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THE REGULATION OF mTORC1 SIGNALING THROUGH MULTIPLE SIMULTANEOUS STIMULATORY INPUTS TO THE TSC1/2 COMPLEX

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ABSTRACT

The mammalian target of rapamycin (mTOR) protein kinase exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 signaling pathway is involved in numerous cellular processes, including cell growth and protein synthesis, as well as various physiological and pathophysiological conditions, including muscle hypertrophy, inflammation, cancer, and diabetes. Upstream of mTORC1 the tuberous sclerosis complex (TSC), comprised of TSC1 and TSC2, functions to inhibit mTORC1 signaling. This inhibition can be relieved by hormonal and lipid inputs, which induce the phosphorylation of TSC2 resulting in the inhibition of the TSC1/2 complex. In fact, the activation of mTORC1 is tightly regulated by nutrients, hormones, and lipids, particularly the branched chain amino acid (AA) leucine, insulin, and phosphatidic acid (PA) respectively. While the insulin-mediated activation of mTORC1 is largely elucidated, the mechanism through which PA and AAs function is not currently understood. Additionally, although the individual effect of each agonist on mTORC1 signaling has been studied, further research is required to identify the relative contribution of each input.

Therefore, the first aim of this work was to identify the mechanism through which PA functions to stimulate mTORC1 activity. The present studies demonstrate that PA functions through a receptor based mechanism that stimulates the extracellular signal-regulated kinase (ERK) cascade. The inhibition of ERK signaling blocked the PA-mediated stimulation of mTORC1 signaling, clearly demonstrating that the primary

mechanism utilized by PA to stimulate mTORC1 is activation of the ERK cascade. Additionally, AAs and PA signal to mTORC1 through parallel pathways, as mTORC1 signaling increased additively in response to leucine and PA treatments. This is significant since the literature suggests that AAs signal to mTORC1 through the production of PA.

The second aim examined mTORC1 signaling in response to the activation of two signaling cascades that are involved in muscle hypertrophy, the ERK and protein kinase B (PKB) pathways. It was demonstrated that ERK and PKB, also called Akt, increased mTORC1 signaling through parallel pathways. The inhibition of either pathway reduced mTORC1 signaling partially in response to lysophosphatidic acid (LPA) and insulin treatment, which activate ERK and Akt respectively. The inhibition of both pathways completely blocked mTORC1 signaling in response to either agonist. Further, in the absence of TSC2, neither ERK nor Akt regulated mTORC1 signaling. This suggests that a functional TSC1/2 complex is required for the ERK- and Akt-mediated regulation of mTORC1 signaling.

Overall, the data presented herein demonstrate that the combination of multiple stimulatory inputs increase mTORC1 signaling in an additive fashion. This is due to both TSC1/2 dependent and independent mechanisms, as LPA and insulin signal to mTORC1 through the inactivation of TSC1/2 while it is known that the leucine-mediated stimulation of mTORC1 signaling does not require TSC1/2.

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LIST OF ABBREVIATIONS

1-BuOH	primary butanol
4E-BP1	eukaryotic initiation factor 4E binding protein 1
AA	amino acid
ARF	ADP ribosylation factor
caAkt	constitutively active Akt
caMEK1	constitutively active MEK1
caRheb	constitutively active Rheb
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDG	Endothelial Differentiation Gene
eEF2K	eukaryotic elongation factor-2 kinase
eIF	eukaryotic initiation factor
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FKBP	FK506-binding protein
FKBP12	12-kDa FK506-binding protein
FKBP38	38-kDa FK506-binding protein
FRAP	FK506-binding protein 12-rapamycin associated protein
FRB	FKB12-rapamycin binding
GAP	GTPase-activating protein
GβL	G protein β-subunit-like protein
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HEK293	Human embryonic kidney 293 cell line
IB	Immunoblot
IGF-1	Insulin-like growth factor 1
IP	Immunoprecipitation
IRS-1	Insulin receptor substrate 1
LPA	Lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
MAPK	Mitogen-activated protein kinase
MAP4K3	Mitogen-activated protein kinase kinase kinase kinase-3
MEF	Mouse embryonic fibroblast cell line
MEK	Mitogen-activated protein kinase kinase
mLST8	mammalian lethal with SEC13 protein 8
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1

mTORC2	mammalian target of rapamycin complex 2
$p90^{RSK}$	p90 ribosomal s6 kinase
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PC	Phosphatidylcholine
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLA	Phospholipase A
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PRAS40	Proline-rich Akt Substrate of 40-kD
PtdBuOH	Phosphatidylbutanol
RAPT1	Rapamycin target protein 1
Raptor	Regulatory associated protein of mTOR
Rat2V25	Rat2 fibroblast cell line expressing a dominant negative PLD1
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of TOR
RNAi	Ribonucleic acid interference
S6K1	p70 ribosomal protein S6 kinase 1
siRNA	small interfering ribonucleic acid
SKAR	S6K1 aly/REF-like target
TSC	Tuberous sclerosis complex
Vps 34	Vacuolar protein sorting 34
WT	Wild type

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"Whoever corrects a mocker invites insult; whoever rebukes a wicked man incurs abuse. Do not rebuke a mocker or he will hate you; rebuke a wise man and he will love you. Instruct a wise man and he will be wiser still; teach a righteous man and he will add to his learning."

- Proverbs 9:7-9

CHAPTER I

INTRODUCTION

1.1 Overview of mTOR

The mammalian target of rapamycin (mTOR), also known as FK506 binding protein 12-rapamycin associated protein (FRAP) and rapamycin target protein-1 (RAPT1), is a serine-threonine protein kinase of 289-kD which contains 2549 amino acids. It was first cloned and identified in 1994 [1; 2], following the discovery of yeast genes *TOR1* and *TOR2* in *Saccharomyces cerevisiae* [3]. Importantly, mutations in *TOR1 or TOR2* conveyed rapamycin resistance to yeast cells [4]. Rapamycin, an inhibitor of mTOR signaling, was originally found in the soil bacterium *Streptomyces hygroscopius* discovered on the Easter Island, Rapa Nui, from which rapamycin obtained its name [5]. Rapamycin potently inhibits mTOR through binding FKBP12, a FK506-binding protein, which then binds to the FKBP12-rapamycin binding (FRB) domain on mTOR [6]. The inhibition of mTOR revealed its role as a nutrient sensor as well as its involvement in a wide range of cellular processes including cell growth, proliferation, metabolism, autophagy, and survival [7; 8].

It should be noted that mTOR does not function alone but assembles into two multi-protein complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Fig. 1). The two complexes are similar in that both contain mTOR and mammalian lethal with SEC13 protein 8 (mLST8 aka GβL), but are distinguished by the presence of raptor (regulatory associated protein of mTOR) in mTORC1 and rictor (rapamycin-insensitive companion of TOR) in mTORC2 [9; 10; 11]. As the names of raptor and rictor suggest, the two mTOR complexes also differ in their sensitivity to

rapamycin, the activity of mTORC1 being largely inhibited, whereas that of mTORC2 is not affected acutely [12]. Because of this, more is known about mTORC1 than mTORC2, and it has been shown that mTORC1 activity is regulated by hormonal, nutrient, and lipid signaling pathways [13].

Since mTOR regulates a variety of cellular processes, maintaining proper regulation of the activity of the protein kinase is critical. Dysregulated mTOR can lead to various diseases in humans, including neurological disorders such as the familial cancer neurofibromatosis type 1, autism spectrum disorders, and Alzheimer's disease [14]. Also, the inhibition of mTOR with rapamycin is commonly used in therapy for multiple types of human cancer [15], and since mTOR functions as a nutrient sensor, it has also been implicated in diabetes and obesity [16].

A common element in diabetes and obesity is muscle atrophy and mTOR is known to be involved in the maintenance of muscle mass. Examples of increased muscle hypertrophy are observed in various models, including resistance exercise [17; 18], synergist ablation [19], insulin-like growth factor 1 (IGF-1) infusion [20], and treatment with clenbuterol, a β−adrenergic agonist [21]. In all cases examined, hypertrophy is blocked by rapamycin [22; 23; 24], indicating mTOR involvement.

The mTORC1 signaling pathway has been the focus of intense study, and it is known that increased mTORC1 signaling regulates cell growth and stimulates protein synthesis in part through phosphorylation of downstream targets such as eukaryotic initiation factor

(eIF) 4E binding protein 1 (4E-BP1) and the p70 ribosomal protein S6 kinase 1 (S6K1). When 4E-BP1 is in a hypophosphorylated state it binds eIF4E, which prevents eIF4E from binding eIF4G, thereby inhibiting the assembly of the active mRNA cap binding complex eIF4F, a crucial step in translation initiation. However, in a hyperphosphorylated state 4E-BP1 no longer binds eIF4E. In this way, by allowing cap dependent mRNA translation to occur, the phosphorylation of 4E-BP1 by mTORC1 relieves the inhibition of eIF4E [13]. The phosphorylation of S6K1 leads to the phosphorylation of additional proteins involved in mRNA translation such as ribosomal protein S6, eIF4B, S6K1 aly/REF-like target (SKAR), and eukaryotic elongation factor 2 kinase (eEF2K) [7; 8]. Because 4E-BP1 and S6K1 are directly phosphorylated by mTORC1, they are commonly used as a measure of mTORC1 activity.

The phosphorylation of 4E-BP1 and S6K1 by mTORC1 is tightly controlled through the regulation of mTORC1 activation. In fact, there are multiple proteins upstream of mTORC1 that are involved in its regulation. Directly upstream of mTORC1, its activation is mediated by the small GTPase Rheb (Ras homolog enriched in brain), which was discovered through identifying genes induced by synaptic activity, where it was found that the GTP binding domain was conserved between Rheb and Ras1 in yeast [25]. When Rheb is in the GTP bound state it binds to and potently activates mTORC1, but when it is associated with GDP its ability to stimulate mTORC1 is substantially reduced [26]. The GTPase activity of Rheb is governed by the tuberous sclerosis complex 1 and 2 (TSC1/2), with TSC2 functioning as a GTPase-activating protein (GAP) and thus as a negative regulator of mTORC1 signaling (Fig. 2) [27; 28; 29].

An autosomal dominant mutation in either TSC1 or TSC2, results in the inactivation of the TSC1/2 complex, which is the cause of tuberous sclerosis, a disease characterized by tumor growth in the skin, and the heart, and is also the cause of various conditions including autism, epilepsy, and renal cell carcinoma [30; 31]. Since TSC1/2 serves as a major site for the integration of multiple signaling pathways upstream of mTORC1, including those mediating the actions of growth factors, cellular energy state, glucagon, glucocorticoids, and hypoxia [32; 33; 34; 35; 36], it is logical that its inactivation would have deleterious effects. In support of this, mouse embryonic fibroblast (MEF) cells, which lack TSC2, have very high levels of mTORC1 activation [30].

Efforts to understand the role of TSC1/2 in the stated conditions have revealed that the complex is regulated by signaling pathways such as the protein kinase B (PKB) and extracellular signal-regulated kinase-1, -2 (ERK1/2) pathways. PKB, also known as Akt, is a serine-threonine protein kinase located upstream of TSC1/2, and has been shown to be an important regulator of muscle hypertrophy [37; 38]. Akt must translocate to the plasma membrane for activation, and it does this through the binding of phosphatidylinositol-3,4,5-triphosphate (PIP_3) to the pleckstrin homology (PH) domain of Akt [39]. Additionally, to be fully activated, Akt must be phosphorylated at threonine 308 (T308) and serine 473 (S473) [40]. 3-phosphoinositide-dependent kinase-1 (PDK1), which is also recruited to the plasma membrane through binding PIP_3, is responsible for the phosphorylation of T308 while mTORC2 is responsible for the phosphorylation of S473 [41]. These sites are often used to demonstrate the activation of Akt. Once

activated, Akt inactivates TSC1/2 by phosphorylating TSC2 at S939, S981, S1130, S1132, and T1462 [42].

1.2 Akt and ERK mediated regulation of mTORC1 through the TSC1/2 complex

It has been demonstrated that when the Akt sites on TSC2 are mutated, mTORC1 signaling is no longer increased in response to insulin treatment [42]. The insulin-mediated activation of mTORC1 has been studied extensively, and insulin increases the phosphorylation of the downstream targets of mTORC1, S6K1 and 4E-BP1 [43; 44]. This activation is inhibited in the presence of rapamycin, demonstrating the involvement of mTORC1 [44; 45]. When the insulin receptor (IR), which possesses tyrosine kinase activity, binds insulin, it recruits and phosphorylates various substrates, particularly insulin receptor substrate 1 (IRS-1) [46]. Once activated, IRS-1 activates phosphoinositide 3-kinase (PI3K) which in turn phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) at the plasma membrane generating PIP_3 [47]. PIP_3, as previously stated, binds Akt at the plasma membrane enabling the activation of Akt, the subsequent phosphorylation of TSC2, and the inactivation of TSC1/2. This inactivation results in increased Rheb-GTP levels, which leads to the activation of mTORC1 (Fig. 3). Akt also phosphorylates the mTORC1 repressor Proline-rich Akt Substrate of 40-kD (PRAS40), preventing the inhibition of mTORC1 [48]. PRAS40 directly binds to and inhibits mTORC1, but when it is phosphorylated at threonine 246 (T246) it no longer inhibits mTORC1, although the lack of inhibition itself does not activate mTORC1, as further signals are required [48], particularly increased levels of Rheb-GTP, as mentioned.

While it is known that Akt inactivates the TSC1/2 complex it is not known exactly how this inactivation occurs. It is thought that phosphorylation of TSC2 on S939 and S981 allows 14-3-3 to bind to TSC2 and shuttle it to the cytosol, preventing the formation of the TSC1/2 complex at the membrane, where TSC1 is found [49]. Indeed, it has been demonstrated that upon phosphorylation of the Akt sites on TSC2, TSC2 is translocated from the membrane to the cytosol, and that mutation of the Akt sites prevents the translocation, keeping TSC2 at the membrane, where it is bound to TSC1 [49]. This model suggests that Akt increases mTORC1 activity through the dissociation of the TSC1/2 complex, although other models have been suggested, namely where 14-3-3 binds to TSC2 resulting in an increase of mTORC1 signaling, but that it does not cause the dissociation of the complex [50]. In addition to Akt promoting the association of TSC2 and 14-3-3, it has been suggested that Akt is also involved in the AMPK mediated inhibition of mTORC1. Particularly, it is known that AMPK phosphorylates TSC2, which activates the TSC1/2 complex, subsequently inhibiting mTORC1 activity. AMPK is activated in response to low levels of cellular ATP, and functions to reduce the cellular energy expenditure. In low energy conditions, protein synthesis is too costly, and mTORC1 activity must be inhibited. Studies have shown that Akt is involved in the regulation of ATP cellular content, and that in the presence of constitutively active Akt that intracellular ATP levels remain high, resulting in mTORC1 signaling [51]. Additionally, the same group demonstrated that Akt1/Akt2 double knock out cells had reduced intracellular ATP levels, as well as mTORC1 activity. Interestingly, when a dominant negative AMPK is expressed in Akt1/Akt2 DKO cells, mTORC1 activity is no longer repressed, indicating that Akt is regulating mTORC1 activity through an energy

sensing mechanism, which ultimately activates AMPK, which then inhibits mTORC1 signaling by phosphorylating TSC2 and activating the TSC1/2 complex [51]. Ultimately, further research is needed to determine exactly how Akt inactivates the TSC1/2 complex, whether it be through the dissociation of the complex, the inhibition of AMPK, or a combination of both.

