GPCR. An appropriate way of addressing such a question would be through direct interactions of a GPCR and an RGS protein. There is growing evidence that seven transmembrane receptors directly bind to RGSs. Studies

providing proof of interactions have been done *in vitro*, using GPCR fragments [63] and overexpressed RGS proteins [64].

RGS proteins are widely expressed, albeit some exceptions [65]. Many tissues express multiple RGS proteins, although some show tissue specificity. RGS5 protein was found to be expressed only in heart and skeletal muscle [66], although its mRNA was also found in the brain [67]. Within the cell, most RGS proteins are localized in the cytoplasm and translocate to the membrane upon receptor activation [58]. Nuclear pools of RGSs have also been described [68]. Translocation of RGS proteins to their cellular regulatory sites may provide a level for regulation of RGS function. Other cellular mechanisms involved in regulating RGS functions involve phosphorylation [58]. RGS genes have also been shown to be regulated transcriptionally while splicing of many RGS genes increases the repertoire of RGS proteins [61].

Because they are important modulators and integrators of G-protein signaling, RGSs have emerged as potential new therapeutic targets [58, 69, 70]. Knockout studies have revealed a novel role for RGS2 in hypertension [71]. A role for RGS4 and RGS9 in schizophrenia and drug addiction respectively has also been proposed [64, 72]. Altered RGS protein expression has also been seen during hypertrophy and heart failure [66, 73], sepsis [74] and many other diseases [73].

1.3 LYSOPHOSPHATIDIC ACID

1.3.1 Introduction

Lysophosphatidic Acid (LPA) is a bioactive and naturally occurring phospholipid with diverse biological actions. Although originally known as a key intermediate in lipid biosynthesis, LPA is now considered an important extracellular lipid mediator which exerts growth factor-like responses. LPA can be produced by a wide variety of cell types including adipocytes [75], leukocytes [76], fibroblasts [77], neuronal cells [78], ovarian cancer cells [79] and others [76], but the best characterized cellular sources of LPA are blood platelets, which release LPA in the extracellular environment upon activation. Consequently, physiologically significant levels (1μM) of bioactive LPA are present in serum where it is found bound to albumin [80-82] and other proteins such as gelsolin [83], liver fatty acid binding protein (LFABP) [84] and 15 and 28 KDa proteins still as yet unidentified [81]. In humans, local LPA concentrations were found to increase within 24h of blood clotting from approximately 1μM to 6μM [85]. LPA has also been found in various other body fluids such as saliva, follicular fluids and ascitic fluid from certain cancer patients [86].

1.3.2 Metabolism of LPA

Although LPA can be produced by multiple enzymatic pathways, four major ones have been described which probably account for the vast proportion of this lipid mediator that is found either in serum or blood plasma. Indeed, LPA production and degradation involves a number of complex biochemical pathways

which have been reviewed in a many papers [87-89]. Extracellular lyso-PLD activity has long been detected in rat plasma, where it contributed to LPA production using LPC as substrate [86]. Recently, plasma lyso-PLD was found to be identical to autotoxin [90, 91], a 125KDa ectophosphodiesterase previously identified as a melanoma cell-motility factor acting in an autocrine fashion [92] and implicated in nucleotide metabolism [93, 94]. Autotaxin/Lyso-PLD belongs to the family of ecto-nucleotide pyrophosphatases/phosphodiesterases (ecto-NPPs) and is widely expressed.

1.3.3 Signaling induced by LPA

LPA-specific cell surface receptors mediate its signaling and responses. Receptors for LPA belong to the well known family of G-Protein-Coupled Receptors (GPCR) and were originally named Endothelial-cell-Differentiation Genes (EDG) [95]. To date, four known LPA receptors have been identified. EDG-2 (LPA1), EDG-4 (LPA2), EDG-7 (LPA3) share sequence identity with other receptor families such as the cannabinoid receptor family. The fourth receptor $P2y_9$/GPR23 (LPA4) (20-24% amino acid identity with LPA1-3) is more similar to the platelet-activating factor (PAF) and purinergic receptor families than to the other LPA receptors [96]. LPA1-3 receptors are widely expressed in embryonic as well as adult tissues and upregulated in various cancer cells, whereas LPA4 expression is restricted to the ovary and to a much lesser extent the kidney and skeletal muscle [97].

Non-GPCR targets for LPA have been reported, such as the Peroxisome Proliferator-Activated Receptor-γ (PPARγ) which was proposed to be an

intracellular receptor for LPA [98]. This area of research is currently under investigation, with a few reports suggesting the possible significances of LPA binding to PPARγ [99] or LPA effect on PPARγ expression and function [100].

Like all GPCRs, LPA receptors signal through G proteins, notably the Gαi, Gαq and Gα12/13 family of G proteins to activate a wide range of signaling cascades. LPA-mediated Rho activation occurs through LPA receptor coupling to the Gα12/13 family of G proteins and has been known to induce proliferation and cytoskeleton responses such as cell adhesion, stress fiber formation [101], cell rounding and neurite retraction [102]. Recently, Yamada et al. [103] reported physical and functional interactions of LPA receptors with the PDZ domain of RhoGEFs to activate RhoA. LPA receptors coupling to Gαi family of G proteins results in inhibition of adenylyl cyclase with concomitant activation of the Ras-MAPK cascade and PI3K to mediate proliferation and survival against apoptosis in a variety of cells [101, 104, 105], as well as hypertrophy of neonatal cardiac myocytes [106]. Finally, LPA receptors coupling to Gαq family of G proteins activates PLC with subsequent effect on calcium homeostasis and cell proliferation. LPA-induced transactivation of receptor tyrosine kinase has also been reported [107]. Finally, LPA-mediated activation of other signaling molecules has been investigated, notably p38 and JNK MAP Kinases [108], nuclear transcription factor CREB [109] and mammalian Target Of Rapamycin (mTOR) [110].

1.3.4 Biological responses elicited by LPA

The very first responses elicited by LPA which suggested that this lipid molecule may be a bioactive molecule were platelet aggregation, smooth muscle contraction, and changes in blood pressure. We now know that LPA is capable of inducing a plethora of common biological responses in a wide variety of mammalian cells including proliferation [111] and even apoptosis [112]. Also, pleiotropic responses to LPA have been suggested [97, 113], which further highlights the plethora of biological responses elicited by such a simple lipid molecule. Finally, a role for LPA has been proposed in physiological processes and diseases.

1.3.5 LPA in physiological processes

1.3.5. a) Role of LPA in wound healing

Because it is secreted by thrombin-activated platelets during the wound healing process and injured fibroblasts, LPA has for long been referred to as the "wound hormone" [114], stimulating tissue repair events such as vasoconstriction, matrix metalloproteases production, proliferation and migration of epithelial cells. Furthermore, LPA has been shown to induce wound healing when applied to the skin [115].

1.3.5. b) Role of LPA in development

LPA signaling has also emerged as an important process which occurs during development of vertebrates and simple organisms such as nematodes and the slime mold [88]. Indeed, LPA has been implicated in the development of cardiac, vascular, immune and neuronal systems to name a few. Recently, Ye X.

et al. [116] has proposed a role for the EDG-7 (LPA3) receptor in embryo implantation and spacing.

1.3.6 LPA in the pathology of diseases

1.3.6. a) Role of LPA in cancer

The finding that autotaxin acts as a melanoma cell-motility factor may provide a link between LPA production and the progression of cancer. Indeed, the LPA-mediated migration of both tumor cells and non-cancerous cells such as mouse skin fibroblasts [117] may generate an invasive environment and thus contribute to metastasis during cancer. The idea of LPA being a key contributor to the metastatic cascade and to cancer is thoroughly being investigated. The role of LPA in ovarian cancer is by far the best studied, as LPA is a well-known growth factor for ovarian cancer cells and elevated LPA levels have been found in plasma of patients suffering from this type of cancer. Indeed, ovarian cancer cells produce significant amounts of LPA [79] and LPA present in ovarian cancer ascites has been shown to mediate cell growth, invasion, prevent apoptosis and sensitivity to the chemotherapeutic agent cisplatin [118]. The role of LPA in ovarian cancers is still under intense investigations. It was found that LPA-mediated ovarian cancer cell proliferation occurs through induction of granulin-epithelin precursor (GEP), a growth and survival factor for ovarian cancer [119]. A role for LPA has also been proposed in other types of cancers. The EDG-4 (LPA2) receptor was found to mediate mitogenic signals in colon cancer cells [120], possibly through the β-catenin pathway [121]. Furthermore, LPA promotes the survival of lymphocytic leukemia cells through activation of Akt/PKB [122]. LPA mediating growth and

survival of prostate, breast, lung, brain and pancreatic cancer cells have also been reported [123].

1.3.6 b) Role of LPA in atherosclerosis

Increased LPA production which occurs during platelet aggregation following vascular injuries may have deleterious effects on cardiovascular diseases. Atherosclerosis is a pathological condition characterized by endothelial dysfunction and accumulation of oxidized low density lipoproteins (LDL) [124]. Bioactive LPA has been shown to accumulate within mildly oxidized LDL seen in atherosclerotic lesions and possibly represent the active ingredient responsible for endothelial inflammation, permeability and impairment during atherosclerosis [124, 125]. LPA was also found to stimulate proliferation of vascular smooth muscle cells and fibroblasts, thus contributing to endothelial instability, as well as induce chemotaxis of lymphocytes and monocytes seen in the inflammation process during atherosclerosis [124].

1.3.6 c) Role of LPA in CNS injury

Astrogliosis, a process believed to interfere with neuronal repair and axonal regeneration, occurs during CNS injury when astrocytes become hypertrophic and start to proliferate [126]. LPA receptor activation was found to induce astrogliosis in vivo and proliferation of cultured murine cortical astrocytes [127]. Also, the fact that LPA is able to mediate hyperphosphorylation of Tau protein has led investigators to suggest a role for this lipid molecule in Alzheimer's disease [128].