While TSC1/2 is phosphorylated and inactivated by Akt, it is also inhibited by the mitogen-activated protein kinase (MAPK) pathway, namely the ERK1/2 cascade. ERK1/2 phosphorylates TSC2 at serine 664 (S664) and serine 540 (S540) [52; 53]. When the serines are mutated to alanine (S664A/S540A) ERK1/2 no longer inactivates TSC1/2 through phosphorylating TSC2 [52; 53]. It was found that ERK phosphorylation of TSC2 resulted in the dissociation of the TSC1/2 complex, and that the inhibition of ERK activity increased TSC1/2 activity and inhibited mTORC1 signaling [52]. It is not known whether ERK phosphorylation of TSC2 promotes the dissociation of the complex due to 14-3-3 binding to TSC2, although it has been shown that the S540A mutation does not inhibit 14-3-3 binding [50], suggesting that that 14-3-3 is not involved in the ERK mediated activation of mTORC1. ERK1/2 is thought to be involved in cancer growth and tumorigenesis, and ERK1/2 activity is increased in various tumor cell lines [54]. Importantly, it has been shown that there is increased ERK1/2 activation in hamartomatous lesions due to TSC [55]. Because of these observations it has been postulated that the dysregulation of ERK1/2, specifically its hyperactivation, could lead to tuberous sclerosis and cancer [53].

In addition to its upregulated activity in certain cancers, ERK1/2 signaling, which is known to be involved in cellular proliferation and differentiation [56], has also been implicated in muscle hypertrophy [57; 58; 59]. ERK1/2 phosphorylation, as well as mitogen-activated protein kinase kinase-1, -2 (MEK1/2) phosphorylation, the upstream kinase of ERK, is increased in response to exercise in human skeletal muscle [60]. ERK1/2 can be activated in response to both growth factors and G-protein coupled receptors (GPCR), which activate the small GTPase Ras. Ras then phosphorylates Raf, which translocates to the plasma membrane when bound by the lipid second messenger phosphatidic acid (PA) [61; 62]. The translocation of Raf to the membrane is a critical step in the proliferation of the ERK cascade, as it has been demonstrated that when Raf cannot localize to the membrane that ERK signaling is blocked [62]. However, when Raf localizes to the membrane it phosphorylates MEK1/2, which in turn phosphorylates and activates ERK1/2 (Fig. 4), which in turn inhibits the TSC1/2 complex and increases mTORC1 signaling. Recent work from the lab of Dr. Karyn Esser supports that activation of mTORC1 requires the activation of ERK, as it was shown in skeletal muscle that ERK activation corresponded with the phosphorylation of TSC2 due to mechanical overload [63].

1.3 Nutrient- and lipid-mediated regulation of mTORC1 signaling

While the activation of mTORC1 can be regulated in a TSC1/2 dependent manner in response to hormonal inputs such as insulin, a pathway that is largely elucidated, a signaling pathway that is not yet fully understood which activates mTORC1 is one that

senses amino acid (AA) sufficiency, though this appears to be through a TSC1/2 independent mechanism. This observation is supported by studies that show AA-mediated mTORC1 activation in the absence of TSC2 [64]. However, the relevant signaling pathway is not well characterized. Studies implicate a number of possible targets that respond to AAs, including MAP kinase kinase kinase kinase-3 (MAP4K3) [65], the type III PI3-kinase vacuolar protein sorting 34 (Vps 34) [66], and the Ras-related GTPases Rag A, Rag B, Rag C, or Rag D [67; 68]. Rag proteins are of particular interest, as it has been suggested that they function as a docking site for mTORC1, and that in the presence of AAs they translocate mTORC1 to Rheb-GTP, increasing mTORC1 activity [69]. Additionally, a pathway sensing AA sufficiency has been reported to act through a mechanism in which Rheb binds and inactivates the endogenous inhibitor of mTORC1, FK506 binding protein (FKBP) 38 [70; 71], although more recent studies [72; 73; 74; 75] have disputed the role of FKBP38 in regulating mTORC1 activity.

The fact that AAs increase mTORC1 signaling independently of TSC1/2 emphasizes the extensive regulation of mTOR signaling in response to stimuli. Not all AAs increase mTORC1 activation equally and it has been shown that branched chain AAs stimulate mTORC1 signaling to a greater extent than other AAs. Leucine, in particular, is a potent activator of mTORC1 [76; 77; 78], and it has been demonstrated that leucine induces protein synthesis in skeletal muscle in an mTORC1 dependent manner, as the addition of rapamycin inhibits leucine-induced protein synthesis [77].

In addition to AAs and hormones, mTORC1 activity is also regulated by lipids. The lipid most often implicated in mTORC1 signaling is PA, which is produced from three distinct pathways [79]. It has been mentioned that PA is involved with the ERK signaling cascade, and it also increases mTORC1 signaling, though how this is accomplished is not currently understood. It was first demonstrated that PA could increase mTORC1 signaling in 2001 [80]. 4E-BP1 phosphorylation and S6K1 kinase activity is increased upon the addition of exogenous PA to human embryonic kidney 293 (HEK293) cells [80], and this increase is blocked by the addition of rapamycin [80]. Following this study, other groups showed that four separate fluoromethylene phosphonate analogs of PA increase S6K1 kinase activity to levels similar to those obtained in the presence of exogenous PA. Indeed, some of the analogs increased kinase activity to a greater extent than exogenous PA [81].

Additional research has implicated PA-mediated mTORC1 signaling in the regulation of inflammatory responses through the modulation of the S6K1 pathway [82]. PA levels are often increased in various types of cancer, and it has been suggested that PA signaling to mTOR is partly responsible for increased survival of cancer cells [83]. Recently, it has been demonstrated that PA levels are increased due to mechanical stimulation, such as passive stretch and eccentric contractions [84; 85], suggesting a role for PA in muscle hypertrophy. While studies have implicated PA in mTORC1 activation, it has never been demonstrated that PA directly increases mTORC1 kinase activity, which complicates the interpretation of mTORC1 signaling in response to PA. In fact, when the PA-binding

domain of mTOR is mutated, preventing the binding of PA, the kinase activity of mTORC1 is unaffected [80].

To elucidate how PA is involved in mTORC1 signaling, a better understanding of PA is required. It is known that PA is a negatively charged phospholipid composed of a glycerol backbone (Fig. 5A), two fatty acid chains, and a phosphate group (Fig. 5B). Typically, one of the fatty acid chains is unsaturated while the other is saturated. PA represents a minor component of cell membranes, and its concentration is less than 5% in comparison to phosphatidylcholine [81], a phospholipid that is very prominent in the cell membrane. However, PA intracellular levels are increased in response to various stimuli, which will be discussed. Due to its structure and charge, PA possesses numerous second messenger properties, and it is known that it is involved in cell signaling in animals as well as plants [79; 86]. For example, in animals it is known that PA is involved in numerous cellular functions including cell survival, cell proliferation, secretion, endocytosis, vesicular trafficking, cytoskeletal rearrangement, and cell differentiation [79]. It has already been noted that PA is crucial for the translocation of Raf to the cell membrane [61; 62], and in addition to this function, it has been shown that Ca^{2+} levels in cell culture models are increased in response to PA treatment [87]. Indeed, it has been reported that PA increases intracellular levels of free Ca^{2+} and is involved in cardiac muscle hypertrophy [88; 89; 90].

Given the important role of PA as a second messenger, it would be predicted that its production would be tightly regulated. This prediction is accurate, as PA is produced

from three distinct mechanisms, namely the phospholipase D (PLD), lysophosphatidic acid acyltransferase (LPAAT), and the diacylglycerol kinase (DGK) pathways (Fig. 6). Of these pathways, it is thought that PLD and DGK are primarily responsible for the production of PA [79].

LPAAT, which is expressed in numerous tissues and is mostly located in microsomes and the plasma membrane, functions through the removal and transfer of an acyl group from acyl-CoA to lysophosphatidic acid (LPA) [91], producing PA (Fig. 7). LPA is similar in structure to PA being composed of a glycerol backbone and a phosphate group, although it does differ from PA, having only one fatty acid chain as opposed to two (Fig. 5C). Though not the primary source of PA, the LPAAT pathway is known to play an important role in PA production [91; 92; 93]. The inhibition of certain forms of LPAAT induces tumor cell apoptosis, implicating LPAAT in mechanisms of tumor cell survival [94]. Additionally, LPAAT activity is linked to the activation of mTORC1, as the overexpression of LPAAT in a transformed HEK293 cell line increases phosphorylation of both S6K1 and 4E-BP1 [95].

Another source of PA production is the phosphorylation of the second messenger diacylglycerol (DAG) (Fig. 5D) by DGK. PA can also be converted back to DAG through the action of phosphatidic acid phosphatase (PAP) [96] (Fig. 8). In addition to LPAAT activity, the production of PA from DAG is linked to mTORC1 signaling as the overexpression of DGK increases S6K1 phosphorylation and rapamycin blocks this increase [97].

Of the three pathways involved in PA production PLD is of particular interest, as it is often highly active in various cancers, such as breast and colon cancer [98; 99]. PLD has multiple isoforms, and the two mammalian isoforms, PLD1 and PLD2, which are ~50% homologous [100], are the most studied. Both PLD1 and PLD2 produce PA through the hydrolysis of phosphatidylcholine, resulting in PA and free choline (Fig. 9). It has been demonstrated that PLD1 and PLD2 are present in skeletal muscle and are involved in the mechanical stimulation of mTORC1 [84]. PLD1 is mostly located in perinuclear vesicles and the Golgi apparatus, and in some cells it is localized to the plasma membrane [79; 101]. In fact, studies have shown that PLD1 cycles between the plasma membrane and perinuclear vesicles [102]. PLD2 on the other hand is primarily localized to the plasma membrane [79], although it may also cycle through cellular vesicles [103]. The PLD-PA pathway has been closely studied in relation to mTORC1 signaling, and it has been shown that rapamycin effectively blocks PLD-mediated activation of mTORC1, although cells with overactive PLD demonstrate a level of rapamycin resistance [104; 105].

PLD is activated in response to certain agonists, including LPA, phorbol esters such as phorbol 12-myristate 13-acetate (PMA) [106; 107; 108], as well as various stimuli such as growth factors, hormones, and mechanical stimulation [84; 100; 109]. Particularly, PLD is activated by protein kinase C (PKC), as well as small GTPases in the Ras superfamily such as Rho and ADP-ribosylation factor (ARF) [100]. Rho proteins, which are active when bound to GTP, require PIP_2 as a cofactor in order to activate PLD, and it has been shown that PLD activation in response to phorbol esters is blocked in the presence of a dominant negative Rho [110]. ARF proteins also activate PLD in response

to phorbol esters [108]. In some cell types, such as HIRcB cells, which overexpress human insulin receptors, insulin increases PLD activity through ARF proteins [111], though the activation of PLD by insulin seems to be cell type specific. PKC is known to increase PLD activity, and is also stimulated by phorbol esters in cell culture. In fact, the activation of PKC and Rho leads to a synergistic increase in PLD activity, and the inhibition of PKC decreases PLD activity [112]. PKC also regulates PLD activity through the phosphorylation of serine and threonine residues. These phosphorylation events correlate with the downregulation of PLD activity, and are thought to be important for its regulation [113].

Besides Rho-, ARF-, and PKC-mediated activation of PLD, it has recently been shown that PLD is activated in response to mechanical stimulation, which increases the intracellular content of PA [84]. This correlates with an increase in mTORC1 signaling, which is blocked by increasing amounts of rapamycin or the primary alcohol 1-butanol [84]. Primary alcohols, such as butanol and ethanol, are commonly used to inhibit the PLD-mediated increase of intracellular PA. In addition to the hydrolysis activity of PLD, it also possesses transphosphatidylation activity. The transphosphatidylation reaction facilitates the removal of phosphatidyl groups from phospholipids and the subsequent transfer to alcohols, resulting in a phosphatidylalcohol (Fig. 9) [114]. This process is extremely useful in the study of PLD, and is the basis for measuring PLD activity. By activating PLD with a given agonist in the presence of alcohols, the production of the resulting phosphatidylalcohol can be measured, demonstrating the effect of the agonist on PLD activity. Additionally, 1-butanol, in particular, is commonly used to reduce PA

content due to the action of PLD [106; 115]. In this manner, 1-butanol is useful in distinguishing the contribution of PLD to PA accumulation in comparison to LPAAT and DGK.

While it is known that the PA produced from LPAAT, DGK, and PLD activity can function to increase mTORC1 activity, the exact mechanism by which PA does this is not currently elucidated. It is thought that PA directly binds to mTOR at the FRB domain and stimulates signaling through this interaction. Studies have characterized the interaction of PA with mTOR, demonstrating that PA does bind directly to mTOR [116], although exactly how this increases mTOR signaling is unknown. It has been demonstrated that high levels of PLD activity confers rapamycin resistance [104]. Also, groups have shown that 1-butanol inhibits mTORC1 signaling in response to PLD activity, and that the addition of exogenous PA [105] overcomes this inhibition. In fact, one of the first studies connecting PA and mTOR signaling shows that mitogen stimulation of mTORC1 is inhibited by 1-butanol, suggesting that PA is responsible for the mitogen-mediated activation of mTOR. In addition, it also shows that adding exogenous PA to cells in culture increases S6K1 activity [80]. However, the same group showed that the binding of PA to mTOR does not increase mTOR kinase activity, as the mutation of the PA binding site on mTOR does not diminish its kinase activity [80]. This raises questions regarding how PA increases mTORC1 signaling, particularly since a study demonstrated that PA is important for mTOR complex formation, and that the inhibition of PA production weakens both the mTORC1 and mTORC2 complexes [105]. This suggests that PA may not be responsible for mTORC1 activation, but is instead

important for the formation of functional mTOR complexes. Ultimately, while groups have attempted to demonstrate that PA stimulates mTORC1 signaling through binding to mTOR, direct evidence for this hypothesis is lacking, and further research is required.

An additional caveat to the hypothesis that PA activates mTOR through directly binding it is that PA is quickly hydrolyzed to LPA in the presence of phospholipase A (PLA) (Fig. 10) [117; 118], which is present in serum [119; 120], and is secreted by cultured cells [121; 122]. Since it is a common practice to study mTOR signaling in response to exogenously added PA, it is possible that what is actually being studied is an LPA-mediated effect. Indeed, it has been shown that LPA does increase mTOR signaling [106], and that LPA mediates its effects through binding to a cell surface receptor. This presents an alternative path through which PA could be stimulating mTOR in studies utilizing exogenous PA, specifically that it does not enter the cell but instead works through a receptor based mechanism.

The activation of mTORC1 by LPA is mediated by receptors and LPA has three known cell surface receptors encoded by the endothelium differentiation gene (EDG), which are EDG-2, -4, and -7 [123]. These receptors are also referred to as LPA1, LPA2, and LPA3 respectively. They are coupled to G proteins, which are responsible for propagating the signal initiated by LPA. Besides LPA conversion to PA through LPAAT activity, and its hydrolysis by PLA, LPA can also increase the intracellular content of PA through the G coupled protein mediated increase in PLD activity. Once LPA binds EDG, Rho is activated in response to G coupled protein signaling, which in turn activates PLD. In

addition to PLD, LPA also activates MAPK signaling through the G coupled protein mediated activation of Ras, which ultimately propagates ERK1/2 signaling, which could in turn increase mTORC1 signaling through the inactivation of TSC1/2 [52]. It is possible that the LPA-mediated increase of PA is important for the propagation of ERK1/2 signaling, as it has been shown that PA is important for the translocation of Raf to the plasma membrane [61; 62], as previously mentioned.

LPA signaling is involved in multiple processes including cell survival, proliferation, neurogenesis, tissue remodeling, and reproductive functions [124; 125; 126]. LPA, which is present in serum at concentrations ranging from 2μM to 20μM [127], and is present in individual cells at approximately 154 pmol [123], is released from platelets [128] and is generated as an intermediate in phospholipid biosynthesis [127]. LPA levels are increased in various cancers, including ovarian and colorectal [129; 130; 131], and it is involved in inflammatory processes [132; 133]. Additionally, LPA induces cardiac hypertrophy, which is blocked in the presence of MEK inhibitors [134], indicating a link between LPA, ERK, and muscle hypertrophy.

1.4 The increase of mTORC1 signaling in response to simultaneous stimulatory inputs

While the importance of PA, LPA, AAs, and insulin in the regulation of mTORC1 signaling has been discussed, these individual inputs also appear to work together to increase mTORC1 activity. For instance, mTORC1 signaling in skeletal muscle is

increased synergistically in response to the addition of leucine and insulin [135]. Additionally, the combination of insulin with AAs increases protein synthesis and is needed for the assembly of the translation initiation complex [136]. While it has been suggested that AAs and PA activate mTORC1 through parallel pathways [80], later studies indicated that they work through the same pathway [137].

One of the first studies that suggested PA and AAs function through parallel pathways was performed in 2001. The study shows that mTOR signaling increases in response to the addition of PA, though signaling increases much more when PA and AA treatments are combined [80] (Fig. 11A). In 2008, seven years after the parallel pathway model was presented, an alternate model was suggested by the same group [137] (Fig. 11B). The new model effectively contradicts the previous model by submitting that AAs signal indirectly through PLD, by the activation of Rheb. It postulates that Rheb binds to and activates PLD1, which subsequently produces the PA that activates mTOR. In support of this model, it was demonstrated that Rheb binds PLD1 and that this interaction is GTP dependent [137]. Additionally, the deletion of TSC2 results in increased PLD activity [137]. During this time another group demonstrated that Rheb activates mTOR by binding FKBP38, the endogenous inhibitor of mTOR. The interaction between Rheb and FKBP38 was shown to be AA and GTP dependent [71]. This discovery led to the hypothesis that Rheb may activate mTOR by supplying active PLD1 and relieving the FKBP38-mediated inhibition [137]. However, subsequent studies suggest that the involvement of FKBP38 is not as clear since the interaction between Rheb and FKBP38 could not be duplicated [74], indicating that FKBP38 does not play a major role as an

endogenous antagonist of mTORC1. Still, this does not negate that Rheb may increase mTORC1 signaling through the activation of PLD1.

1.5 Introduction of dissertation project

Despite the extensive work that has been done on mTORC1, questions remain regarding how signaling is affected by lipid inputs, specifically PA and LPA. For example, does PA bind to mTOR and upregulate its activity or does it function through an alternative mechanism? Studies examining PA-mediated activation of mTOR typically utilize the addition of exogenous PA to cell culture, and it is possible that exogenous PA does not enter the cell, but rather works through a receptor based mechanism to increase cellular signaling. Since increased PA content and mTORC1 activity correlates with physiological and pathophysiological conditions, such as muscle hypertrophy and cancers respectively, it is important to understand the relationship between PLD, PA, and mTORC1 activation. Further, since it is debated whether AAs signal to mTOR through a PLD-mediated mechanism or through a parallel pathway that is independent of PA production, the relationship of lipid signaling and AAs requires further elucidation.

Regarding muscle growth, it has been demonstrated that ERK signaling increases muscle hypertrophy, and because it is known that PA signaling is involved in the ERK cascade, the connection between PA, ERK, and mTORC1 activation also requires further study. The involvement of the mTORC1 signaling pathway in muscle hypertrophy in general raises questions. Since it is known that TSC1/2 is an important upstream inhibitor of

mTORC1 that is inhibited by ERK and Akt signaling, which are both involved in muscle hypertrophy, it is tempting to postulate that the combined activation of ERK and Akt could lead to greater mTORC1 signaling through the inhibition of TSC1/2 than either could alone.

Therefore, to begin to answer the stated questions and to further our understanding of mTORC1 regulation, the following proposed aims were tested:

1) To determine the mechanism of PA-mediated mTORC1 signaling and whether the activation is due to PA directly binding to mTOR or if PA is working through a receptor based mechanism. Additionally, the involvement of PLD signaling in the AA-mediated activation of mTORC1 will be tested (Chapter III).

2) To determine whether ERK and Akt signal to mTORC1 through parallel pathways, and whether they increase mTORC1 signaling through the inactivation of TSC1/2. Additionally, the combined effect of hormonal, lipid, and AA treatments on mTORC1 signaling will be examined (Chapter IV).

1.6 Chapter I: Figures and legends

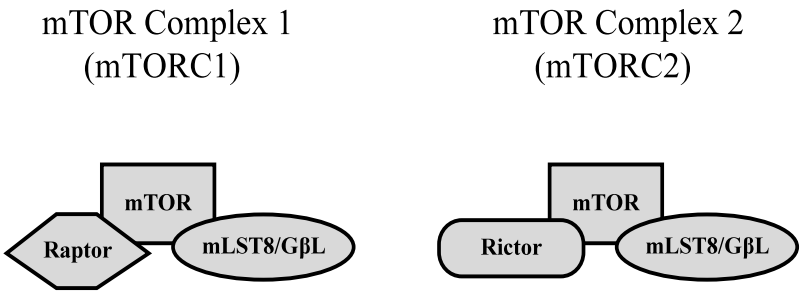

Figure 1. mTOR exists in two distinct complexes as mTORC1 and mTORC2. mTORC1 is composed of mTOR, raptor, and GβL, while mTORC2 is composed of mTOR, rictor, and GβL. Each complex functions differently, and much more is known about mTORC1 than mTORC2.

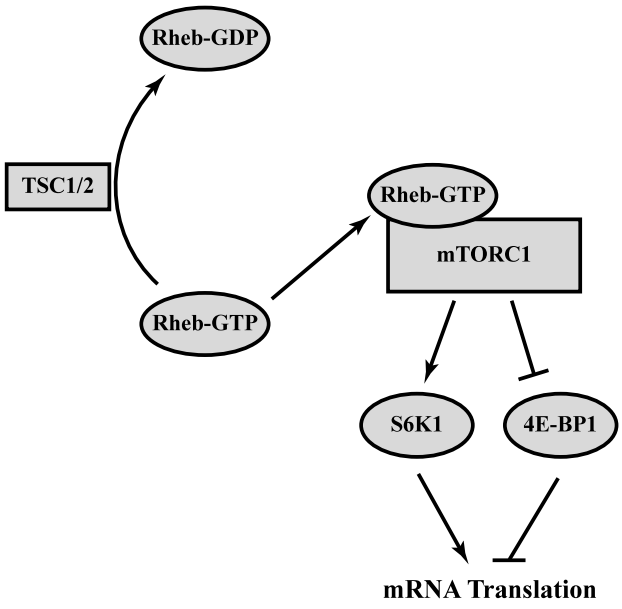

Figure 2. TSC1/2 functions as an upstream inhibitor of mTORC1. Rheb-GTP binds to and potently activates mTORC1, although it does not significantly increase mTORC1 signaling when it is bound to GDP. When bound to TSC1, TSC2 functions as a GAP for Rheb. The activation of the GTPase activity of Rheb leads to the cleavage of GTP to GDP, decreasing mTORC1 signaling.

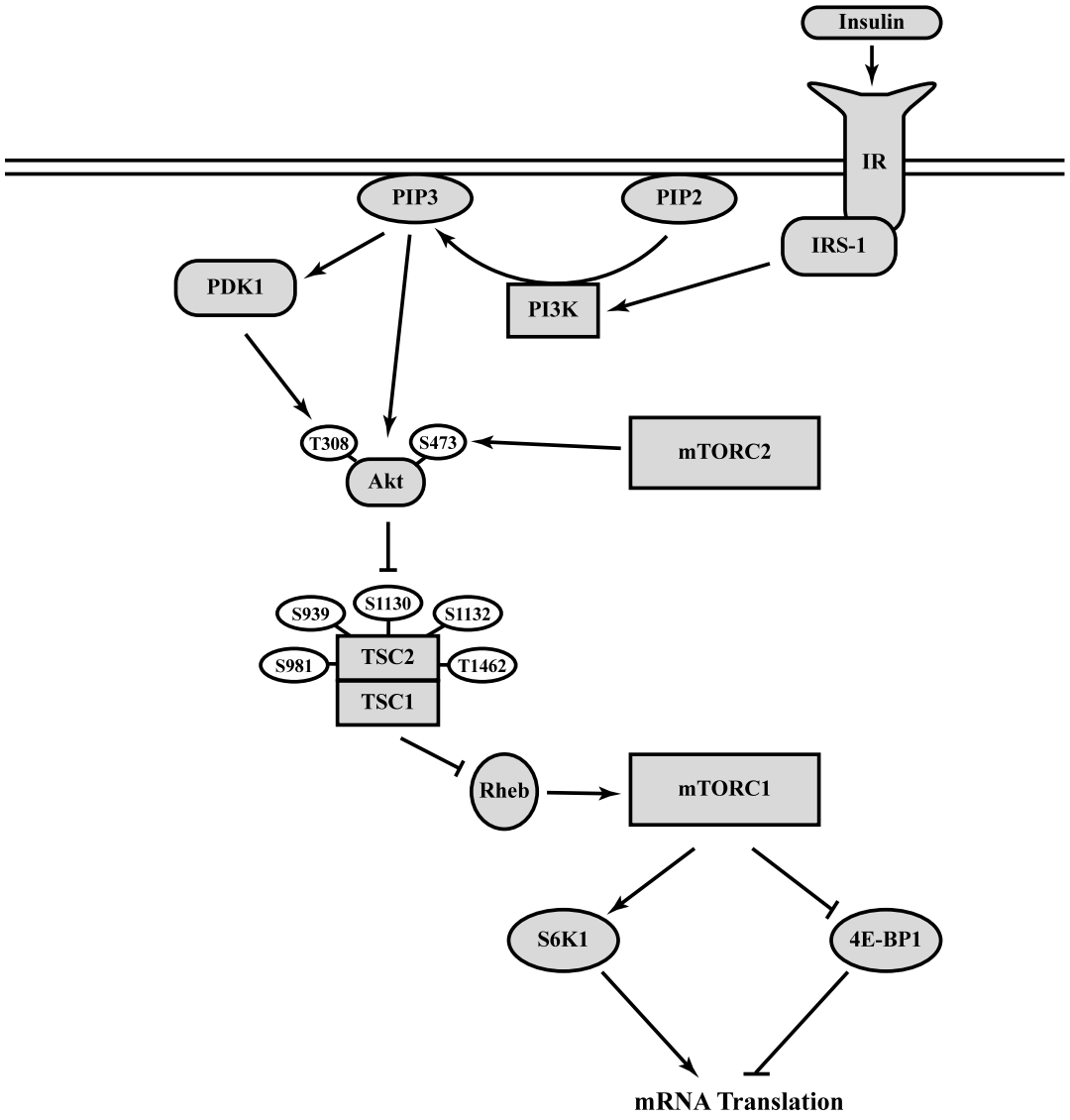

Figure 3. Insulin-mediated activation of mTORC1 through the Akt pathway. Insulin increases mTORC1 signaling through binding IR, which then recruits IRS-1. IRS-1 then activates PI3K, which phosphorylates PIP_2 generating PIP_3. Akt and PDK1 bind PIP_3 and localize to the plasma membrane. PDK1 and mTORC2 phosphorylate Akt, which then inactivate TSC1/2 through phosphorylation of TSC2, ultimately increasing mTORC1 signaling due to greater levels of Rheb bound to GTP.