Chapter 2. INTRODUCTION TO MY STUDIES

2.1 Rationale and Objectives of my studies

The observation that GPCR-mediated signaling can be dampened at the level of the G-protein by the Regulators of G-protein Signaling (RGSs) has attracted a lot of interest, especially in the cardiovascular field, where many pathophysiological conditions are characterized by diminished responsiveness of GPCRs to agonist stimulation. One typical example is provided by Rogers et al. [129] who reported that RGS4 overexpression resulted in decreased contractile and hypertrophic responses mediated by the endothelin-1 (ET-1) receptor in the myocardium, resulting in heart failure. This defect in ET-1-stimulated cell contraction and the possible involvement of RGS4 has also been reported by another group [130]. Altered expression of a number of RGSs has also been seen in cases of human congestive heart failure and during sepsis [73]. Because they overexpressed constitutively activated human β2-adrenergic receptors (β_2-ARs), TG4 mice represent a widely used model of heart failure, since these mice exhibit altered β_2-AR-mediated signaling in the atria [131]. Using an RT-PCR based approach, our lab previously identified RGS5 as being upregulated in the atria of TG4 mice, which was further confirmed by Northern blot analysis. To further assess the role of RGS5 in β_2-AR-mediated signaling, our lab generated an anti-serum against RGS5. I have used this antibody to characterize the expression of RGS5 protein in a variety of tissues and cell lines and to study the upregulation of RGS5 in another model of constitutive activity of β_2-AR-signaling. Furthermore,

based on my observation that RGS5 protein was expressed in skeletal muscle, we questioned the role it played in this tissue. Because most RGSs are promiscuous regarding which Gα subunit they bind to, and because a degree of specificity might be directed by the coupled receptor, we reasoned that it would serve to regulate the signaling of a subset of GPCRs in this tissue. A number of GPCRs, such as receptors for Lysophosphatidic Acid (LPA), and accessory proteins have been reported to be expressed in skeletal muscle [37]; the exact signaling cascades they activate and how they are regulated remain to be defined. In order to gain insights into GPCR signaling and responses in skeletal muscle and any possible regulation by RGS5, I have used the skeletal muscle cell line C2C12.

Chapter 3. RESULTS

3.1 Introduction

During the course of my masters, I was involved in two sets of projects. The first project, which is depicted in section 3.2, studies the regulation and expression of RGS5. The second one found in section 3.4 analyzes the responses elicited by LPA, a GPCR agonist in skeletal muscle cells.

3.2 Manuscript #1: β-adrenergic receptor-mediated atrial specific upregulation of RGS5

3.2.1 Introduction

Regulators of G-protein Signalling (RGS) are identified by the presence of a conserved 120-125 amino acid motif, which is referred to as the RGS box [61, 73, 132]. RGS containing proteins have been classified into six subfamilies. Most RGS containing proteins are relatively large proteins (> 300 residues) and they possess a number of previously identified sequence motifs that suggest specific functions. For example, GRK2 has a kinase domain that serves to phosphorylate GPCRs while its RGS box is capable of inhibiting $G_{\alpha q}$ [133]. In contrast, the R4 subfamily of RGS containing proteins is small (ca. 200 residues). These RGSs negatively regulate G-Protein Coupled Receptor (GPCR) signalling mainly by decreasing the levels of the receptor activated G_{α}-proteins [61, 73]. Since this inhibition occurs without affecting the receptor per se, RGSs are strong candidates to be involved in mediating decreases in GPCR responsiveness that are not due to the internalisation of cell surface receptors. This role is strongly supported by the fact that a number of RGSs are induced in response to a wide variety of stimuli and that increased levels of RGSs lead to significant decreases in GPCR responsiveness. Evidence now exists that the basal cellular level of RGS proteins will also serve to regulate the responsiveness of GPCRs to agonist stimulation. For example, Heximer et al. [71] have demonstrated an increased responsiveness of the P2Y receptor in smooth muscle cells of mice lacking the

RGS2 gene. These animals develop a number of abnormalities, including hypertension, indicating that basal control of cardiovascular GPCR responses by RGS2 is of critical importance for regulating signaling.

The ß adrenergic receptors ($ß_1$- and $ß_2$AR) are the most powerful receptors for stimulating an increase in cardiac output in the failing heart [134]. Therefore the loss of ßAR responsiveness that occurs in chronic heart failure is a prominent feature in the progression of the disease. Although decreased ßAR responsiveness in some forms of cardiac failure is due to a loss of $ß_1$AR on the cell surface, there is no decrease in cell surface receptors associated with reduced ßAR responsiveness in sepsis and other forms of heart failure [135, 136]. Up-regulation of RGSs may account for the hyporesponsiveness to vasoactive GPCR agonists that occur in cardiovascular tissues during sepsis and in the heart during heart failure. For example, RGS1, RGS4, RGS7 and RGS16 mRNA levels have been shown to increase in animal models of sepsis [135, 137, 138] while RGS3 and RGS4 levels are elevated in failing human hearts [130, 139].

A number of transgenic animal models that overexpress adrenergic receptors in the heart have been developed [140]. In addition to serving as models of cardio pathologies, the study of these animals has also generated insight into the basic physiology of cardiac AR signaling. TG4 mice, which have a 195-fold increase in cardiac $ß_2$AR levels, are a widely used model [131, 141, 142]. The overexpressed $ß_2$AR are partly constitutively active and, consequently, the heart rate of these animals remains maximal even at rest. In spite of these observations, there is experimental evidence to suggest that most of the overexpressed $ß_2$AR do not properly couple to G_α-proteins [131]. Since RGSs are capable of functionally

uncoupling GPCRs and a number of RGSs are induced in response to GPCR stimulation, we hypothesized that some RGS(s) may be up regulated in the heart of TG4 animals. Using a degenerate RT-PCR based screen, we have identified RGS5 as being up regulated in the atria but not in the ventricles of TG4 mice. We further demonstrate that the chronic administration of the ßAR agonist isoproterenol to rats is sufficient to cause an increase in the levels of RGS5 in the atria.

3.2.2 Materials and Methods

Animals and tissues. TG4 and their non-transgenic littermates were generated and identified as previously described [131]. Sprague Dawley (male, 280-320g) rats were used to study the effects of isoproterenol on RGS5 gene expression. The animals were given a daily intra-peritoneal (i.p.) injection of isoproterenol (2.4 mg/kg) for 14 days and the animals were sacrificed 4 hours after the last treatment [143]. Control animals were injected with the same volume of physiological saline. Tissues were collected by dissection, quick frozen in dry ice and stored at -80^{0}C. All protocols conformed to the McGill University Animal Care and Research Ethics Committees.

Degenerate RT-PCR. Ventricles and left atria of ten TG4 mice and 12 of their non-transgenic littermates were rapidly dissected and snap-frozen in liquid nitrogen. RNA was isolated and pooled, reverse transcribed and amplified by PCR using degenerate RGS specific oligonucleotides as previously described [137]. The 240 base pair PCR fragments generated were cloned and divided into different groups based on the patterns obtained with a variety of restriction

endonucleases. Amino acid sequences deduced from the DNA sequences were used to search the protein sequence database at GenBank [144].

Northern Blot Analysis. Ten μg of total atrial RNA or twenty μg of total ventricular RNA isolated from TG4 mice and their non-transgenic littermates were separated on a 1.5% agarose/formaldehyde gel and transferred onto a nylon membrane. The cloned 240 bp mouse RGS5 PCR product, corresponding to an internal portion of the RGS motif of RGS5 [137], was used as a specific probe to detect the RGS5 transcript, while the $ß_2$AR cDNA used as a probe was previously described [145]. Preparation of the (α-^{32}P)-dCTP radiolabelled DNA probes as well as the hybridization conditions used were previously described [137].

Production of RGS5 and RGS16 anti-serum. The entire coding sequences of human (h) RGS5 and hRGS16 were amplified using the previously described RGS5 and RGS16 containing plasmids [137]. The following forward 5'-GGGATCCCCATGTGCAAAGGACTTGCAGCT -3' and reverse 5'-CTCGAGCTACTTGATTAACTCCTGATA-3' primers were used for RGS5 and the following forward 5'-GGGATCCCCATGTGCCGCACCCTGGCCGCC-3' and

reverse 5'-GCTCGAGTCAGGTGTGTGAGGGCTCGTC-3' primers were used for RGS16. The PCR products of the coding sequences of RGS5 and RGS16 were individually subcloned as *BamH1-Xho1* fragments into pGEX-5X-3 (Amersham Bioscience). The plasmids were introduced into *E. coli* and recombinant GST-RGS5 and GST-RGS16 were produced and purified using glutathione-sepharose beads as recommended by the manufacturer (Sigma-Aldrich). The recombinant GST-RGS fusions were concentrated using Centricon

filtration centrifugation (Millipore), emulsified in incomplete Freud's adjuvant and used to immunize rabbits. The anti-sera were collected and used 10 days following a third injection of GST-RGSs. The anti-RGS5 serum was further purified by antigen affinity chromatography. Briefly, purified GST-RGS5 protein was first fixed to Affi-Gel 15 agarose beads as described by the manufacturer (BioRad). The RGS5 anti-serum was then bound to the RGS5 bound agarose beads and subsequently eluted using 0.1M glycine pH 2.5. The eluted anti-serum was neutralized to pH 7.5 with 1M Tris pH 9.0 and subsequently used for western blot analyses. Although the affinity purified anti-sera specifically recognizes RGS5, it nevertheless retained its ability to recognize bacterial GST on a western blot.

Protein extraction and western blot analysis. Soluble protein was extracted from yeast by directly heating the NaOH treated cells to 95^0C in SDS-PAGE loading buffer as previously described [145]. Soluble protein was extracted from tissues using a Polytron in 2-3 volumes of ice-cold homogenizing buffer (50mM Tris-HCl, (pH 7.4), 0.2M sucrose, 1mM EDTA, 1mM PMSF, 1mM DTT, 1mM Benzamidine and 1% NP-40). Soluble protein was extracted from cultured cells using 1 ml of ice-cold lysis buffer per 60mm dish (50mM Tris-HCl (pH 8.0), 150mM NaCl, 0.1% SDS, 100μg/ml PMSF, and 1% NP-40). Twenty to fifty μg of soluble proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with different primary anti-serum [146]. Commercially available rabbit anti-p44/p42 MAP kinase (Cell Signaling Biotechnology) was used as described by the manufacturer. HRP-conjugated secondary anti-serum was used and signals were subsequently detected with ECL plus

(AmershamBioscience) followed by exposure to X-ray film (Kodak X-Omat). The relative expression levels were determined using Multi-Analyst software (BioRad).

Mammalian cultured cells. C2C12 (CRL-1772) and HEK293A cells were grown in Dulbecco's modified Eagle medium (DMEM) media containing 10% fetal bovine serum (FBS). HL-1 cells were grown in Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 10% FBS [147]. HEK293A cells were transfected with pcDNA3.1 control vector or the pcDNA3.1 plasmid expressing human RGS5 [148]. Stable cell lines were selected for and maintained by the addition of 400 μg/ml of Geneticin (G418). C2C12 cells that reached 80% confluency were differentiated into myotubes by placing them in differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum.