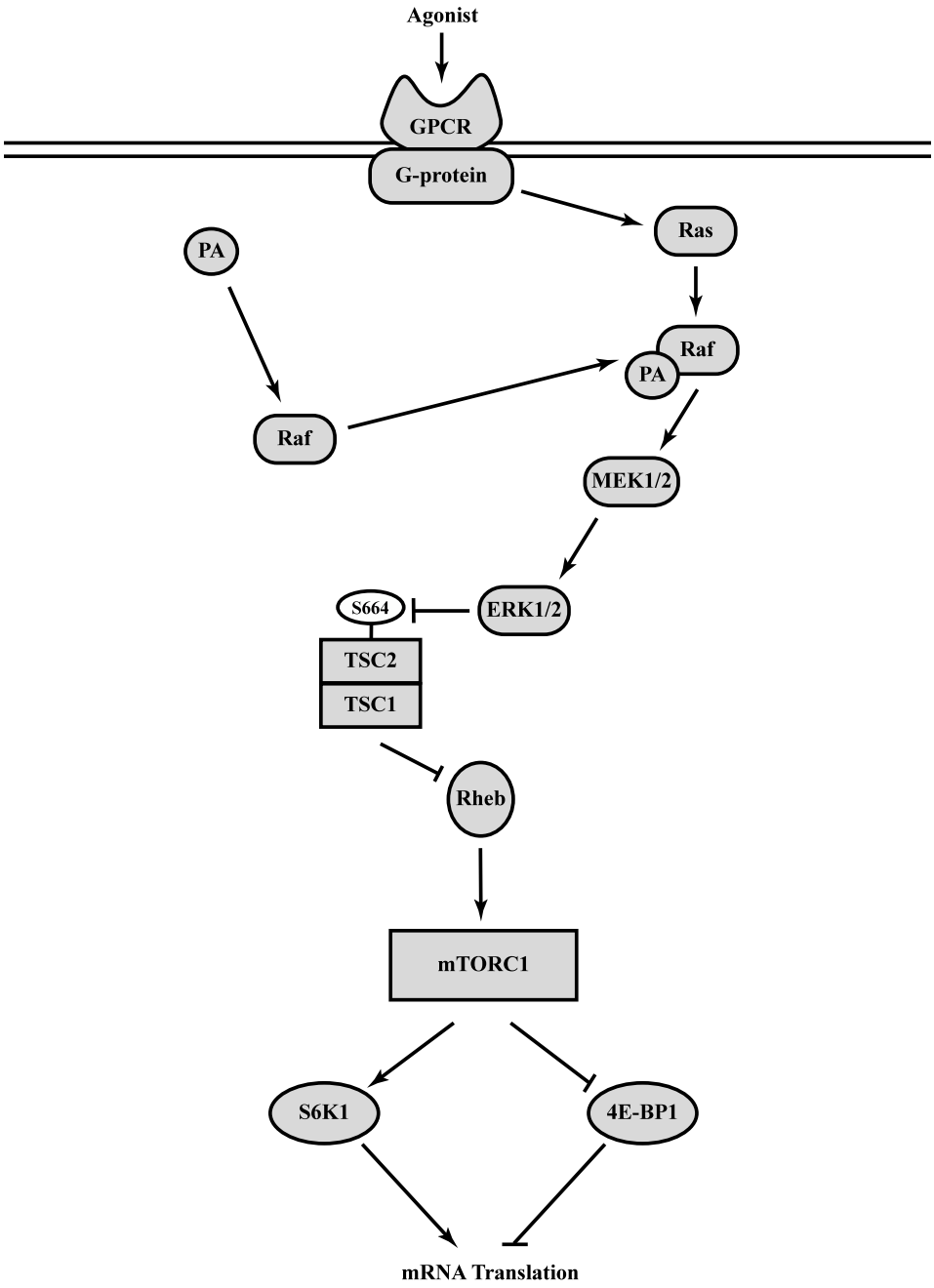

Figure 4. The upregulation of ERK1/2 signaling through a GPCR. Upon stimulation with an agonist, Ras is activated through a GPCR. Ras cannot proliferate the ERK1/2 cascade until Raf binds PA, enabling its translocation to the plasma membrane where Ras phosphorylates and activates Raf, which phosphorylates MEK1/2, which phosphorylates ERK1/2. ERK1/2 then inactivates TSC1/2 through the phosphorylation of TSC2 at S664, ultimately resulting in increased levels of Rheb bound to GTP, which binds to and increases mTORC1 signaling.

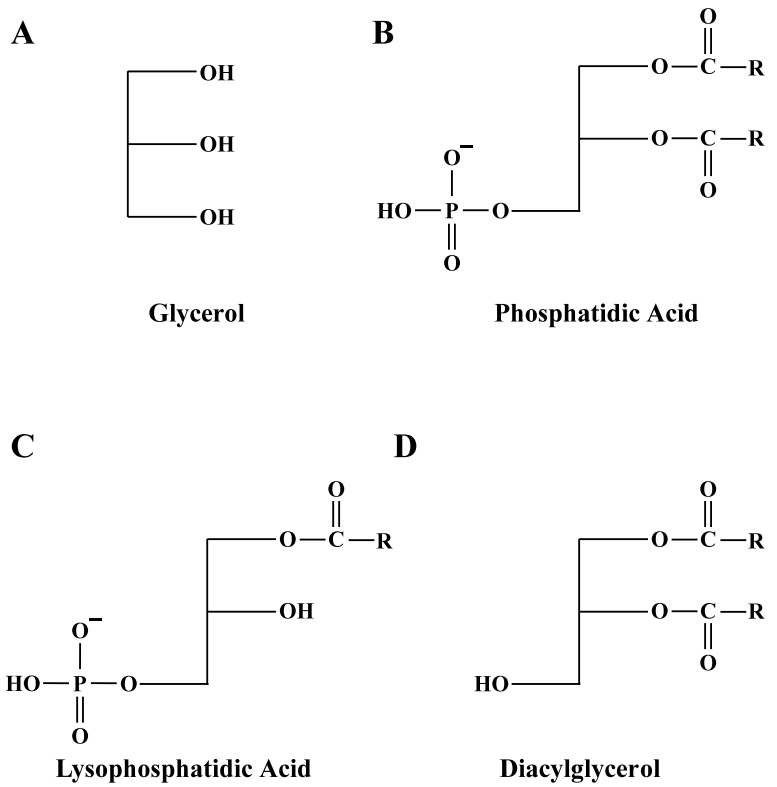

Figure 5. Structures of the glycerol backbone and subsequent phospholipid products. Lipids function as important second messengers in cell signaling pathways. (A) Glycerol serves as a precursor for numerous phospholipids including (B) phosphatidic acid, (C) lysophosphatidic acid, and (D) diacylglycerol. In B, C, and D, R denotes an acyl chain which could be saturated or unsaturated.

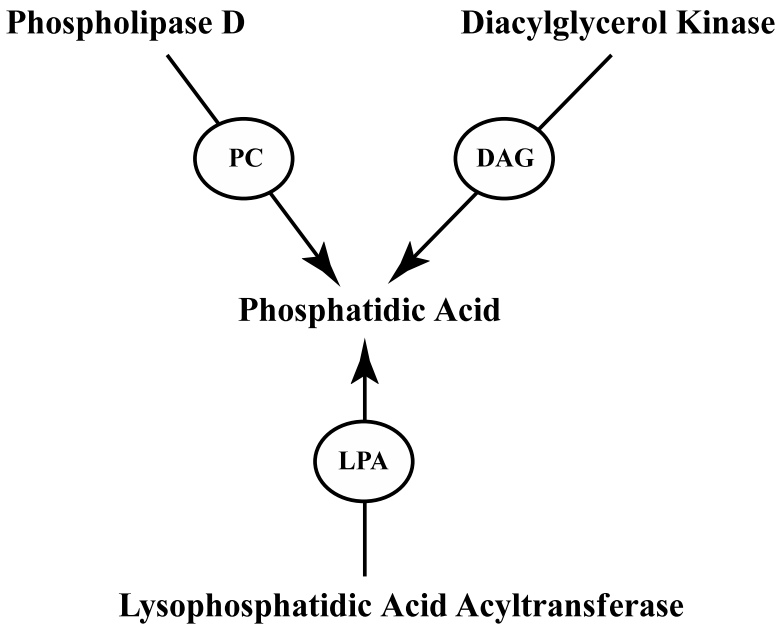

Figure 6. Phosphatidic acid is produced through multiple pathways. PA production is regulated by three distinct pathways, namely PLD, DGK, and LPAAT, which modify PC, DAG, and LPA respectively to generate PA.

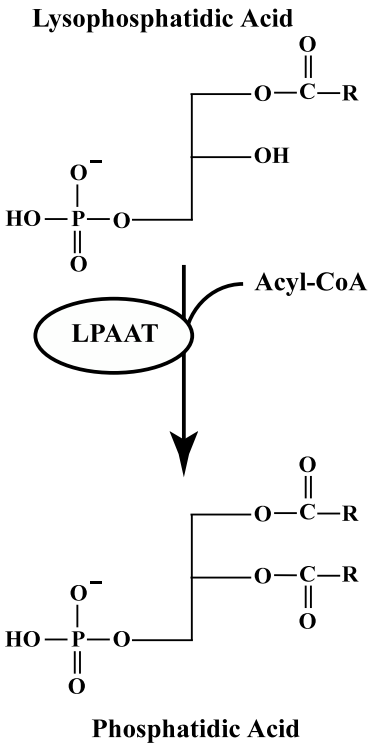

Figure 7. LPAAT production of phosphatidic acid. LPAAT increases intracellular phosphatidic acid content through the transfer of an acyl chain from Acyl-CoA to lysophosphatidic acid, yielding phosphatidic acid. R denotes the presence of an acyl chain which could be saturated or unsaturated.

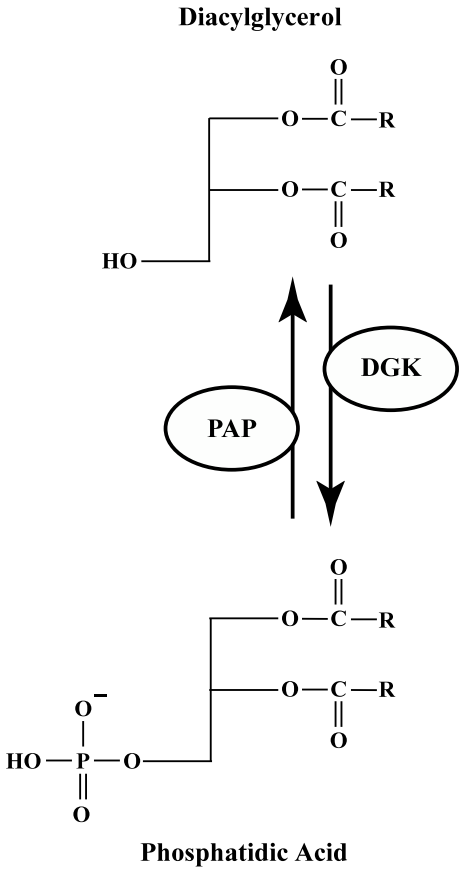

Figure 8. DGK production of phosphatidic acid. DGK phosphorylates diacylglycerol yielding phosphatidic acid. PAP can regenerate diacylglycerol from phosphatidic acid by removing the phosphate group. R denotes the presence of an acyl chain which could be saturated or unsaturated.

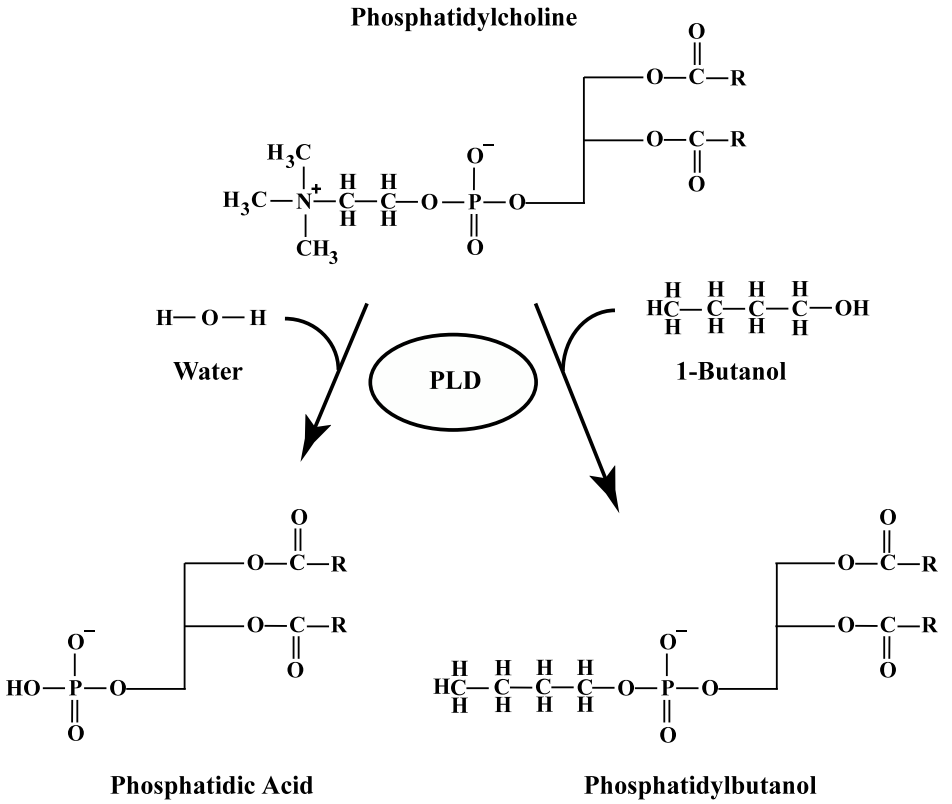

Figure 9. PLD production of phosphatidic acid. PLD increases intracellular phosphatidic acid content through hydrolyzing phosphatidylcholine, yielding choline and phosphatidic acid. Primary alcohols, such as 1-butanol, compete with water in the hydrolysis reaction, and PLD greatly prefers to utilize 1-butanol in the reaction, resulting in the production of phosphatidylbutanol. Therefore, 1-butanol serves as an inhibitor of PLD mediated phosphatidic acid production, and the resulting phosphatidylbutanol does not function as a second messenger. Additionally, PLD activity can be measured by the amount of phosphatidylbutanol produced in response to an agonist. R denotes the presence of an acyl chain which could be saturated or unsaturated.