3.2.3 Results

RGS5 is up-regulated in the atria of TG4 mice

A degenerate RT-PCR based strategy was used in order to identify RGSs that may be up regulated in the hearts of transgenic TG4 mice that overexpress the $ß_2$AR. We have previously used the same strategy to identify RGS1 and RGS16 as being up regulated in the heart of septic animals [137]. Total RNA from the atria and ventricles of TG4 mice as well as their non-transgenic littermates was isolated, reverse transcribed into DNA, amplified by PCR using degenerate RGS-specific oligos and the resultant RGS containing fragments

cloned. The nucleotide sequence of twenty 240-bp RGS containing clones was determined from each group. Eight of the 20 clones analyzed from the ventricles of control animals were identified as RGS5. In contrast, RGS5 was not identified in the 20 clones isolated from the atria of control animals. Nine RGS5 sequences were identified in ventricles while 5 RGS5 sequences were identified in atria in the clones obtained from TG4 mice. These results suggested that RGS5 is specifically up regulated in the atria of TG4 mice. To confirm these results, northern blot analysis was performed using total RNA isolated from each tissue. A single band corresponding to a 4.4 kb transcript was detected using RGS5 as a probe in the atria and ventricles of TG4 animals (Fig.1A). Densitometric analysis, using 18S rRNA (Fig. 1B) to control for loading, revealed a 3.2-fold increase in the basal levels of RGS5 levels in the atria of TG4 mice when compared to the levels observed in the atria of control animals. In contrast, there was no observable difference in the levels of RGS5 mRNA in ventricles of both animals. As a control, the blot was re-probed with a $ß_2$AR cDNA (Fig. 1C). The two characteristic transgene $ß_2$AR transcripts [142] were observed only in the RNA of TG4 animals. These results indicate that RGS5 is specifically up regulated in the atria of TG4 mice.

Generation and characterization of RGS5 specific anti-serum

In order to further characterize RGS5 expression, we generated an RGS5 specific anti-serum in rabbits using purified recombinant GST-RGS5 fusion protein. The resultant anti-serum was further enriched by affinity column chromatography. As a first step towards characterizing the RGS5 anti-serum we used extracts of yeast cells expressing RGS5 and RGS16, since we had previously

demonstrated that these RGSs could be functionally expressed in these cells [137, 145]. A prominent band having an estimated molecular weight (MW) of 23 kDa is detected in extracts prepared from yeast cells expressing RGS5 (Fig. 2A, left panel). The size of the band corresponds to the calculated MW of 20,9 kDa for human RGS5. In addition, the anti-serum is specific for RGS5 since it did not recognize RGS16 in extracts of yeast cells expressing RGS16. As a control, we used an RGS16 anti-serum to demonstrate that a 25 kDa protein having the appropriate size for RGS16 (calculated MW of 22,6 kDa) is present in the extracts of yeast expressing RGS16 (Fig. 2A, right panel). Since RGS16 shares a high degree of sequence identity to both the RGS box and N-terminal non-RGS region of RGS5 [73, 137], these results suggest that the RGS5 anti-serum is specific for RGS5. As a further control, we demonstrate that the RGS5 protein is also detected by the anti-serum in extracts prepared from HEK293A cells that are transfected with a pcDNA vector expressing RGS5 but not in extracts from the same cells transfected with pcDNA alone (Fig 2B). In contrast, the non-affinity purified RGS16 anti-serum is of limited use since it recognizes RGS5 on a western blot (not shown).

Tissue distribution of RGS5 protein

Although the mRNAs and proteins for a number of RGSs have been shown to be present in the heart, the tissue distribution of the RGS5 protein has not yet been reported. In order to determine the distribution of RGS5 protein, western blot analysis was carried out using extracts prepared from a variety of different rat tissues. RGS5 was detected in heart and skeletal muscle but not in brain, lung, spleen or kidney (Fig. 3). These results are consistent with the

previously published tissue distribution of RGS5 mRNA [67]. To control for possible loading artifacts, the level of total p44/42 Map kinase was also determined in the same extracts. Although p44/42 is not usual control for loading, it is widely used as control when examining changes in the levels of the phosphorylated forms of this Map kinase. Densitometric analysis using p44/42 Map kinase as an internal standard revealed that the heart expresses 9 fold more RGS5 than skeletal muscle. Because RGS5 is expressed in blood vessels, including aorta, pericytes, endothelial and vascular smooth muscle cells [73, 149-151] we used cultured cell lines to ascertain if RGS5 is expressed in muscle cells. A 23-kDa protein that has the same MW as RGS5 expressed in yeast was detected in skeletal muscle C2C12 cells and cardiomyocyte HL-1 cells but not in the vascular smooth muscle A10 cell line (Fig. 4). This suggests that RGS5 is indeed expressed in skeletal and cardiac muscle cells.

C2C12 cells grow as myoblasts that are capable of fusing in order to develop into non-growing terminally differentiated myotubes [152]. Tsai et al. [153] have previously reported that overexpression of $G_{s\alpha}$ delays the development of C2C12 myotubes. Given that RGSs are involved in regulating heterotrimeric G-protein signaling, we examined the expression of RGS5 in developing C2C12 cells. Protein extracts were prepared prior to and up to 8 days following the initiation of development and the levels of RGS5 protein was determined by western blot analysis (not shown). Although RGS5 levels were higher at day 1 and day 8 of development, the changes were very modest compared to other developmentally regulated genes such as myostatin [152]. RGS5 may well serve to regulate heterotrimeric G-protein signaling during muscle myotube formation

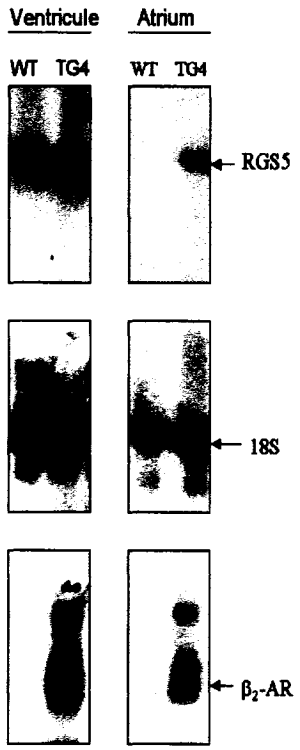
but its observed regulation suggests that elevated levels of the protein are not required.

Atrial specific up regulation of RGS5 protein by the ßAR agonist isoproterenol

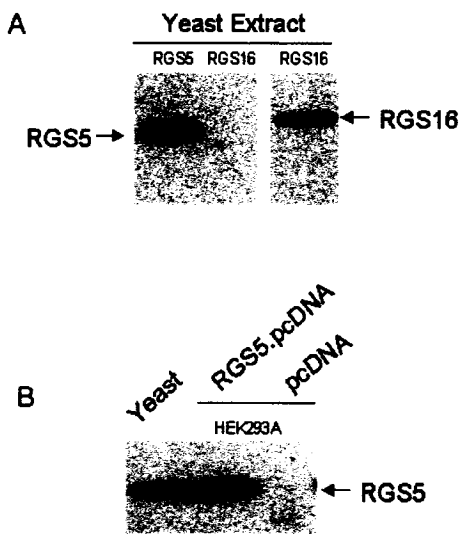
The results of our northern blot analysis suggested that the levels of RGS5 in the atria of TG4 mice with constitutive activity of the $ß_2$AR-signalling cascade were up regulated (Fig. 1). To determine if chronic ßAR stimulation was sufficient for the observed increase, western blot analysis was used to examine the levels of RGS5 protein in the atria and ventricles of rats that had been chronically treated with the ßAR agonist, isoproterenol (2.4mg/kg-day), for 14 days. In these animals, the level of RGS5 protein was found to be increased in atria but not in the ventricles (Fig. 5A). Densitometric analysis using p42/p44 MAP kinase as an internal control, revealed that there was a 4 fold increase in the levels of RGS5 in the atria of isoproterenol treated animals (Fig. 5B).

3.2.4 Figures

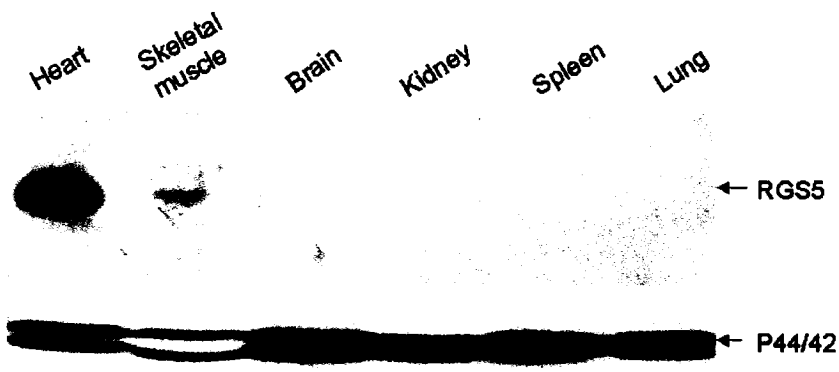
3.2.4 a) Up-regulation of RGS5 in the atria of TG4 transgenic mice with cardiac overexpression of $ß_2$AR. The hearts of TG4 mice and their non-transgenic littermates were separated into atria and ventricles. Total RNA was isolated and pooled from the left atria and ventricles of 10 non-transgenic (WT) and from 12 transgenic (TG4) mice. Northern blot analysis was then performed using 10 µg of atrial and 20 µg of ventricular RNA. The blot was sequentially hybridized with (A) RGS5, (B) 18S rRNA and (C) $ß_2$AR specific (^{32}P)-labeled DNA probes.