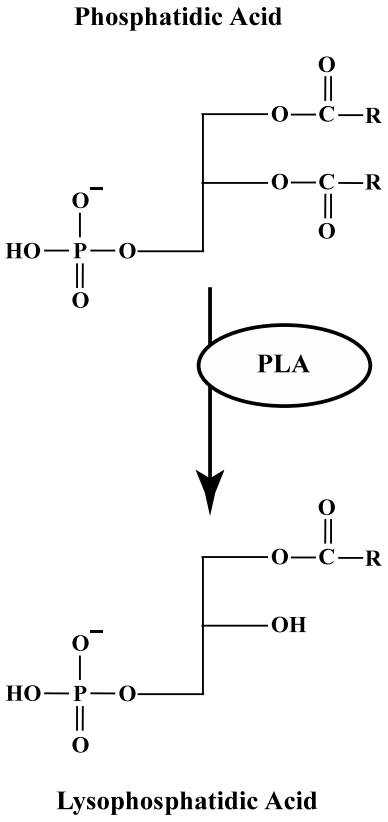

Figure 10. Phosphatidic acid production of lysophosphatidic acid. PA is quickly hydrolyzed by PLA in serum, yielding LPA. LPA can either induce signaling through a cell receptor, or it can be converted back to PA through LPAAT activity. R denotes the presence of an acyl chain which could be saturated or unsaturated.

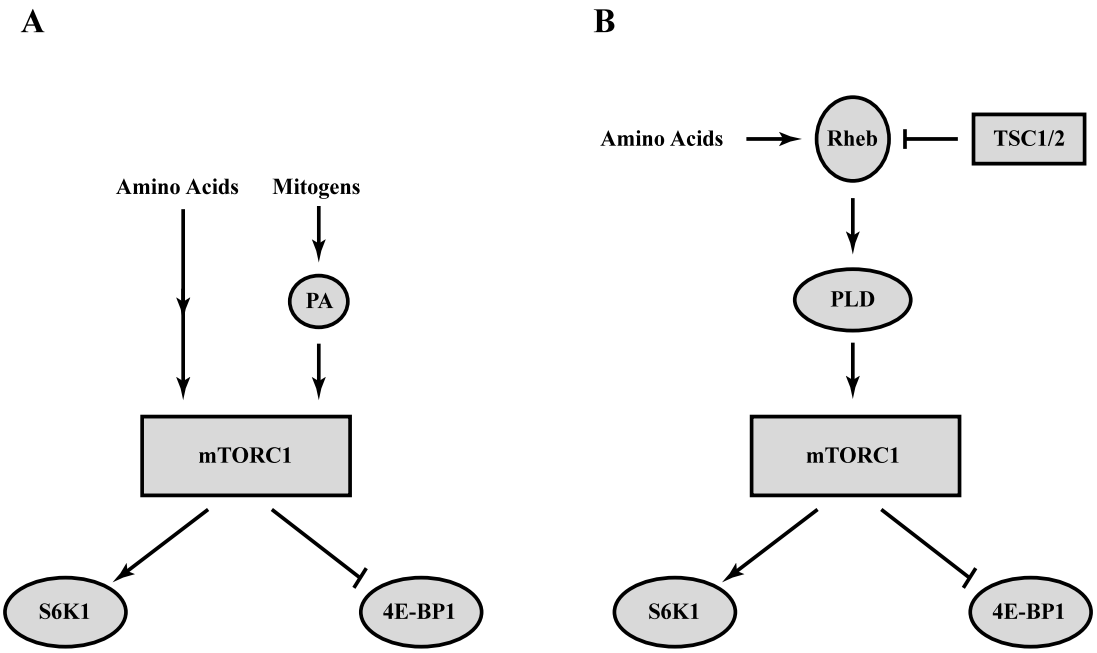

Figure 11. Possible interactions between lipid and nutrient signaling. (A) In this proposed pathway, PA is produced in response to mitogens. The addition of AAs then increases mTORC1 signaling in an additive fashion with PA. (B) In this postulated pathway, AAs activate Rheb, which then binds to and activates PLD, increasing intracellular PA content, which subsequently increases mTORC1 signaling.

CHAPTER II

MATERIALS AND METHODS

Cell culture studies

Cell maintenance – Rat2 fibroblasts (ATCC, Manassas, VA, USA), TSC2 KO (TSC2-/-, p53-/- MEF cells) [30] [graciously provided by Dr. David Kwiatkowski (Harvard Medical School)], and wild type (WT) MEF cells [a kind gift from Drs. David Ron and Heather Harding (NYU Langone Medical Center)] were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) lacking sodium pyruvate (Gibco/Invitrogen Life Sciences, Carlsbad, CA, USA), 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO, USA), and 1% Pen Strep (Gibco/Invitrogen Life Sciences, Carlsbad, CA, USA). Rat2V25 cells [138] [kindly provided by Drs. John Exton and Yoonseok Kam (Vanderbilt University Medical Center)] were maintained in high glucose DMEM containing 0.5mg/mL of G418 (Gibco/Invitrogen Life Sciences, Carlsbad, CA, USA). All cells were maintained at 37°C with 5% CO_2 and grown to ~75% confluence before seeding. On the day of study, medium was aspirated, cells were washed with sterile phosphate buffer saline (PBS), and experimental medium was added, which was a custom formulated cell culture medium (Atlanta Biologicals, Lawrenceville, GA, USA) lacking leucine, histidine, and pyruvate. Histidine (Sigma, St. Louis, MO, USA) was added prior to use. Each cell type used was ~75% confluent when medium was aspirated. FBS was supplemented as per experimental conditions, which are noted in the figure legends.

MEK and Akt inhibitor studies – Rat2 cells were incubated for 2.25h in experimental medium containing 0.5% FBS. During the last 15min, 20μM PD-98059 (Calbiochem,

San Diego, CA, USA) suspended in dimethyl sulfoxide (DMSO), 10μM U0126 (Promega, Madison, WI, USA) in DMSO, and/or 0.4μM Akt1/2 Kinase Inhibitor (Sigma, St. Louis, MO, USA) in DMSO, were added to the medium prior to treatment with 0.76mM leucine (Sigma, St. Louis, MO, USA) suspended in PBS, 10nM insulin (Novolin®R) (Novo Nordisk Inc., Princeton, NJ, USA) suspended in sterile water, 22μM 18:1 lysophosphatidic acid (LPA) [1-oleoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt)] (Avanti Polar Lipids, Alabaster, AL, USA), and/or 100μM 16:0-18:1 phosphatidic acid (PA) [1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (monosodium salt) (POPA)] (Avanti Polar Lipids, Alabaster, AL, USA).

EDG-2 receptor antagonist and rapamycin studies – In Rat2 studies involving inhibition of the EDG-2 receptor, cells were incubated for 2.5h in experimental medium containing 0.5% FBS. During the last 30min, 10μM Ki16425 (Cayman Chemical, Ann Arbor, MI, USA) suspended in DMSO was added to the medium prior to treatment with 100μM PA. In the Rat2 study involving rapamycin (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) inhibition of mTOR, cells were incubated for 2h in experimental medium. During the last 15min, 100nM rapamycin was added to the medium prior to treatment with 22μM LPA.

MEF, Rat2V25, and additional Rat2 studies – Either Rat2 or Rat2V25 cells [138], were incubated for 2h in experimental medium prior to treatment with 0.76mM leucine, 22μM LPA, 50mM 1-butanol (Fisher Scientific, Waltham, MA, USA), and/or 50mM *tert*-

butanol (Reagent Plus) (Sigma, St. Louis, MO, USA). TSC2 KO and WT MEF cells were incubated in experimental medium containing 0.5% FBS for 2h prior to treatment with 0.76mM leucine and/or 22μM LPA.

Constitutively active construct studies – For the study involving the overexpression of MEK1 cDNA (activated) in pUSEamp (S218D/S222D double mutant) (Upstate Biotechnology, Lake Placid, NY, USA) and HA-Akt CA (myr-HA-Akt) in pCMV5 (Addgene, Cambridge, MA, USA), cells were incubated in experimental medium containing 0.5% FBS for 2h prior to sample collection. For the study where pRK5-myc-Rheb (S16H) [kindly provided by Dr. Kun-Liang Guan (University of California, San Diego)] was overexpressed, Rat2 cells were incubated in medium containing 0.5% FBS for 2h prior to treatment with 0.76mM leucine and/or 22μM LPA.

Transfections

Transfection studies utilized FuGENE® HD Transfection Reagent (Roche, Indianapolis, IN, USA) following the instructions of the manufacturer. Cells were approximately 80% confluent at the time of transfection. Transfection complexes were prepared at a concentration of 2μg plasmid DNA/100μL high glucose DMEM containing 10% FBS and 1% Pen Strep. 8μL FuGENE® HD Transfection Reagent was then added, at a ratio of 8:2 transfection reagent to plasmid DNA. The resulting transfection reagent:DNA complex solution was vigorously mixed for 2sec and incubated for 15min at room temperature. 100μL of the final complex solution containing pCMV5 [graciously

provided by Dr. David Russell (UT Southwestern)], pUSEamp (Upstate Biotechnology, Lake Placid, NY, USA), activated MEK1, myr-HA-Akt, pRK5-myc-Rheb (S16H) or pRK7 (Addgene, Cambridge, MA, USA), was added to the cells, which incubated for 6h with the transfection reagent, after which the media was removed and high glucose DMEM containing 10% FBS and 1% Pen Strep was added. 16h to 24h post-transfection the cells were incubated in either experimental medium containing 0.5% FBS for an additional 2h or experimental medium lacking FBS for an additional 30min.

PA treatments

PA, suspended in chloroform (EMD Chemicals, Gibbstown, NJ, USA), was dried by either vacuum centrifugation in polypropylene microfuge tubes (Greentree Scientific, Bloomfield, NY, USA) (for PA dose response) or dried under N_2 gas in glass tubes (Supelco, Bellefonte, PA, USA) (for all other PA experiments), then suspended at a final concentration of 10mM in sterile filtrated buffer containing 150mM NaCl, 10mM Tris-HCl, pH 8. The solution was mixed vigorously for 2min, incubated at 37°C for 10min, and mixed again for 2min. PA was promptly added to culture dishes at a final concentration of 100μM. Because of its relatively short half life in aqueous solution, fresh PA was added at 45min intervals.

LPA treatments

LPA was prepared as suggested by the manufacturer. Specifically, the LPA powder was suspended in 1:1 water:100% ethanol at 100mM, incubated at 37°C for 5min, and then sonicated for 5min using an Aquasonic Model 50T water bath sonicator (VWR, West Chester, PA, USA). An aliquot of the LPA solution was then added to PBS solution to obtain a stock solution of 10mM, which was then added to cell culture to obtain a final concentration of 22μM.

PLD activity assay

Cells were grown to ~60% confluence in 6-well dishes, and then radiolabeled with [^{3}H]-palmitate (5μCi/mL) (PerkinElmer, Waltham, MA, USA) for 16h. The cells were then washed with PBS and the medium was replaced with either experimental medium or complete DMEM with 10% FBS, and the cells were returned to the incubator. Two hours later, either 50mM 1-butanol or 50mM *tert*-butanol was added to the medium, followed by either 0.76mM leucine or 22μM LPA, and the dishes were returned to the incubator for 30min. Cells were collected in 1mL of ice-cold methanol and transferred to disposable glass culture tubes (Fisher Scientific, Waltham, MA, USA). Chloroform (1mL) and 10mg of phosphatidylbutanol (PtdBuOH) [1,2-dioleoyl-sn-glycero-3-phosphobutanol (sodium salt)] (Avanti Polar Lipids, Alabaster, AL, USA) were added to each tube and the solution was mixed thoroughly and then incubated at room temperature for 20min. Following the incubation, 0.9mL of 0.88% KCl in H_2O was added, the

solution was mixed, and then centrifuged at 1000g for 5min at room temperature. The organic phase was removed and dried via vacuum centrifugation. The sample was resuspended in 50µL of chloroform, and an aliquot (10µL) was analyzed using the LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Brea, CA, USA) using Formula 989 scintillation cocktail (PerkinElmer, Waltham, MA, USA). The remainder of the sample (40µL) was spotted on a thin-layer chromatography plate (Whatman, Kent, UK - Cat #: 4865-821), using a solvent consisting of ethyl acetate (Sigma, St. Louis, MO, USA), 2,2,4-trimethylpentane (Sigma, St. Louis, MO, USA), acetic acid (J.T. Baker, Phillipsburg, NJ, USA), and purified H_2O (11:5:2:10 by volume). Following chromatography, PtdBuOH bands were detected by iodine staining.

Lipid extraction and mass spectrometry

Cells were deprived of leucine and serum for 2h, followed by treatment with leucine or LPA for 30min, as described above. Cells were collected in 10mM Tris-base, pH 7.2. Protein concentration was determined using a detergent compatible (DC) Protein Assay Kit (BioRad Laboratories, Hercules, CA, USA) as per the manufacturers instructions. An aliquot of sample containing 1mg of protein was added to 4mL of a 1:1 chloroform:methanol solution, followed by addition of 5% acetic acid (2mL) and 500pmol di14:0 PA [1,2-dimyristoyl-*sn*-glycero-3-phosphate (sodium salt)] (Avanti Polar Lipids, Alabaster, AL, USA), which served as an internal standard. The samples were mixed and then centrifuged at 2000rpm for 10min, and the organic layer was removed and placed in a disposable glass tube. The aqueous phase was re-extracted with 2mL of

chloroform, and the organic phases were combined. The sample was then dried under a stream of nitrogen gas, resuspended using chloroform:methanol containing 5% acetic acid, and then re-extracted with chloroform. Samples were resuspended in chloroform and filtered using Puradisc Syringe Filters (Whatman, Kent, UK) into glass conical tubes, dried under N_2 gas, and then resuspended in 1:1 chloroform:methanol. Samples were analyzed using an ABI 4000 Q Trap Mass Spectrometer [139] (Applied Biosystems, Carlsbad, CA, USA), after being diluted into 1:1 chloroform:methanol. Precursor ion scanning for 153 (glycerolphosphate) was utilized to identify PA [140].