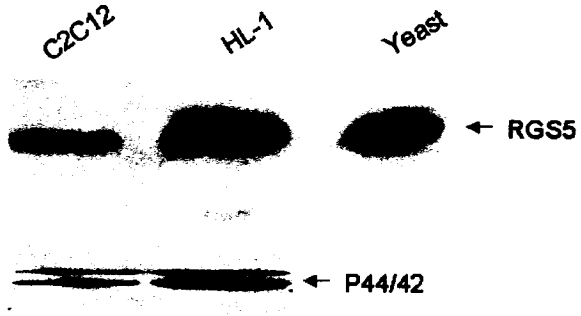
3.2.4.b) Analysis of RGS5 anti-serum in yeast and HEK293A cells. (A) Western blots using extracts prepared from yeast cells expressing RGS5 or RGS16. The extracts were challenged with either the RGS5 anti-serum (left panel) or with RGS16 anti-serum (right panel). (B) Western blot analysis using protein extracted from HEK293A cells that were transfected with the mammalian expression pcDNA alone or pcDNA expressing a hRGS5 cDNA. Twenty μg of soluble protein were separated by SDS-PAGE, transferred to nitrocellulose and challenged with an RGS5 anti-serum. As a control, 0.5 μg of protein extract from yeast cells expressing hRGS5 (Yeast) was included on the gel. The location of the RGS5 and RGS16 proteins are indicated by arrows.



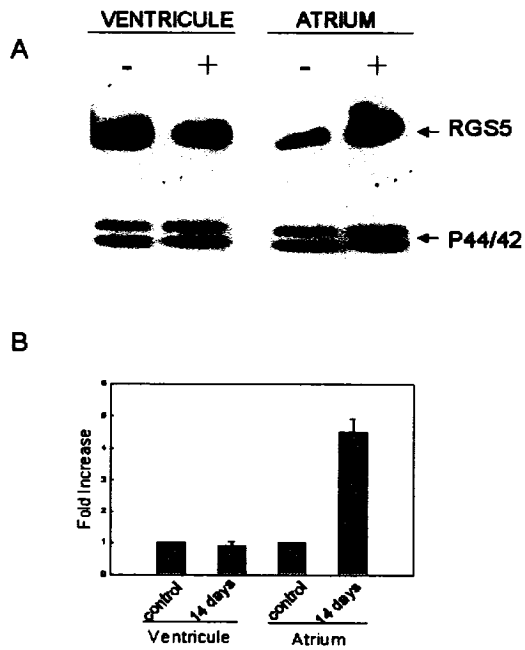
3.2.4.c) Tissue distribution of RGS5 protein. Western blot analysis using anti-RGS5 was performed on protein extracts prepared from a variety of different rat tissues. The level of p44/42 Map Kinase was also determined in the same extracts. This served as control for loading. Similar results were obtained in 3 separate experiments.



3.2.4.d) Analysis of the expression of RGS5 in cultured muscle cell lines. Western blot analysis of extracts prepared from skeletal muscle C2C12 and cardiomyocyte HL-1 cell lines using the RGS5 anti-serum. Extract of yeast cells expressing hRGS5 (Yeast) was included as a control. As a control, western blot analysis was also carried with the same extracts using p44/p42 MAP kinase anti-sera.



3.2.4.e) ß adrenergic receptor mediated up-regulation of RGS5 in cardiac atria. (A) Sprague Dawley rats received a daily intra-peritoneal injection of the ß adrenergic agonist isoproterenol (lanes labeled +) while the control animals received saline alone (lanes labeled -). Fourteen days after treatment, the animals were sacrificed, their hearts surgically removed, separated into atria and ventricles and soluble protein extracts were prepared. RGS5 levels were determined by western blot using the RGS5 anti-serum. As a control, western blot analysis of total p44/p42 MAP kinase was also performed. (B) Densitometric analysis was performed to determine the average levels of RGS5 protein normalized to the levels of p44/p42 MAP kinase. Data represent the mean ± S.D. of three independent experiments.



3.2.5 Discussion

RGS5 is a member of the R4 subfamily of RGS containing proteins. These RGSs are small (ca. 200 residues) and are characterized by the presence of an N-terminal region consisting of an amphipathic α-helix [154, 155]. Overexpression of RGSs in cultured mammalian cells has revealed that these RGSs are capable of inhibiting signalling by acting as a GTPase Accelerating Protein (GAP) for GPCR activated $G_{q\alpha}$ as well as $G_{i\alpha}$ proteins [151, 156]. In spite of these observations, it is largely assumed that the different RGSs will have specific functions [157]. Differential tissue-specific gene expression is an important factor that will serve to limit the role of the different RGSs. For example, RGS5 is expressed in the heart, skeletal muscle, smooth muscle, pericyte and a variety of sub-regions within the brain (Fig. 3) [65, 67, 73, 149, 150]. Therefore, RGS5 will be limited to regulating GPCR within these cell types. Further, the specificity of function may also reside in the ability of different RGSs to complex with different GPCRs, G-proteins and effectors [158, 159]. Clues as to the specificity of RGS5 function come from ribozyme knock down experiments in cultured smooth muscle cells [149]. A decrease in RGS5 levels was specifically associated with an increase in Angiotensin II receptor signalling. On the other hand, a number of other studies suggest that heterologously expressed RGS5 can also regulate a variety of different GPCRs, including the endothelin 1 receptor, the somatostatin receptor 5 and the muscarinic receptor M2 [145, 151, 156, 160].

The levels of a number of RGSs are increased by a variety of stimuli, including GPCR agonists [61, 135, 137, 161-163]. Many of the RGSs are induced in specific subsets of different cell types. For example, a number of RGSs are

differentially regulated in a variety of regions of the rat brain following the induction of electroconvulsive seizures or the injection of amphetamine [164, 165]. Here we show that RGS5 is specifically increased in the atria in response to overexpression of the β_2AR and to the chronic stimulation of βARs (Fig.1, 5). Both βARs typically couple to $G_{s\alpha}$ leading increases in cAMP and activation of PKA [134]. Under certain circumstances, such as in response to chronic agonist stimulation, both receptors can switch coupling specificity and activate $G_{i\alpha}$ proteins leading to the activation of different signalling pathways such as p42/44 Map kinase [Martin, 2004 #1273]. Activation of either pathway may be responsible for the increase in RGS5 levels. A parathyroid hormone mediated increase in cAMP appears to be largely responsible for mediating its ability to increase in RGS2 levels in cultured murine osteoblasts [Tsingotjidou, 2002 #1274]. Nevertheless, signalling pathways activated by other heterotrimeric G-proteins have also been shown to lead to increases in RGS2 levels in multiple cell types [Grant, 2000 #1275; Tsingotjidou, 2002 #1274]. The mechanism responsible for the observed atrial specific increase in RGS5 remains at present unknown.

An increase in the levels of RGSs such as RGS5 is expected to lead to a decrease in GPCR signaling. The simplest interpretation is that an increase in RGS levels in response to the stimulation of a given GPCR will serve as a negative feedback loop to limit signaling responses to the GPCR itself [163]. Conversely the increase in RGS levels may lead to decreased responsiveness for other GPCRs thereby serving to contribute to the phenomenon of heterologous desensitization [Tsingotjidou, 2002 #1274]. RGS5 can serve as a GAP for $G_{q\alpha}$ as

well as $G_{i\alpha}$ therefore the observed increase in RGS5 will likely serve to inhibit GPCRs coupled to these G-proteins. An increase inhibition of βARs mediated $G_{i\alpha}$ signalling will likely lead to enhanced βAR mediated $G_{s\alpha}$ responses and may therefore serve to counterbalance $G_{s\alpha}$ to $G_{i\alpha}$ class switching following prolonged of βAR stimulation. Although R4 RGSs cannot serve as GAPs for $G_{s\alpha}$ proteins, some of RGSs can nevertheless functionally inhibit $G_{s\alpha}$ signaling by preventing G-protein mediated activation of effector proteins (effector antagonism) [166]. A recent report suggests that the ability of RGS5 to inhibit GPCR signaling is likely to be limited to its ability to act as a GAP for inhibit G_{α} proteins since it lacks effector antagonist activity [Anger, 2004 #1268]. This suggests that RGS5 will not serve to inhibit βARs $G_{s\alpha}$ mediated responses. It nevertheless remains to be determined which of the many GPCRs and/or G_{α} proteins that are known to be expressed in the heart and skeletal muscle are regulated by RGS5. The endogenous expression of RGS5 in the HL-1 cardiomyocyte and C2C12 skeletal muscle cell lines will permit the use of techniques such as RNAi to explore the specificity of this RGS (Fig. 4).

The tissue specific ßAR-mediated increase in RGS5 that we observe in the atria (Fig. 1,5) suggests that RGS5 may be involved in regulating cell type specific signaling events, such as the GPCR-mediated release of ANF [167] or the decrease in GPCR responsiveness in diseases such as atrial fibrillation [168].

3.3 Introduction to my second study

Because our lab has recently identified a novel anti-apoptotic gene [169], and because of the known survival and apoptotic properties of LPA in many cells, we became interested in identifying which type of responses does LPA induce in C2C12 skeletal muscle cells. Very few studies have analyzed LPA responses in skeletal muscle, even though apoptosis accounts for several diseases in this tissue [170, 171]. Moreover, our previous finding that RGS5 was upregulated in the atria of both TG4 mice and chronically stimulated β-ARs-containing mice suggest that this protein may account for the decrease in GPCR responsiveness seen during certain cases of heart failure. Therefore, potential regulation of LPA-mediated apoptotic responses and possibly other GPCRs by RGS5 can be used in therapy of skeletal muscle diseases. The following study reports our findings regarding the signaling pathways and responses activated by LPA in C2C12 cells.

3.4 Manuscript #2: Lysophosphatidic Acid mediates pleiotropic responses in skeletal muscle cells

3.4.1 Introduction

G-Protein Coupled Receptors (GPCRs) represent the largest class of cell surface receptors [172], which regulate different physiological processes in response to a wide diversity of agonists. Skeletal muscle tissue is of great interest when it comes to GPCR biology since it expresses a rich diversity of different GPCRs [37]. In contrast to what is known about the role of many GPCRs in other tissues such as the heart, the function of the majority of GPCRs expressed in skeletal muscle remains largely unknown [173]. C2C12 is a commonly used cell culture model to study various aspects of skeletal muscle biology [37]. A number of groups have also used these cells to characterize GPCR mediated signaling pathways. For example, it was recently demonstrated that S1P mediates cytoskeletal rearrangements, such as the appearance of stress fibers, by the activation of a variety of intracellular effectors including Rho and phospholipase D [174].

Lysophosphatidic acid (LPA) is a GPCR agonist that is a potent effector of cell growth, survival and differentiation [89, 175]. In ovarian cells, LPA may serve in the development of cancerous cells [176]. In cardiomyocytes, LPA induces hypertrophy and appears to protect these cells from hypoxic induced cell death [106]. In some neuronal cells, LPA induces apoptotic mediated cell death

[177]. LPA receptors are expressed in skeletal muscle [37] and they have been implicated as negative regulators of development [178]. Nevertheless, its potential involvement in other aspects of skeletal muscle function as well as the specific signaling pathways that it activates has not been examined. Here, we have examined the signaling pathways activated by LPA in cultured C2C12 myoblasts. These cells were found to express multiple LPA receptors that are involved in activating both mitogenic and apoptotic signaling cascades.

3.4.2 Materials and Methods

Reagents. Pertussis Toxin (PTX), LPA, L-phenylephrine, 5-hydroxytryptamine (serotonin, 5-HT) and TNF-α were obtained from Sigma-Aldrich. The inhibitors PD98059 and Wortmannin were purchased from Calbiochem (La Jolla, CA). All primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated rabbit secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. C2C12 and HEK293 cells were grown in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum and 1% mixture of penicillin-streptomycin at 37°C in 5% CO_2. To assay for ERK1/2, Akt, JNK and p38 phosphorylation, sub-confluent C2C12 cells were serum-deprived in DMEM containing 0.1% BSA before stimulation with LPA.

Reverse transcriptase polymerase chain reaction. RNA was extracted from tissue samples and cultured cells using RNAzol essentially as described [169]. Equal

aliquots of reverse transcribed RNA were used to amplify the different LPA receptor transcripts using the following conditions: 94°C for 45sec, 55°C for 30sec, 72°C for 45sec for a total of 33 cycles. The following oligos were used: LPA_1, forward: 5'-ATCTTTGGCTATGTTCGCCA-3' and reverse: 5'-TTGCTGTGAACTCCAGCCA-3'; LPA_2, forward: 5'-TGGCCTACCCTTCCTCATGTTCCA-3' and reverse: 5'-GACCAG TGAGTTGGCCTCAGC-3'; LPA_3, forward: 5'-GAGGATGAGAGTCCACAG-3' and reverse: 5'-GCACAGCAGATCATCTTC-3'; LPA_4, forward: 5'-TGAAGGCTTCTCCAAACGTGTCTG-3' and reverse: 5'-GTTCAGAGTTGCAAGGCACAAGGT-3'. β-actin mRNA was amplified using the forward: 5'-GTGGGCCGCCCTAGGCACCAG-3' and reverse: 5'-CTCTTTGATGTCACGCACGATTTC-3' oligos [169]. No bands were observed in PCR reactions containing RNA that was not reverse transcribed. An aliquot of each PCR reaction was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized and photographed under UV illumination. A hot start of 2 min at 94°C as well as a final incubation of 10 min at 72°C was carried out for all PCR reactions.

Preparation of protein lysates and Western Blot analysis. Following treatments, cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and lysates containing soluble proteins were prepared as described [66]. 30 to 50μg of soluble protein was subjected to 12% SDS-PAGE, transferred to PVDF membranes and western blot was carried out as described previously [66].

Assay of cell proliferation and viability. To assess cell proliferation, both C2C12 and HEK293 cells were seeded at low density in 6-well plates and incubated with or without LPA for a total of 72 h. At the end of the incubation period, cells were washed with ice-cold PBS, trypsinized and whole cell number was determined microscopically. To assess cell viability, 3×10^4 C2C12 cells were seeded in 6-well plate prior to treatment with LPA or TNF-α. At the end of treatment period, cells were washed with ice-cold PBS, trypsinized and the cell suspension was collected. Cells were then mixed with trypan blue dye at a 1:1 ratio for 3 minutes. A minimum sample of 300 cells containing both viable and dead cells was counted microscopically. Dead cells were distinguished from viable cells by the ability of the latter to exclude the dye.

DNA fragmentation assay. PBS washed cells were trypsinized and cell number determined microscopically. Aliquots containing 10^6 cells were resuspended in lysis buffer containing 10 mM Tris-Cl,pH 7.4, 10mM NaCl, 10mM EDTA, 0.5 % SDS, and 0.1 mg/ml proteinase K and incubated at 37°C overnight. The DNA was extracted by sequential treatment with phenol, phenol and chloroform and chloroform followed by precipitation with 2.5 volumes of cold ethanol. The DNA

was resuspended in 100 µl of sterile water and treated with 50µg/ml RNAse A at 37°C for 1 h.

3.4.3 Results and Discussion

C2C12 myoblasts express multiple LPA receptors

As a prelude to investigating the signaling responses activated by LPA, we used RT-PCR analysis to determine which of the four known LPA receptors are expressed in C2C12 cells. RT-PCR analysis revealed the presence of LPA_1, LPA_2 and LPA_4 mRNA in C2C12 myoblasts (Fig. 1A). We also examined the pattern of LPA receptor expression in other cell lines including HEK293 and atrial HL-1 myocyte cells, as well as heart and skeletal muscle tissues obtained from mouse. The level of β-actin mRNA was also determined and served as a loading control. Our results indicate that the cells and tissues examined express at least two different LPA receptors with distinct cell and tissue specific patterns. Densitometric analysis was used to determine the relative expression levels of mRNA in the different cells and tissues (Fig. 1B). These results reflect the reported diversity of LPA receptor expression [121, 122].

LPA-mediated activation of ERK1/2 and Akt is PTX sensitive in C2C12 cells

The capacity of individual GPCR agonists to mediate MAPK activation, in particular ERK1/2 phosphorylation, has been reported to depend on the cell type [17]. Indeed, stimulation with LPA resulted in ERK1/2 activation in a number of cells but not in C2C12 myoblasts [179, 180]. In an attempt to delineate

the potential mitogenic properties of LPA in C2C12 myoblasts, we decided to re-investigate the effects of LPA on ERK1/2 activation in these cells. C2C12 cells were incubated in serum-free medium for 24h prior stimulation with 1 μM LPA for 5 minutes. Under these conditions, the addition of LPA resulted in a modest 3-fold increase in the phosphorylated forms of ERK1/2 when compared to control cells treated with vehicle alone (Fig. 2A). As a control, we also monitored the level of total ERK1/2 in all experiments in order to ensure that the observed differences in the levels of phosphorylated ERK1/2 was not due to loading artifacts. To rule out the possibility that the modest ERK1/2 activation was specific to LPA, serum-deprived C2C12 cells were also stimulated with specific agonist to other GPCRs that are known to be expressed in skeletal muscle [37]. Likewise, little or no increase in ERK1/2 phosphorylation was observed upon stimulation of C2C12 cells with 1 μM of the α-adrenergic receptor agonist phenylephrine, with the serotonin receptor agonist 5-hydroxytryptamine or by the addition of serum (5-HT) (Fig. 2A).

Prolonged periods of serum deprivation are necessary to observe receptor-mediated responses in certain cells [181]. To examine this possibility for C2C12, the cells were incubated in serum-free medium for a 48h period prior to being stimulated with LPA, phenylephrine, 5-HT or serum. Under these conditions, all three agonists as well as serum induced a noticeable increase in ERK1/2 phosphorylation compared to unstimulated control cells while the levels of total ERK1/2 remained essentially unchanged (Fig. 2B). Densitometric analysis using total ERK1/2 levels as a loading control showed that there was a respective 14, 150, 17 and 15 fold increase in the levels of phospho-ERK1/2 with LPA,

serum, phenylephrine and 5-HT. These conditions were therefore adopted for all subsequent experiments designed to monitor LPA-mediated activation of MAP Kinases.

LPA receptors have been shown to activate ERK1/2 via $G_{q\alpha}$ and $G_{i\alpha}$ subunits [96]. To examine the possibility that a $G_{i\alpha}$ subunit may be involved, C2C12 cells were pre-treated with Pertussis Toxin (PTX) prior to stimulation with LPA for either 2 or 5 min. Western blot analysis revealed that PTX largely abolished ERK1/2 phosphorylation after 2 or 5 min of LPA stimulation (Fig. 2C). The levels of total ERK remained unchanged by PTX treatment. The ability of LPA to activate ERK1/2 is often accompanied by a concomitant activation of the PI3K/Akt pathway [32, 182]. To examine the possibility that LPA may also activate Akt in C2C12 cells, extracts prepared from cells stimulated with LPA for 5 min were analyzed by western blot using phospho-specific Akt antibody. The level of phosphorylated Akt increased in response to LPA (Fig. 2D). Pre-treatment of cells with PTX, largely abolished the increase in Akt phosphorylation (Fig. 2D). As a control for loading, total Akt levels were also monitored (Fig. 2D). LPA therefore activates both ERK1/2 and Akt in a PTX sensitive manner in C2C12 cells.

LPA is not a mitogen and instead activates apoptotic-signaling pathways in C2C12 cells

Given that LPA activates both ERK1/2 and Akt, we decided to test the possible mitogenic effects of LPA in C2C12 cells. C2C12 cells were plated at low density in complete medium with or without LPA. After 48 h, there was no

difference in cell number, in control cells compared to cells grown in the presence of LPA (not shown). We also found that LPA did not stimulate an increase in cell number even when it was added to C2C12 cells placed in serum-free medium (data not shown). As a control, we examined the ability of LPA to stimulate proliferation of serum grown HEK293 cells. We found that there was a 23% increase in cell number in cultures grown in the presence of LPA compared to untreated cells (not shown). A similar effect on the proliferation of HEK293 cells was observed if the cells were treated with LPA in serum free media (not shown). These results suggest that, although LPA activates the usually mitogenic ERK1/2 proteins in C2C12 cells, it does not induce growth in these cells.

In a number of cells, LPA mediated ERK1/2 and/or Akt activation does not lead to a mitogenic response, instead they often promote survival [32, 182]. To examine the possibility that LPA may promote the survival of C2C12 cells, we determined the effects of LPA on these cells undergoing apoptosis in response to prolonged serum-deprivation (72 h). To monitor apoptosis, western blot analysis was performed using PARP specific antibody, since the appearance of an 89 kDa cleaved PARP fragment is a commonly used marker of apoptosis. The results obtained show that most of the detectable PARP in C2C12 cells growing in 10% FBS is in the uncleaved form (Fig. 3A). There was a noticeable increase in the level of the cleaved PARP fragment in cells placed in serum free media for 72 h compared to cells grown in 10% FBS (Fig. 3A). Surprisingly, we detected an apparent increase in the levels of the cleaved PARP fragment in serum-deprived cells grown in the presence of LPA (Fig. 3A). This suggests that LPA does not

prevent starvation-induced PARP cleavage in C2C12 cells; instead LPA may be enhancing the apoptotic effect caused by serum deprivation.