Western blot analysis

For Western blot analysis, cells were scraped into 1X Laemmli buffer. Hyperphosphorylation of S6K1 and 4E-BP1 were assessed by gel-shift analysis following sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The acrylamide concentration used for S6K1 was 7.5% and the concentration used for 4E-BP1 was 15% as previously described [141; 142]. Following gel electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (PALL Life Science, Port Washington, NY, USA). All other analyses were performed using Criterion Tris-HCl 4-15% gels (BioRad Laboratories, Hercules, CA, USA), and following transfer to PVDF membranes, the membranes were incubated in 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) for 1h with gentle rocking. The membranes were then rinsed briefly in TBST and washed for 10min with TBST prior to being incubated with primary antibody overnight at 4°C. All primary antibodies (Table 1) were purchased

from Cell Signaling technology, Inc. (Danvers, MA, USA) with the exceptions of: anti-S6K1 (Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-4E-BP1 (Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-phospho-PRAS40(T246) (Invitrogen, Carlsbad, CA, USA), anti-Actin (Sigma, St. Louis, MO, USA), anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). Following the overnight incubation with primary antibody, the membranes were washed in TBST for 10min, followed by incubation for 1h at room temperature and rocking in either goat anti-rabbit IgG (Bethyl Laboratories, Inc., Montgomery, TX, USA) or goat anti-mouse IgG (Bethyl Laboratories, Inc., Montgomery, TX, USA) secondary antibody. Membranes were developed using ECL Plus Western Blotting Detection System (GE Healthcare-Amersham, Buckinghamshire, UK) by rocking in developing solution for 5min, and then imaged using a GeneGnome HR Bioimaging System and GeneTools software (Syngene, Frederick, MD, USA). After development, blots probed with anti-phospho-S6K1(T389), anti-phospho-ERK1/2(T202/Y204), or anti-phospho-Akt(S473) antibody were washed and rocked in harsh stripping buffer containing 62.5mM Tris-HCl, 69.35mM SDS, 18.3µM β-mercaptoethanol, pH 6.7, for 30min at 55°C. Blots were quickly rinsed three times in purified H_2O followed by TBST, then thoroughly washed three times in TBST then rocked for 5min to ensure that no harsh stripping buffer was present. Membranes were then incubated in 5% nonfat dry milk in TBST for 1h with gentle rocking and washed as stated above. Finally, membranes were reprobed with either polyclonal anti-actin or monoclonal anti-GAPDH antibody for loading control. Values for phospho-S6K1(T389) and phospho-ERK(T202/Y204) were normalized to either actin or GAPDH.

Values for phospho-Akt(S473), phospho-PRAS40(T246), phospho-TSC2(T1462), and phospho-TSC2(S939) were normalized to GAPDH.

TSC2 immunoprecipitation

Rat2 fibroblasts were grown in 140mm^2 plates and rinsed twice with PBS prior to incubation in experimental medium, containing 0.5% FBS and lacking leucine, for 2h. Cells were then treated with 22μM LPA and/or 10nM insulin for an additional 2h. The cells were then washed two times in PBS and samples with similar treatments were pooled together in 850μL of homogenization buffer (40mM HEPES (pH 7.5), 120mM NaCl, 1mM EDTA, 10mM sodium pyrophosphate, 10mM β-glycerophosphate, 50mM NaF, .3% CHAPS, 1.5mM sodium vanadate, 1μM microcystin-LR, and 10μL/mL Sigma Protease Inhibitor), incubated at 4°C for 20min while rocking, and then centrifuged at 1000g for 3min at 4°C. An aliquot of supernatant containing 0.5mg protein, which was determined through a DC Protein Assay Kit per the manufacturers instructions, was added to a mixture containing 500μL of BioMag® Goat Anti-Rabbit beads (Qiagen, Valencia, CA, USA), that had been incubated overnight in homogenization buffer, with 10μL of polyclonal tuberin (C-20) (anti-TSC2-T) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). The samples were then rocked for 2h at 4°C, and sequentially washed with homogenization buffer, after which 100μL of 1X Laemmli buffer was added and the samples were heated at 100°C boiled for 5min, and then centrifuged. The supernatant was transferred to a new tube prior to electrophoresis on a Criterion Tris-HCl 4-15% gel, as described previously.

Statistics

Data were analyzed by one-way ANOVA using the GraphPad Prism 5 software program. If a significant difference was detected, data were analyzed further by unpaired t-test. Mass spec data was analyzed by one-sample t-test. $P<0.05$ was considered statistically significant.

Table 1: Primary antibodies used for protein quantitation and immunoprecipitation

Antibody	Company	Catalog #
anti-4E-BP1	Bethyl	A300-501A
anti-Actin	Sigma	A5060
anti-phospho-Akt(S473)	Cell Signaling	4060
anti-total-Akt	Cell Signaling	9272
anti-phospho-ERK(T202/Y204)	Cell Signaling	9101
anti-total-ERK	Cell Signaling	9102
anti-GAPDH	Santa Cruz	sc-32233
anti-HA-tag	Cell Signaling	2367
anti-phospho-PRAS40(T246)	Invitrogen	44-1100G
anti-total-PRAS40	Cell Signaling	2691
anti-S6K1	Bethyl	A300-510A
anti-phospho-S6K1(T389)	Cell Signaling	9205L
anti-phospho-TSC2(S939)	Cell Signaling	3615
anti-phospho-TSC2(T1462)	Cell Signaling	3611
Tuberin (C-20)	Santa Cruz	sc-893
anti-total-TSC2	Cell Signaling	3612

All primary antibodies were used at the concentration recommended by the manufacturer.

CHAPTER III

PHOSPHATIDIC ACID MEDIATES ACTIVATION OF mTORC1 THROUGH THE ERK SIGNALING PATHWAY

3.1 Abstract

The lipid second messenger, PA, represents one positive input to mTORC1, and it is thought to act by binding directly to mTOR, thereby enhancing the protein kinase activity of mTORC1. Support for this model includes findings that PA binds directly to mTOR and addition of PA to the medium of cells in culture results in activation of mTORC1. In contrast, the results of the present study do not support a model in which PA activates mTORC1 through direct interaction with the protein kinase, but instead show that the lipid promotes mTORC1 signaling through activation of the ERK pathway. Moreover, rather than acting directly on mTORC1, the results suggest that exogenous PA must be metabolized to LPA, which subsequently activates the LPA receptor EDG-2. Finally, in contrast to previous studies, the results of the present one demonstrate that leucine does not act through PLD and PA to activate mTORC1, and instead show that the two mediators act through parallel upstream signaling pathways to activate mTORC1. Overall, the results demonstrate that leucine and PA signal through parallel pathways to activate mTORC1, and that PA mediates its effect through the ERK pathway, rather than through direct binding to mTOR.

3.2 Introduction

Because of its central role in regulating various anabolic pathways, activation of mTORC1 is critical in mediating the anabolic response to various perturbations that promote tissue hypertrophy. For example, 4E-BP1 and S6K1 phosphorylation are

increased in skeletal muscle in various animal models of resistance exercise [143]. Additionally, S6K1 phosphorylation is increased in humans after exercise [17], showing that mTORC1 signaling is increased by exercise. Treatment with the selective mTORC1 inhibitor rapamycin dramatically attenuates both exercise-induced mTORC1 activation and muscle hypertrophy [24; 38; 144]. The mechanism through which exercise promotes activation of mTORC1 is incompletely defined and may be multifactorial. A recent study [85] shows that, in vivo, electrically-induced muscle contraction resulted in activation of PLD leading to increased PA production. Moreover, inhibition of PLD, but not PI3K, attenuated contraction-induced mTORC1 activation. Based on other studies [80; 116] showing that PA binds directly to mTOR, a model was proposed [85] in which muscle contraction results in activation of PLD and increased PA production, with subsequent binding of PA to mTOR leading to its activation. In support of such a model, addition of PA to cells in culture also resulted in activation of mTORC1 [80; 82; 85]. In those studies it was assumed that addition of PA to the culture medium led to its intracellular accumulation, thereby resulting in mTORC1 activation. A caveat to this assumption is that PLA_2 present in the cell culture medium due to either secretion from cells and/or its presence in serum added to the culture medium, may lead to hydrolysis of PA to produce LPA. LPA is known to bind to specific receptors on the plasma membrane, specifically EDG-2, -4, and -7, leading to activation of multiple intracellular signaling pathways, including PLD and ERK1/2 [145]. Moreover, both PLD and ERK1/2 have been shown to activate mTORC1 [52; 146]. Thus an alternative explanation for the activation of mTORC1 by exogenous addition of PA is that the lipid is hydrolyzed to LPA, which

subsequently binds to its receptor, leading to activation of one or more signaling pathways upstream of mTORC1.

Given the uncertainties noted above, the study described herein was designed to assess the mechanism through which PA acts to govern the activation state of mTORC1. We show that, although PA may associate directly with mTOR, it primarily signals through the ERK signaling pathway to activate mTORC1. Moreover, addition of PA to cells in culture leads to mTORC1 activation through a LPA receptor-based signaling pathway. Finally, we show that the PLD pathway and the AA sensing pathway function in parallel, producing an additive effect on mTORC1 activation.

3.3 Results

3.3.1 LPA-mediated activation of PLD increases PA content

LPA is a potent activator of PLD in many cell types [106; 147]. Indeed, as shown in Fig. 12A, LPA caused a greater than 3-fold increase in PLD activity in serum- and leucine-deprived Rat2 cells. LPA also dramatically increased the amount of PA present as the 18:1-18:1 (Fig. 12B), 18:0-18:1 (Fig. 12C), and 16:0-18:1 (Fig. 12D) products. The products generated in response to LPA treatment indicated that it not only caused the expected increase in PLD activity but also may have led to an increase in its flux through LPAAT. That is, the fatty acid side chain of the exogenous LPA was the 18:1 moiety, and the major product measured was 18:1-18:1 PA.

3.3.2 LPA-mediated activation of mTORC1 is inhibited by 1-butanol

To assess the effect of LPA on activation of mTORC1, the phosphorylation state of the two best characterized mTORC1 substrates, S6K1 and 4E-BP1 was assessed. LPA treatment led to a significant increase in phosphorylation of both S6K1 (Fig. 13A) and 4E-BP1 (Fig. 13B), and this effect was attenuated by 1-butanol, but not by *tert*-butanol, suggesting that the lipid was acting to activate mTORC1 in part through PLD. In contrast, 1-butanol treatment was without effect in serum-deprived cells, demonstrating a maximal repression of mTORC1 signaling induced by a combination of serum and leucine deprivation.

3.3.3 LPA-mediated activation of mTORC1 is inhibited by rapamycin

To provide further evidence that LPA was increasing S6K1 and 4E-BP1 phosphorylation by activating mTORC1, cells were treated with the selective inhibitor rapamycin prior to addition of LPA to the culture medium. As shown in Fig. 13C, rapamycin blocked the LPA-induced phosphorylation of S6K1. Rapamycin also blocked phosphorylation of 4E-BP1 (data not shown).

3.3.4 PA-mediated activation of mTORC1 requires serum

To further investigate the mechanism through which PA acts to increase mTORC1 signaling, cells were treated directly with PA. Surprisingly, in contrast to LPA, PA did

not promote significant phosphorylation of S6K1 either 30min or 2h after addition to the culture medium (Fig. 14A). A previous study [105] demonstrating activation of mTORC1 by exogenously added PA used cells incubated in medium containing 0.5% rather than no serum. Therefore, the study in Fig. 14A was repeated, except that the cells were incubated in medium containing 0.5% serum. As observed in the absence of serum, in cells incubated in medium containing 0.5% serum, mTORC1 signaling was not significantly increased 30min after addition of PA, even though LPA was effective at this time point (Fig. 14B). However, mTORC1 signaling was significantly increased 2h after PA addition to cells incubated in 0.5% serum.

3.3.5 PA-mediated activation of mTORC1 is blocked by an EDG-2 receptor antagonist

A plausible explanation for the permissive effect of serum on PA-induced mTORC1 activation is that PLA present in serum hydrolyzed PA to LPA, and that LPA subsequently bound to its receptor, thereby leading to activation of mTORC1. This explanation would also account for the delayed response to PA compared to LPA. To assess this possibility, cells incubated in 0.5% serum were treated with a selective inhibitor of the LPA receptor, Ki16425 [148], prior to addition of PA. As shown in Fig. 14C, the inhibitor completely prevented PA-induced mTORC1 activation, demonstrating that activation of the LPA receptor accounted for the majority of the PA effect.

3.3.6 PA and LPA mediate mTORC1 activation through a similar mechanism

Stimulation of the LPA receptor leads to activation of both the PLD and ERK1/2 signaling pathways [145]. In order to assess the contribution of the two pathways to activation of mTORC1 signaling, we initially examined the individual and combined effects of LPA and PA at maximally effective concentrations (Fig. 15) on ERK1/2 (Fig. 16A) and S6K1 (Fig. 16B) phosphorylation. In cells treated with either LPA or PA, phosphorylation of both proteins was significantly increased compared to controls. In contrast, when cells were treated with a combination of LPA and PA at maximally effective concentrations there was no additive effect on either ERK1/2 or S6K1 phosphorylation, indicating that LPA and PA were acting through the same pathway to activate mTORC1.

3.3.7 ERK1/2 is required for PA- and LPA-mediated activation of mTORC1

To determine whether or not ERK1/2 mediated signaling was required for LPA-induced activation of mTORC1 signaling, two structurally distinct MEK inhibitors, PD-98059 and U0126, were used. Addition of either PD-98059 (Fig. 17A) or U0126 (Fig. 17C) 15min before LPA significantly attenuated ERK phosphorylation. Moreover, addition of either inhibitor effectively blocked LPA-induced S6K1 phosphorylation (Fig. 17B and 17D). Similarly, inhibition of MEK effectively blocked PA-induced phosphorylation of both

ERK and S6K1 (Fig. 18). Thus, both LPA- and PA-induced activation of mTORC1 signaling was primarily mediated by ERK1/2, rather than through the direct binding of PA to mTOR.

3.3.8 LPA and PA signal through a similar mechanism to mTORC1 that is parallel to the leucine pathway

To further explore the role of ERK1/2 in mediating the effect of LPA and PA on the activation of mTORC1 the combined effect of LPA and leucine was assessed. Leucine was chosen for this study because it acts in parallel with TSC1/2 to regulate mTORC1 [76]. Thus, if LPA were acting through ERK1/2 to repress TSC1/2 and activate mTORC1, then combined treatment with LPA and leucine should act in an additive manner to activate mTORC1. However, because a previous study [137] suggested that PLD might lie downstream of AAs in the mTORC1 signaling pathway, we initially assessed the effect of leucine readdition to leucine-deprived cells on mTORC1 signaling in the presence of 1-butanol. Should leucine signal to mTORC1 through PLD, then 1-butanol should effectively attenuate mTORC1 signaling. Additionally, a previous report [80] presented data showing that 1-butanol blocked the serum-induced activation of mTORC1, implicating a role for PLD in mediating the effect. Therefore, as illustrated in Fig. 19, leucine replacement to serum- and leucine-deprived cells led to activation of mTORC1, as assessed by changes in S6K1 phosphorylation, and this effect was blocked by 1-butanol, but not by *tert*-butanol, suggesting that the branched-chain AA might act to activate mTORC1 through PLD.