LPA has been reported to induce apoptosis in some cell types [177, 183]. To examine the possibility that LPA may itself initiate apoptotic signaling, serum fed C2C12 cells were incubated with or without LPA for 72h and the accumulation of both caspase 3 and PARP cleaved fragments was determined. The addition of LPA to growing C2C12 resulted in an increase in the levels of cleaved caspase 3 and PARP proteins when compared to cells not receiving LPA (Fig. 3B). The observed LPA-mediated cleavage of both caspase 3 and PARP proteins was largely prevented when the cells were pretreated for 24 h with PTX (Fig. 3B). This later result indicates that LPA mediated activation of $G_{i\alpha}$ protein is likely responsible. We also examined the temporal pattern of the appearance of cleaved caspase 3 and PARP proteins in response to LPA stimulation in growing C2C12 cells. Both caspase 3 and PARP cleavage products were detectable by 24 h of LPA treatment but the levels of these cleaved fragments were detectably increased after 48 h of incubation with LPA (Fig. 3C). Taken together, these results demonstrate that LPA mediates the activation of apoptotic markers in C2C12 cells and suggests that it may serve to initiate apoptosis in these cells.

LPA activates stress induced MAP Kinases in C2C12

The LPA-mediated activation of caspase 3 and PARP suggests that this agonist may also activate other proteins involved in mediating apoptotic responses. We therefore examined the possibility that LPA may also activate JNK and p38 MAP kinases, which are associated with pro-apoptotic signaling

responses [184]. The levels of the phospho-JNK and -p38 were increased in cells treated with LPA for 5 minutes, as compared to untreated cells (Fig. 3D). Pretreatment of the cells with PTX completely abolished the LPA mediated phosphorylation of JNK but had little effect on the observed increase in phospho-p38 (Fig. 3D), suggesting that unlike the LPA-mediated activation of JNK, ERK1/2, and Akt, a PTX-insensitive Gα protein is responsible for the observed activation of p38 by LPA. As a control, the level of total JNK was determined and was found to remain constant in each sample examined (Fig. 3D). Taken together, these results further establish that both mitogenic and stress responsive pathways are activated by LPA in C2C12 cells. The complexity of the signaling pathways activated by LPA that we demonstrate in C2C12 cells is similar to what is observed in other cell types such as smooth muscle (SM) cells [185]. In these cells, LPA activates ERK1/2 and induces proliferation in spite of the observation that LPA also induces the stress MAP kinases p38 and JNK. The ability of LPA to induce the proliferation of these cells suggests that the cross talk between different MAP kinases serves to balance both mitogenic and pro-apoptotic responses. In T lymphoblasts, LPA is in turn either mitogenic or apoptotic depending on the culture conditions [113]. Thus, LPA appears to straddle the divide between proliferation and death in a number of cell types.

LPA does not induce cell death in C2C12

The apoptotic potential of LPA in C2C12 cells was further examined by assaying for the ability of LPA to induce cleavage of genomic DNA, another

common apoptotic marker. Genomic DNA was isolated from growing serum fed C2C12 cells that were stimulated with LPA for 72 hours as well as untreated growing cells. Although LPA activates caspase3, PARP, phospho-p38 and –JNK (Figure 3) under these conditions, LPA does not induce DNA cleavage since only high molecular weight DNA, with no apparent laddering is observed in LPA-treated cells (Fig 4A). As a control, genomic DNA was also analyzed from C2C12 cells that were treated with the pro-apoptotic cytokine TNF-α. In contrast to what is observed in cells stimulated with LPA for 72 hours, genomic DNA cleavage was observed in C2C12 cells that were treated with TNF-α (lanes 3 and 4, Fig. 4A). We also demonstrate that TNF-α also results in the accumulation of the cleavage products of caspase 3 and PARP supporting the known pro-apoptotic effects of this cytokine in C2C12 cells (Fig. 4B).

Because apoptosis may also occur in absence of DNA cleavage [186], we next examined the effects of LPA on the viability of C2C12 cells. Growing cells were treated with LPA for 72h and cell viability was determined. We found that 95% of growing control cells remained viable, which is comparable to the 94.5% viability that we observed in cells treated with LPA for 72 hours (Fig. 4C). In contrast, cell viability decreased to 61% in growing C2C12 cells treated with TNF-α for 48 hours (Fig. 4C). These results indicate that even though LPA leads to the activation of a number of markers of apoptosis, it does not induce apoptotic cell death in C2C12 cells.

The ability of LPA to induce caspase 3 activation in C2C12 cells in the absence of cell death seems paradoxical. These results are nevertheless in agreement with reports showing that caspase 3 is involved in a variety of cellular

processes that do not require the induction of apoptosis. A commonly observed theme suggests that activation of caspase 3 is required for the differentiation of certain cell types including C2C12 myoblasts [187]. The LPA mediated activation of caspase 3 that we observe here, is unlikely to be involved in C2C12 differentiation since it occurs in growing serum fed cells, a condition that prevents myoblast differentiation [178].

Inhibition of the ERK and PI3K/Akt pathways favors an increase in LPA-mediated cell death

Given the observed dual nature of LPA activated pathways in C2C12 cells, we hypothesized that the inability of LPA to induce cell death may be due to the concomitant activation of both mitogenic and apoptotic signaling pathways. Therefore, we reasoned that the inhibition of mitogenic pathways could result in LPA mediated activation of apoptosis. For example, pharmacological inhibition of Akt and ERK promotes apoptosis upon stimulation of the tumor necrosis factor receptor superfamily member CD40 in carcinoma cells [188]. To test this hypothesis, the effect of LPA on cell viability was examined in C2C12 cells that were pretreated with specific pharmacological inhibitors of the ERK1/2 and PI3K/Akt pathways, PD98059 and wortmannin respectively. Growing C2C12 cells were incubated with or without LPA for 48h and cell viability was determined. We found that only 4.3% of control C2C12 cells were unviable (Fig. 5A). The viability of cells treated with LPA alone, or PD98059 and wortmannin were similar to the viability observed in control cells (Fig. 8A). In contrast, treatment of the cells with LPA in the presence of PD98059 and wortmannin

resulted in a doubling in the percentage of unviable cells to 8.3%. As a control, the ability of the drugs to inhibit these signaling pathways was also determined. The LPA mediated increase in ERK1/2 phosphorylation was abolished in cells pretreated with PD98059 (Fig. 5B) while wortmannin abolished the LPA mediated increase in Akt phosphorylation (Fig. 5C). Modulation of ERK1/2 and/or Akt activation appears to represent a common mechanism that serves to switch cell fate between proliferation and apoptosis. For example, activation of both Akt and ERK1/2 occurs in response to endoplasmic reticulum (ER) stress [189]. Activation of these pathways has been shown to be a counter-regulatory mechanism that allows cells to adapt to ER or other stresses without inducing apoptosis. In contrast, inhibition of ERK1/2 and Akt activation may allow certain agents like cannabinoid agonists to induce apoptosis in some cells [190]. Taken together, our results suggest that the activation of mitogenic and pro-survival signaling pathways by LPA may serve to counteract the deleterious effects resulting from the activation of apoptotic molecules in C2C12 cells.

3.4.4 Figures

3.4.4. a) Expression of LPA receptors in C2C12 cells. (A) Total RNA was extracted from C2C12 cells as well as HEK293 and HL-1 cells, skeletal muscle and heart tissues, reverse transcribed and amplified by RT-PCR using specific

primers to the indicated LPA receptors. A single band corresponding to the calculated sizes of the different LPA receptors were detected (LPA_1, 394 bp; LPA_2, 467 bp; LPA_3, 514 bp; and LPA_4, 340 bp). Transcript corresponding to β-actin (539 bp) was also amplified and served as an internal loading control. PCR products were separated by agarose ethidium bromide gel electrophoresis and photographs of the resultant UV illumination gels are shown. Similar expression profiles were observed in two independent experiments. (B) Densitometric analysis of the results presented in A. The levels of the different RT-PCR products were normalized to the levels of β-actin. The lowest level of RT-PCR product obtained for each receptor was arbitrarily set at 1.

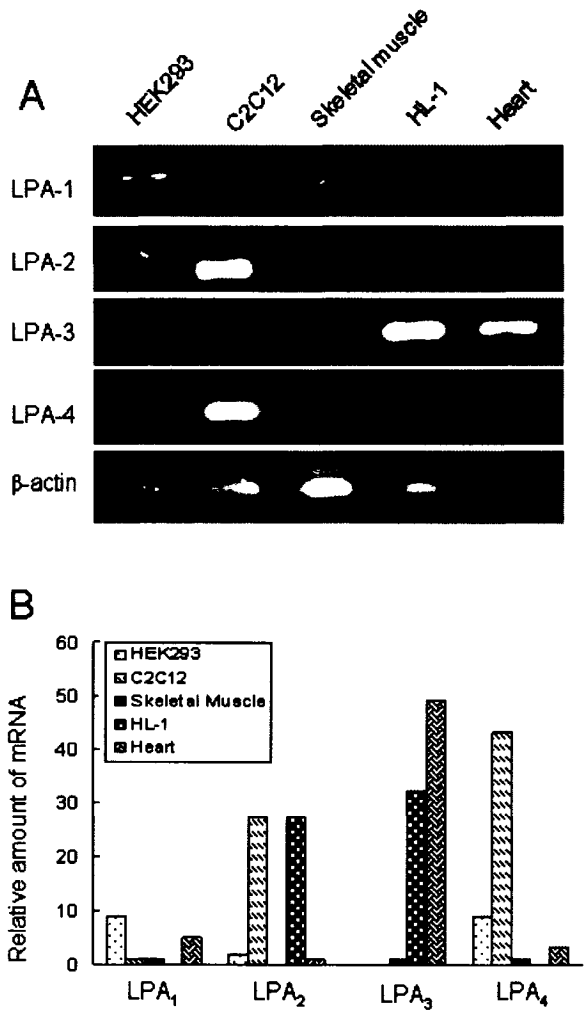

A
HEK293
C2C12
Skeletal muscle
HL-1
Heart
LPA-1
LPA-2
LPA-3
LPA-4
β-actin
B
HEK293
C2C12
Skeletal Muscle
HL-1
Heart
Relative amount of mRNA
60
50
40
30
20
10
0
LPA1
LPA2
LPA3
LPA4

Figure 3.4.4.a)

3.4.4. b) LPA, phenylephrine and 5-HT mediate ERK1/2 activation in C2C12 myoblasts. Subconfluent C2C12 cells were incubated in serum free medium for (A) 24 h and (B) for 48 h and subsequently stimulated with serum or 1 μM of the indicated GPCR agonist for 5 min. Cells that were serum-deprived for 48 h cells were stimulated with (C) LPA for 2 or 5 min without (-) or with (+) PTX pre-treatment or (D) LPA for 5 min without (-) or with (+) PTX pre-treatment. Protein extracts were prepared and analyzed by western blot using phospho-specific ERK1/2 or Akt antibodies as well as antibodies recognizing total ERK1/2 or Akt. The results shown are representative of three independent experiments.

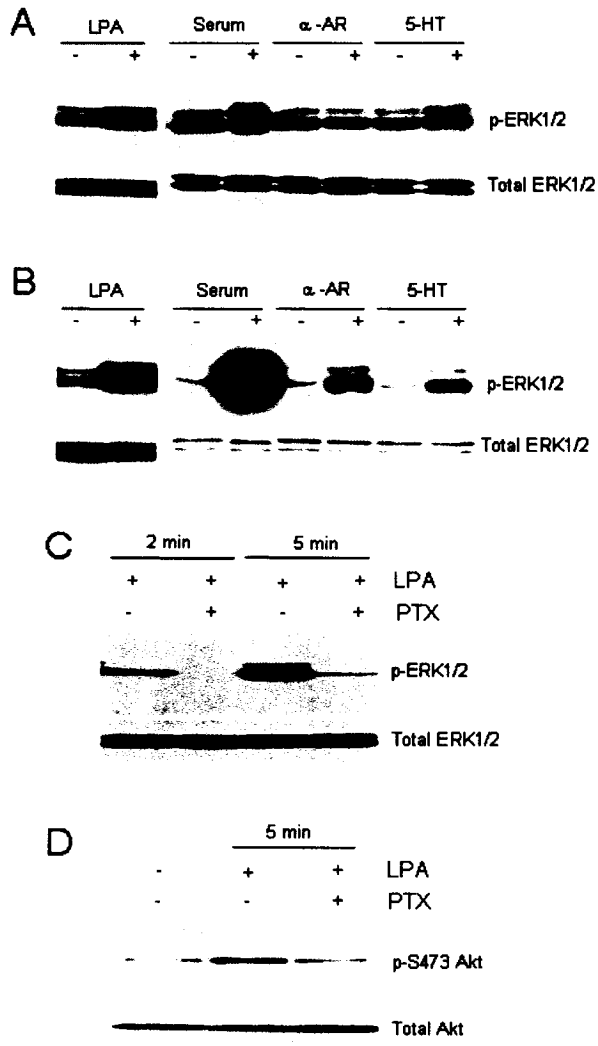

A
LPA
Serum
α-AR
5-HT
- + - + - + - +
p-ERK1/2
Total ERK1/2
B
LPA
Serum
α-AR
5-HT
- + - + - + - +
p-ERK1/2
Total ERK1/2
C
2 min
5 min
+ + + + LPA
- + - + PTX
p-ERK1/2
Total ERK1/2
D
5 min
- + + LPA
- - + PTX
p-S473 Akt
Total Akt

Figure 3.4.4.b)

3.4.4. c) LPA induces caspase 3 and PARP cleavage and activates stress related MAP kinases in C2C12 cells. (A) C2C12 cells were incubated in serum-free medium supplemented with (+) or without (-) LPA for 48h. Control cells were grown in serum-containing medium for 48h. Extracts were prepared and analyzed by western blot using a monoclonal anti-PARP antibody which recognize both the full length (inactive) and cleaved PARP (89 kDa) proteins. (B) Growing C2C12 cells were incubated with (+) or without (-) LPA for 72h. Some cells were also pretreated with PTX (200ng/ml) for 24h prior to the addition of LPA. Western blot were performed using monoclonal caspase-3 and PARP antibodies. The caspase 3 antibody recognizes both the procaspase (35 kDa) as well as the cleaved form of caspase 3 (17 kDa). (C) Growing C2C12 cells were either left untreated (-) or treated (+) with LPA for 24h or 48h. Western blot analysis was performed as described for (B). (D) Serum-deprived C2C12 cells were incubated with or without pertussis toxin (PTX) for 24h prior stimulation with (+) or without (-) LPA for 5 min. Extracts were prepared and analyzed by western blot using phospho-specific JNK and p38 antibodies. Total JNK protein level was also determined as a control.

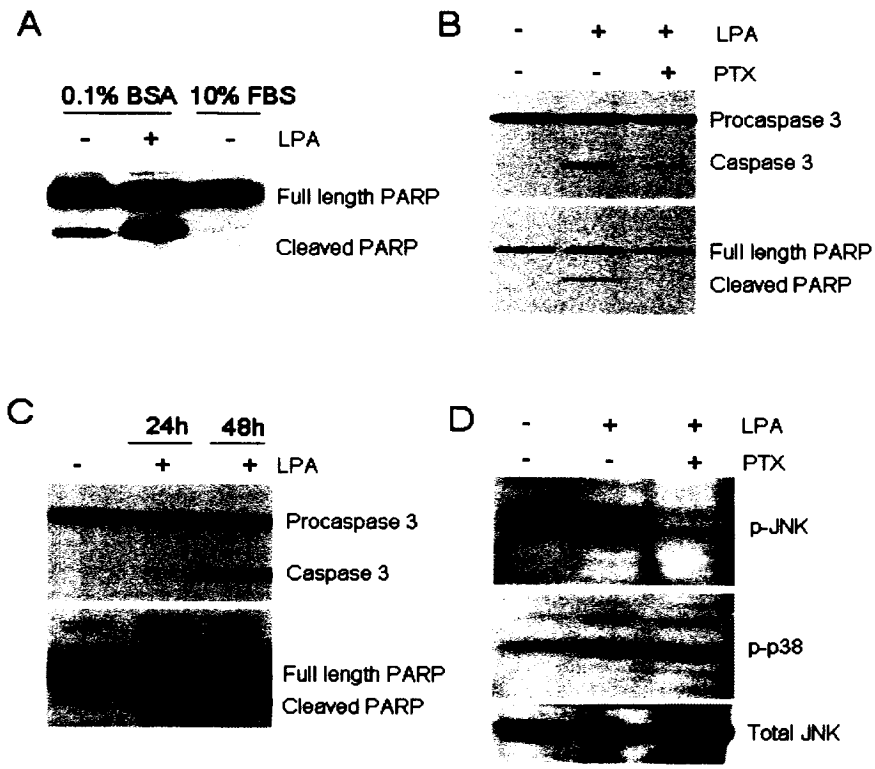

A
0.1% BSA
10% FBS
- + - LPA
Full length PARP
Cleaved PARP
B
- + + LPA
- - + PTX
Procaspase 3
Caspase 3
Full length PARP
Cleaved PARP
C
24h
48h
- + + LPA
Procaspase 3
Caspase 3
Full length PARP
Cleaved PARP
D
- + + LPA
- - + PTX
p-JNK
p-p38
Total JNK

Figure 3.4.4 c)

3.4.4. d) LPA does not induce cell death in C2C12 cells. (A) Growing subconfluent C2C12 cells were incubated with (+, lane 2) or without LPA (-, lane 1) for 48h or with TNF-α (+) for 24 (lane 4) and 48h (lane 3). Cells were then harvested and genomic DNA was isolated, separated by agarose gel electrophoresis, stained with ethidium bromide and a photograph of the UV illuminated gel is shown. (B) C2C12 cells were grown with (+) or without (-) TNF-α for 48h and protein extracts were prepared for western blot analysis using monoclonal caspase-3 and PARP antibodies. (C) Growing C2C12 cells were treated with LPA or TNF-α for 48h and cell viability determined by microscopical examination of cells stained with the vital dye trypan blue. Growing cells left untreated served as a control. The data is expressed as the percentage of cells (mean values ± S.D.) that remained viable. A minimum of 300 cells were scored for each data point and the results shown are representative of three independent experiments.

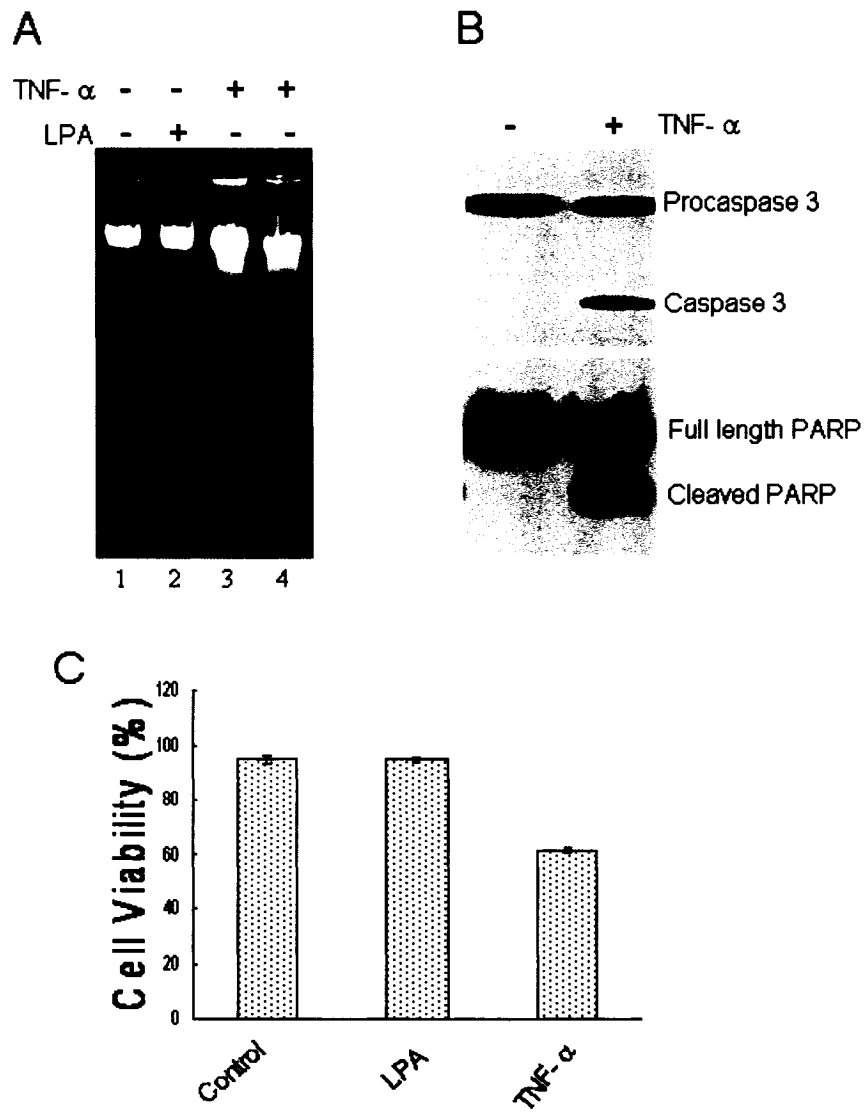

A
TNF- α - - + +
LPA - + - -
1 2 3 4
B
- + TNF- α
Procaspase 3
Caspase 3
Full length PARP
Cleaved PARP
C
Cell Viability (%)
120
100
80
60
40
20
0
Control
LPA
TNF- α