However, having demonstrated that leucine-mediated mTORC1 signaling is attenuated in the presence of 1-butanol, the direct effect of leucine on PLD activity and PA concentration was unknown. Therefore, we also assessed the effect of leucine readdition to leucine-deprived cells on PLD activity and PA concentration. As shown in Fig. 20, leucine had no effect on PLD activity and did not increase PA concentration. In contrast, addition of either leucine or LPA to deprived cells led to an approximate 3-fold increase in S6K1 phosphorylation (Fig. 21A). Moreover, an eight-fold increase was observed when the two were added together. Likewise, PA acted in an additive manner with leucine to activate mTORC1, but not ERK, signaling (Fig. 22). These findings suggest that leucine acts through a pathway parallel to that utilized by LPA and PA to activate mTORC1 signaling. Further evidence showing that leucine acts in a pathway parallel to TSC1/2 to activate mTORC1 is provided by the additive effect of leucine and insulin on mTORC1 activity (Fig. 21B). Like LPA, insulin activates mTORC1 by repressing TSC1/2 function [149], and the additive effect of leucine and insulin suggests that leucine acts in parallel with TSC1/2.

3.3.9 Leucine-mediated activation of mTORC1 is completely inhibited by 1-butanol in Rat2 fibroblasts that express a dominant negative PLD1

To further examine the possible contribution of PLD in the leucine-mediated activation of mTORC1, it was tested whether leucine could increase mTORC1 activity in PLD1 dominant negative Rat2 cells. Rat2V25 cells, in which PLD activity was reduced approximately 50%, were generously donated by Drs. Kam and Exton, who had

previously created the cell line [138]. The cells were originally generated through the expression of inactive rat PLD1 that was tagged with a V5 epitope on its C terminus. It was then demonstrated that the cells no longer formed actin stress fibers in response to LPA treatment, which is known to stimulate stress fiber formation through the activation of PLD1 [138]. Upon stimulation with leucine, mTORC1 signaling in Rat2V25 cells was increased, as seen by increased S6K1 (Fig. 23A) and 4E-BP1 (Fig. 23B) phosphorylation, and the effect was blocked by 1-butanol. These results were curious, as the cells behaved similarly to WT Rat2 cells with fully functional PLD, suggesting that leucine does not signal through PLD, although 1-butanol completely blocked S6K1 and 4E-BP1 phosphorylation in Rat2V25 cells. In comparison, WT Rat2 cells still had partial phosphorylation of S6K1 in response to leucine treatment in the presence of 1-butanol (Fig. 19).

3.3.10 LPA-mediated activation of mTORC1 is completely inhibited by 1-butanol in Rat2V25 cells

It was then tested whether mTORC1 signaling would increase in response to LPA treatment in Rat2V25 cells. Knowing that LPA activates PLD, it was postulated that LPA would no longer increase mTORC1 signaling. Surprisingly, S6K1 and 4E-BP1 phosphorylation increased in response to LPA (Fig. 24), which was similar to the response seen in WT Rat2 cells (Fig. 13). Additionally, 1-butanol blocked mTORC1 signaling to a similar extent as seen in the inhibition of leucine-mediated mTORC1 signaling. It should be noted that *tert*-butanol also partially inhibited mTORC1 signaling

in response to LPA, indicating that *tert*-butanol may have non-specific effects at the concentration used, as PLD does not prefer to utilize tertiary alcohols in the transphosphatidylation reaction that generates phosphatidylalcohols.

3.3.11 Activation of mTORC1 in response to combined leucine and LPA treatments is completely inhibited by 1-butanol in Rat2V25 cells

Having shown that mTORC1 signaling in Rat2V25 cells is increased in response to leucine or LPA treatment, it was next tested if leucine and LPA treatments combined would further increase signaling. Rat2V25 cells were treated with leucine and LPA, which led to a significant increase in mTORC1 signaling, as assessed by S6K1 and 4E-BP1 phosphorylation (Fig. 25). Importantly, treating with both agonists restored S6K1 and 4E-BP1 phosphorylation to the levels seen in their respective positive controls, while 1-butanol completely ablated mTORC1 signaling in response to leucine and LPA. S6K1 phosphorylation was partially reduced due to *tert*-butanol, though this effect was not significant (Fig. 25). 4E-BP1 phosphorylation was significantly reduced by *tert*-butanol, again indicating that it may have non-specific effects.

3.3.12 PLD does not utilize *tert*-butanol in the hydrolysis of phosphatidylcholine

Having shown that *tert*-butanol partially inhibited mTORC1 signaling in certain conditions, it was tested whether PLD could use *tert*-butanol in the hydrolysis of

phosphatidylcholine to yield phosphatidylbutanol. As seen in Fig. 26, LPA potently increased PLD activity in the presence of 1-butanol, indicating that PLD utilized 1-butanol in the transphosphatidylation reaction resulting in increased phosphatidylbutanol levels. However, no increased PLD activity in response to LPA treatment was detected in the presence of *tert*-butanol, demonstrating that PLD does not utilize *tert*-butanol in the transphosphatidylation reaction. Therefore, the slight inhibition of mTORC1 signaling due to *tert*-butanol is likely due to non-specific effects of the alcohol.

3.3.13 PLD activity is significantly reduced in Rat2V25 cells

Having shown that mTORC1 signaling in Rat2V25 cells responded similarly to WT cells when treated with leucine and/or LPA, it was tested whether PLD activity was indeed reduced in the cells as it was reported that a PLD1 dominant negative cell line generated by Drs. Kam and Exton had reverted back to WT [138]. Seen in Fig. 27, PLD activity increased slightly due to LPA treatment in Rat2V25 cells, though this increase was substantially less than that observed in WT cells. Leucine treatment alone had no effect on PLD activity, nor did it lead to a greater increase in activity when combined with LPA, which is consistent with previous data, as leucine did not increase PLD activity in WT cells (Fig. 20).

3.3.14 Conclusions from Rat2V25 cell experiments

In the absence of fully functional PLD1, both leucine and LPA increased mTORC1 signaling, indicating that they are functioning through a pathway other than PLD1, which supports previous data suggesting that LPA primarily increases mTORC1 signaling through the ERK1/2 pathway. Additionally, since PLD2 also produces PA and the Rat2V25 cells only expressed dominant negative PLD1, it cannot be concluded from these experiments that PLD signaling is not involved in mTORC1 activation. While PLD activity was substantially reduced in comparison to WT Rat2 cells, it is possible that a necessary basal level of PA was maintained throughout these experiments due to PLD2 activity.

3.3.15 The additive effect of LPA and leucine on mTORC1 signaling is blocked by MEK inhibitors that restore signaling to levels observed in the presence of leucine alone

Having demonstrated that the leucine-mediated activation of mTORC1 does not require PLD activity, it was next tested what the effect of LPA and leucine combined would have on mTORC1 signaling in WT cells. If LPA were signaling primarily through the ERK pathway, then inhibition of MEK would be expected to attenuate the additive effect of leucine and LPA on the activation of mTORC1, and return S6K1 phosphorylation to the value observed in cells treated with leucine alone. In contrast, because leucine and insulin had no effect on ERK phosphorylation at the time point used for these studies

(Fig. 28), inhibition of ERK should not prevent their additive effect on activation of mTORC1 signaling. To further address this possibility, cells were treated with either PD-98059 (Fig. 29A) or U0126 (Fig. 29B), and then either leucine, insulin, or a combination of both was added to the culture medium. Under these conditions, U0126 had no effect, and PD-98059 had a small, but statistically insignificant effect, on leucine- and insulin-induced activation of mTORC1. Because U0126 was a more effective inhibitor of ERK1/2 phosphorylation compared to PD-98059 (Fig. 17), this result suggests that neither leucine- nor insulin-induced activation of mTORC1 signaling required ERK activation. In contrast, both PD-98059 (Fig. 29C) and U0126 (Fig. 29D) were effective in preventing the additive effect of LPA and leucine, and restored mTORC1 signaling to a value that was not significantly different compared to cells treated with leucine alone. This finding provides further support for the conclusion that LPA, but not leucine, signals primarily through the ERK1/2 pathway to modulate mTORC1.

3.4 Discussion

Over the past decade, numerous studies have implicated a role for an upstream lipid signaling pathway involving PLD and PA in the activation of mTORC1 under a variety of conditions. These conditions include cancer [99; 104; 150], cardiac hypertrophy [88; 89; 151; 152], systemic inflammatory responses [82], and mechanical stimulation of skeletal muscle [84]. The results of those studies showed a positive correlation between changes in PLD activity and/or expression and mTORC1 signaling. Other evidence appearing to link PLD and PA to activation of mTORC1 include the findings that

addition of PA to cells in culture led to increased phosphorylation and/or activation of S6K1 [80; 82; 85; 105] and that PA bound directly to the FRB domain of mTOR [80]. Moreover, in some [85; 105], but not all [80; 82] studies, addition of PA to cells in culture partially attenuated the rapamycin-induced repression of mTORC1. Based on such findings, a model was proposed in which increasing intracellular PA concentrations, either through activation of PLD or by exogenous addition of PA to cells in culture, led to increased binding of the lipid to the FRB domain of mTOR, resulting in activation of mTORC1 [80; 116]. This model has been widely accepted even though PA was shown to have no direct effect on mTORC1 activity in vitro, and mutations in the FRB domain that disrupt PA binding have no effect on mTORC1 kinase activity [80]. Moreover, the mechanism through which PA mediates activation of mTORC1 was made less clear by the observation that PA binds to and activates the mTORC1 substrate S6K1 independently of mTOR [153]. Therefore, studies relying solely on changes in S6K1 activity may mistakenly assume that such changes reflect alterations in mTORC1 activity.

An alternative mechanism through which PA might act to promote activation of mTORC1 involves the MEK-ERK signaling pathway. A number of studies [61; 62; 154] have shown that PA binds to and activates Raf, an upstream component of the ERK signaling pathway, leading to increased ERK phosphorylation and activity. Other studies have shown that preferential activation of the ERK pathway, for example using the phorbol ester PMA [155; 156; 157], leads to phosphorylation of TSC2, and subsequently to dissociation of the active TSC1/2 complex [52]. Mutation of the residues

phosphorylated in response to PMA treatment to ones that cannot be phosphorylated attenuates phorbol ester-induced mTORC1 activation. Moreover, treatment with U0126 dramatically attenuated PMA-induced activation of mTORC1 [155]. Interestingly, TSC2 is phosphorylated both by ERK and by its downstream effector, $p90^{RSK}$ [158]. Consequently, increased intracellular PA concentrations, e.g. occurring as a result of activation of PLD, could lead to activation of mTORC1 through the ERK signaling pathway, rather than through direct binding of PA to mTOR. The results of the present study strongly support this idea. Thus, inhibition of the MEK-ERK pathway using either of two structurally distinct inhibitors blocked both the PA- and LPA-induced activation of ERK and mTORC1. Moreover, although inhibition of MEK-ERK blocked the PA- and LPA-induced activation of mTORC1, it had no effect on the actions of leucine or insulin. Indeed, leucine acted in an additive manner with LPA to activate mTORC1.

The results of the present study also bring into question the assumption that addition of PA to cells in culture leads to mTORC1 activation through internalization of the lipid, and its subsequent direct action on intracellular targets, e.g. Raf and/or mTOR. An alternative mechanism through which exogenous PA activates ERK and mTORC1 is through its hydrolysis to LPA by phospholipases (i.e. PLA) present in the culture medium, followed by subsequent binding of LPA to EDG receptors. Activation of EDG receptors would then lead to increased signaling through the MEK-ERK pathway [123]. PLA is present in serum [120] and is also secreted by cells in culture [121; 122]. The findings in the present study that PA-induced activation of mTORC1 requires serum, and that in the presence of serum PA-induced mTORC1 activation is delayed relative to the

effect of LPA, is consistent with a model in which PA must be hydrolyzed to LPA in order to activate mTORC1. More definitive evidence supporting this idea is provided by the finding that Ki16425, a specific inhibitor of EDG-2 [148], the predominant form of EDG receptor in Rat2 cells [145], blocks PA-induced activation of mTORC1. A caveat to this model is that in some studies [80; 137], addition of PA to serum-starved cells leads to activation of mTORC1. In most of those studies, 16:0-18:1 PA was used, the same lipid used in the current study. However, in those studies, cells were serum deprived overnight prior to the introduction of exogenous PA, as opposed to the current study in which cells were deprived of serum for only 2h. Therefore, it is tempting to speculate that the extended deprivation time permitted accumulation of PLA to levels sufficient to hydrolyze enough PA to LPA to activate EDG-2.

It has been suggested that PLD and PA constitute a signaling pathway upstream of mTORC1 that functions in parallel to an AA signaling pathway [146; 159]. However, more recently, the same authors have concluded that AAs activate mTORC1 through Rheb-mediated activation of PLD [137]. The evidence supporting their conclusion included the findings that short-term AA deprivation attenuated serum-induced activation of PLD and that AA replenishment restored PLD activation by serum. In addition, AA deprivation was found to block the increase in PLD activation associated with repression of TSC2 expression. Because TSC2 acts as an upstream repressor of Rheb function, the latter finding was taken as evidence for it acting through Rheb to promote PLD activation and subsequently to activate mTORC1. In contrast, the results presented herein demonstrate that the readdition of the AA leucine to leucine-deprived cells has no effect

on PLD activity. Additionally, 1-butanol has no effect on PA content in leucine-deprived cells (data not shown), suggesting that in the absence of PLD activation, most of the PA present in the cell originates from an alternative source such as DGK [160] or LPAAT [94]. However, because leucine has no effect on PA content, it is unlikely that the AA activates either DGK or LPAAT.

The results of the present study strongly support the conclusion drawn from earlier studies [80; 159], i.e. that AAs act through a pathway parallel to PLD to activate mTORC1. For example, activation of mTORC1 by a combination of the AA leucine and either LPA or PA was 2-3-fold greater compared to the effect of either one alone, suggesting that the two mediators act through distinct pathways. This conclusion is in agreement with the results of a previous study showing that addition of PA together with a complete mixture of AAs to AA-deprived cells led to a synergistic activation of mTORC1 [80]. Further support for this conclusion is provided by the results presented herein showing that inhibition of the ERK pathway reduced the combined effect of LPA and leucine to the level observed with leucine alone, suggesting that LPA, but not leucine, acts through the ERK pathway to promote activation of mTORC1. The finding that inhibition of the ERK pathway had no effect on the combined effect of insulin and leucine demonstrates that, like leucine, insulin acts through a pathway distinct from ERK to activate mTORC1. Together, the findings reported here provide convincing evidence that leucine and LPA act through distinct mechanisms to activate mTORC1.