Figure 3.4.4 d)

3.4.4. e) Inhibition of ERK and PI3K/Akt pathways results in an increase in LPA-mediated cell death. (A) Growing C2C12 cells either left untreated (-) or treated (+) for 48 h with different combinations of LPA and PD98059 (20μM)/wortmannin (100nM) (PD/Wortmannin). Cells were subsequently harvested and viability determined by microscopical examination of cells stained with trypan blue. The data is expressed as the percentage of dead cells. Mean ± S.E. is from at least three independent experiments. *, $p = 0.004$; **, $p = 0.019$, student *t* test. Serum-deprived C2C12 cells were treated (B) with (+) or without (-) PD98059 or (C) wortmannin for 1h prior stimulation with LPA for 5 min. Cells were harvested and protein extracts analyzed by western blot using phospho-specific ERK1/2 and Akt antibodies. Total protein level for ERK and Akt was also determined to confirm equal loading.

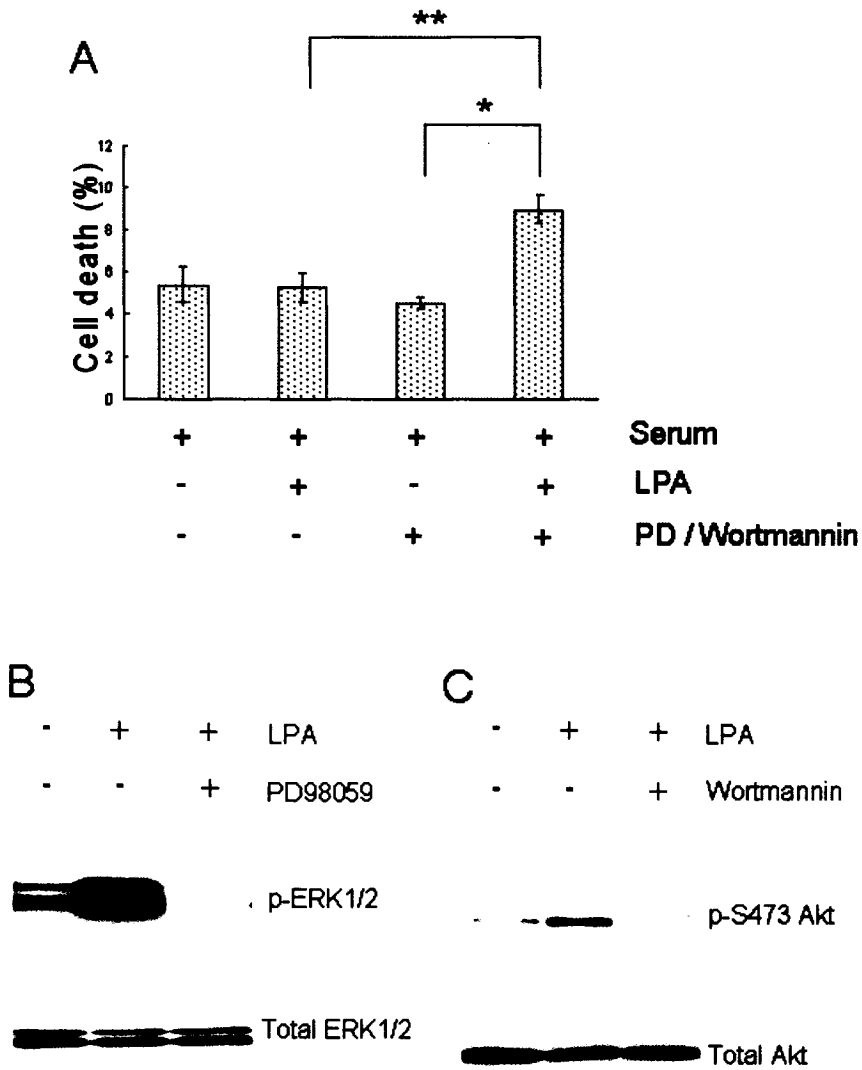

A
**
*
Cell death (%)
0
2
4
6
8
10
12
+ + + + Serum
- + - + LPA
- - + + PD / Wortmannin
B
- + + LPA
- - + PD98059
p-ERK1/2
Total ERK1/2
C
- + + LPA
- - + Wortmannin
p-S473 Akt
Total Akt

Figure 3.4.4 e)

Chapter 4. SUMMARY AND FINAL CONCLUSION

In conclusion, we found that RGS5 protein expression was confined in the heart and skeletal muscle of rat tissues. This tissue distribution of RGS5 protein correlates with the RGS5 mRNA tissue distribution previously published by Chen et al. [67], although these authors found RGS5 mRNA to also be present at low levels in the brain. Our incapacity to detect RGS5 protein in the brain may simply reflect the low level of RGS5 protein present in the brain. Nevertheless, our finding sheds some light into the possible distribution of RGS5 protein which had not been reported before. We also found that, in correlation with the increased mRNA levels of RGS5 in the atria of TG4 mice, RGS5 protein was upregulated in the atria of rats that underwent a 14 day-period of chronic stimulation of β-ARs with isoproterenol. An increase in the levels of RGSs such as RGS5 is expected to lead to a decrease in GPCR signaling. Moreover, many cases of heart failure and sepsis are characterized by decreased responsiveness to GPCRs. Heart failure may be defined, as a mechanical defect of the heart that renders it unable to adequately maintain the cardiac output required for meeting the body's circulatory needs [191, 192]. Activation of neuro-hormonal systems including the renin-angiotensin and sympathetic nervous (adrenergic) systems serve to assist the failing myocardium. These two systems cooperate to increase heart rate, increase cardiac contractility, increase volume preload and increase the number of contractile elements [191, 192]. In the acutely failing heart, these alterations provide support and are thought to be compensatory. In the chronically failing heart, there is a gradual decrease in the responsiveness to the continuous adrenergic stimulation

and cardiac failure eventually ensues. Considering that an increase in RGS levels will lead to decreased GPCR signalling without affecting the receptor, an increase in RGS containing protein(s) may be responsible for the observed decrease in βAR responsiveness during the development of some forms of heart failure. The recent observation that RGS3 and RGS4 are elevated in failing human hearts supports this hypothesis [73, 130, 139]. Sepsis is a complex syndrome that is often triggered by an inappropriate immune response to bacterial infection. If left unchecked, sepsis can lead to hypotension, decreased cardiac response leading to eventual cardiovascular shock [193]. The hypotension persists in spite of the fact that the levels of a number of vasoactive and cardio-stimulatory GPCR agonists are elevated. The condition is associated with refractive responses of multiple different GPCRs in spite of the fact that there is little loss of cell surface receptors. Panetta *et al.* has carried out a degenerate RT-PCR screen in the hearts of a porcine model of sepsis and identified both RGS1 and RGS16 as being up-regulated [74]. Similarly, Wieland's group also found that R4 RGSs including RGS16 and RGS4 were up-regulated in the septic heart [73, 135]. They further demonstrated that overexpression of RGS16 was sufficient to attenuate endothelin-1 signalling in the heart. Given that increased RGS levels diminish multiple GPCR responses, these studies suggest that altered RGS levels may play a role in the refractive hypotension that occurs in sepsis.

Therefore the possible involvements of RGS5 in the pathology of certain cardiac diseases such as heart failure, and probably other tissue-specific diseases where GPCR signaling is attenuated is yet to be determined. Overall, the tissue specific expression of RGS5 suggests that this protein may be involved in

regulating cell type specific signaling events and may account for the decrease in GPCR responsiveness seen in certain diseases of these tissues. Clearly further studies are required to assess the exact role of RGS5 in the heart and skeletal muscle.

We also analyzed the signaling cascades and biological responses elicited by LPA in C2C12 cells. The exact role of various GPCRs, including receptors for LPA, in C2C12 cells and skeletal muscle in general is not well studied. To gain further insights into the role of GPCRs in skeletal muscle, we surveyed the literature but were unable to find a comprehensive review of GPCRs in skeletal muscle. We found that a total of 42 GPCRs and numerous accessory proteins for GPCR signaling have in fact been reported to be expressed in skeletal muscle [37]. GPCRs in skeletal muscle have been associated with processes such as regulation of ion channels and contractility, glucose uptake and development. GPCRs have also been implicated in pathophysiological conditions such as atrophy where increased apoptosis of the muscle fibers occurs [170, 171]. Since LPA is a known regulator of survival and apoptotic responses in a wide variety of cells, we investigated the role of the three LPA receptors that are expressed in C2C12 cells. We found that Gαi-coupled LPA receptors mediate pleiotropic responses in C2C12 cells, suggesting that both mitogenic and apoptotic responses may serve to counterbalance the effects of LPA in C2C12 cells. Although caspase 3 activation has been associated with differentiation of C2C12 cells [187], we have ruled out in our study the possibility that LPA is regulating the differentiation of C2C12 cells, simply because LPA-treated cells were grown in serum-containing medium, a condition which prevents cell differentiation.

However, both the inhibitory and stimulatory effects of LPA on cellular differentiation, including that of C2C12 cells, have been reported [100, 101, 178]. Furthermore, the observation that LPA receptors, under conditions which favor death, can induce apoptosis in C2C12 cells and that several GPCRs are expressed in these cells, allows us to propose this cell line as a model to study GPCR signaling and their possible involvement in skeletal muscle-specific diseases. Finally, because of the endogenous expression of both RGS5 and many GPCRs, studies targeting the role of RGS5 and possibly other RGSs as negative regulators of GPCRs can be assessed in C2C12 cells. Using the new and well used methodology of RNAi, depleting endogenous levels of RG5 protein may reveal unknown roles for this protein in regulating some GPCR signaling in skeletal muscle cells.

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