Overall, the results presented herein support the model depicted in Fig. 30. In this model, exogenous PA is hydrolyzed to LPA, which binds EDG-2, resulting in activation of the PLD and ERK signaling pathways. The finding that 1-butanol attenuates, but does not prevent LPA-induced mTORC1 activation suggests that activation of both PLD and Ras contribute to activation of ERK and subsequently mTORC1. The results are also consistent with the conclusion that leucine acts through a pathway that is parallel to, and independent of, PLD. The components of the leucine signaling pathway and how they lead to mTORC1 activation remain poorly characterized, and provide an area for future studies.

3.5 Chapter III: Figures and legends

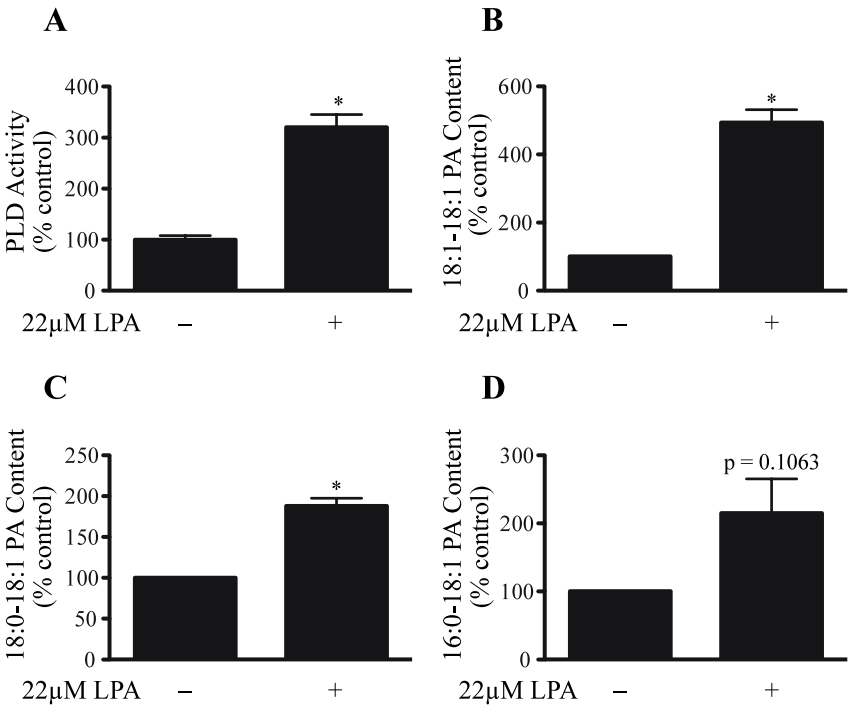

Figure 12. LPA treatment increases PLD activity and PA content. (A) Rat2 cells were deprived of serum and leucine for 2h before addition of 22 μM LPA, which approximated its concentration in serum [127]. PLD activity and PA content were measured as described under Materials and Methods. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. * $p<0.0001$ vs. either the deprived condition or leucine replacement. (B) 18.1-18.1 PA, (C) 18.0:18.1, and (D) 16.0:18.1 PA content was assessed by mass spec analysis as described under Materials and Methods. The results represent the mean ± SEM (n=3). * $p<0.002$ vs. the deprived condition.

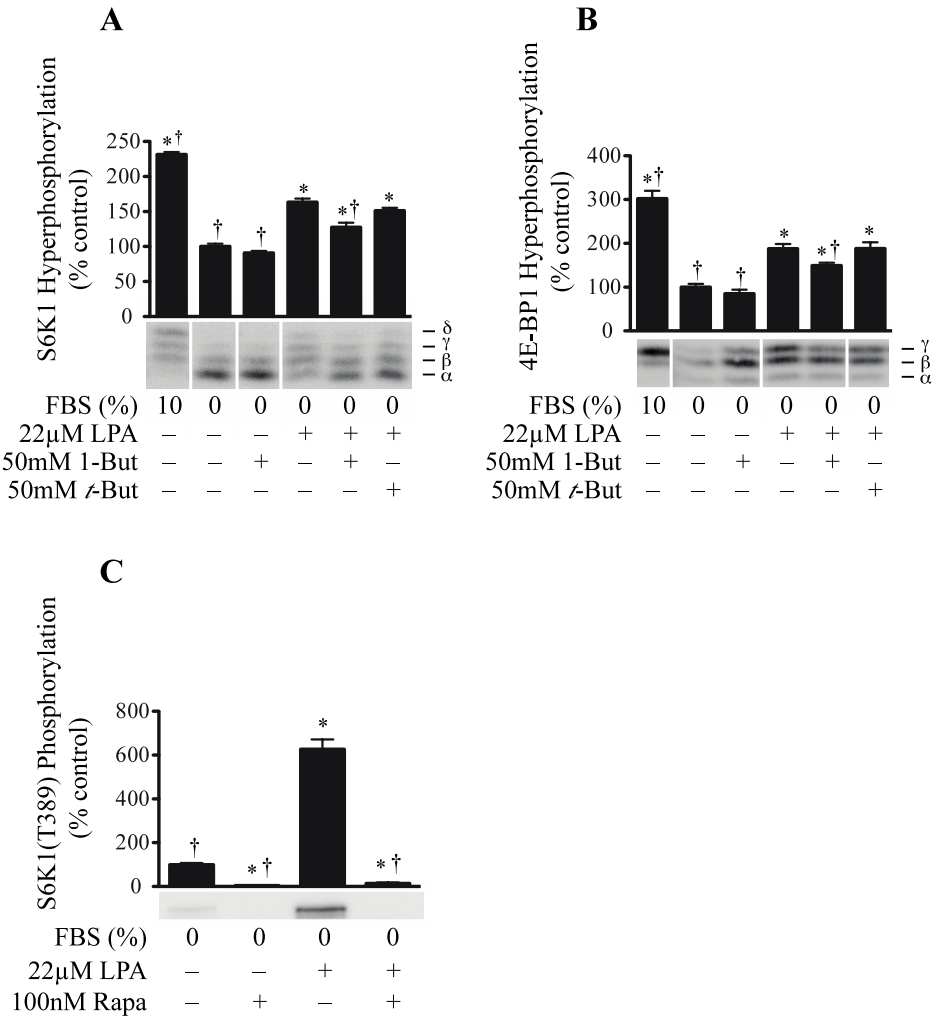

Figure 13. Rapamycin and 1-butanol, but not *tert*-butanol, attenuates LPA-mediated activation of mTORC1. Cells were maintained in DMEM medium containing 10% FBS, or deprived of serum and leucine for 2h. (A and B) 1-butanol, tert-butanol, or (C) rapamycin were added to the medium at the concentrations noted 30min prior to harvest. S6K1 hyperphosphorylation was assessed as (A) the proportion of the protein present in the β, γ, and δ forms relative to the total amount of the protein (α + β + γ + δ) or (C) phosphorylation on Thr389 using an antibody specific for the phosphorylated form of the protein. 4E-BP1 hyperphosphorylation was assessed as (B) the proportion of the protein present in the γ form relative to the total amount of the protein (α + β + γ). Representative blots are shown. In the blots shown in the figure, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. (A and B) * $p<0.003$ vs. cells deprived of serum and leucine; † $p<0.008$ vs. LPA treatment. (C) * $p<0.0001$ vs. cells deprived of serum and leucine; † $p<0.0001$ vs. LPA treatment.

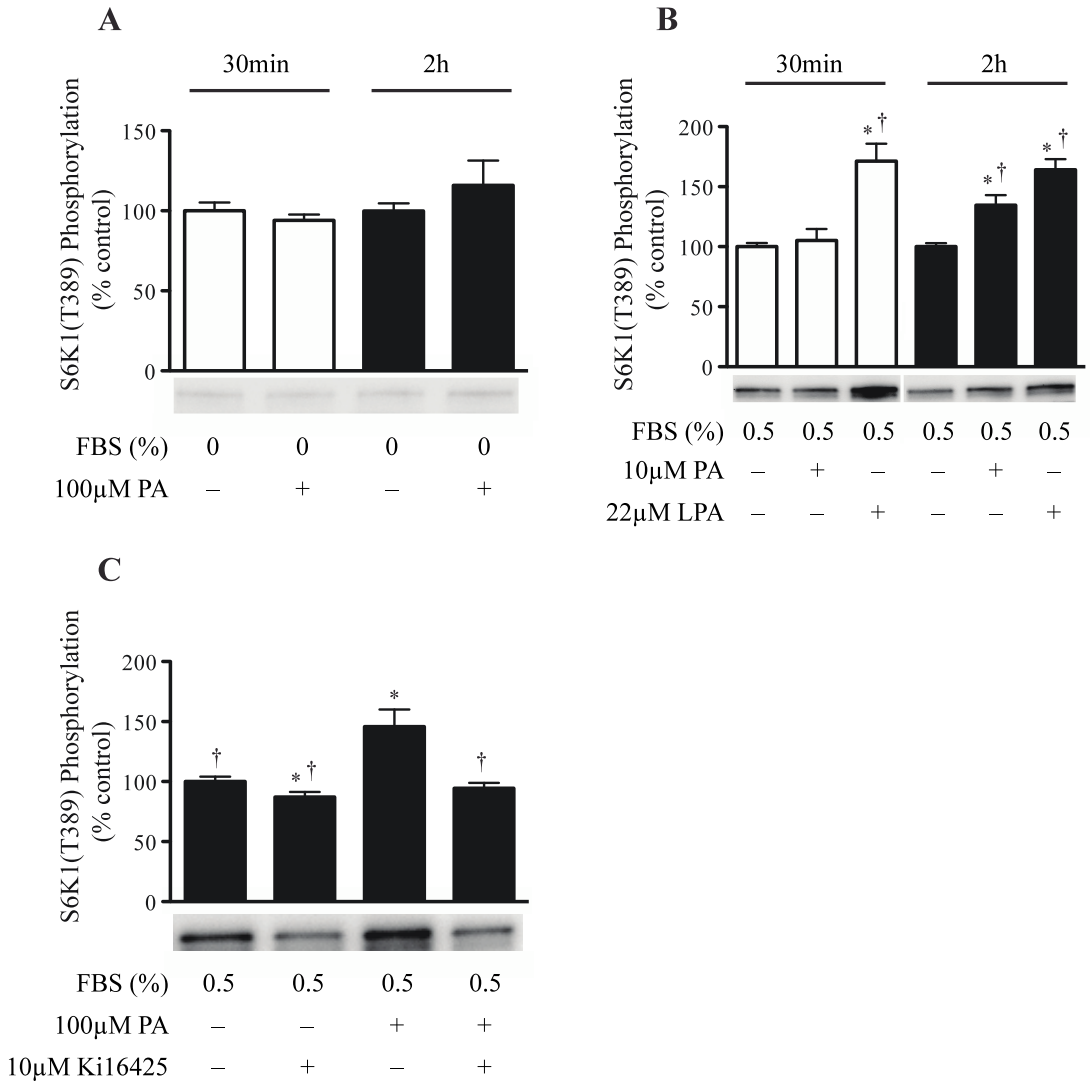

Figure 14. Extracellular PA acts through the LPA receptor to activate mTORC1. Cells were either (A) deprived of serum and leucine or (B and C) incubated in medium containing 0.5% serum without leucine for 2h before addition of either PA or LPA at the concentrations noted in the figure. Cells were harvested 30min (A and B) or 2h (A, B, and C) later. When present, Ki16425 was added to the medium 30min prior to PA. S6K1 phosphorylation on Thr389 was assessed as described in the legend to Fig. 13. Representative blots are shown. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. (B) * $p<0.002$ vs. cells incubated in medium containing 0.5% serum without leucine at the corresponding time point; † $p<0.035$ vs. PA treatment at the 30min time point. (C) * $p<0.05$ vs. cells deprived of serum and leucine; † $p<0.005$ vs. PA treatment.

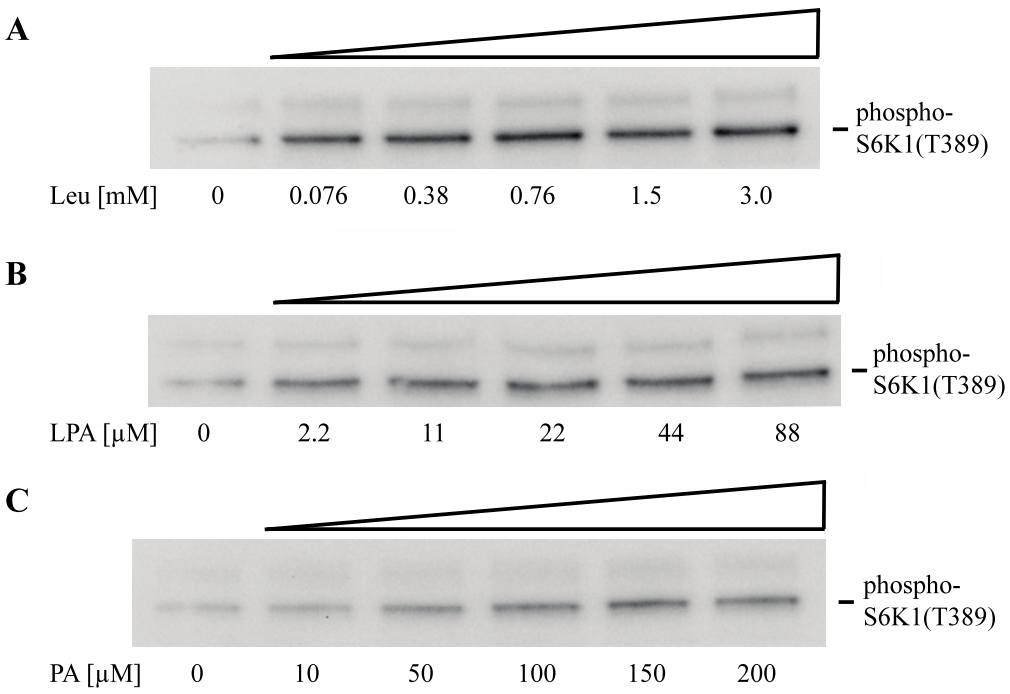

Figure 15. S6K1(T389) phosphorylation is increased by the addition of leucine, LPA, or PA to leucine- and serum-deprived cells. Rat2 cells were incubated in medium containing 0.5% serum without leucine for 2h before addition of either (A) leucine, (B) LPA, or (C) PA at the concentrations noted in the figure. Two h later, S6K1 phosphorylation on Thr389 was assessed as described in Fig. 13. Blots representative of three experiments are shown.

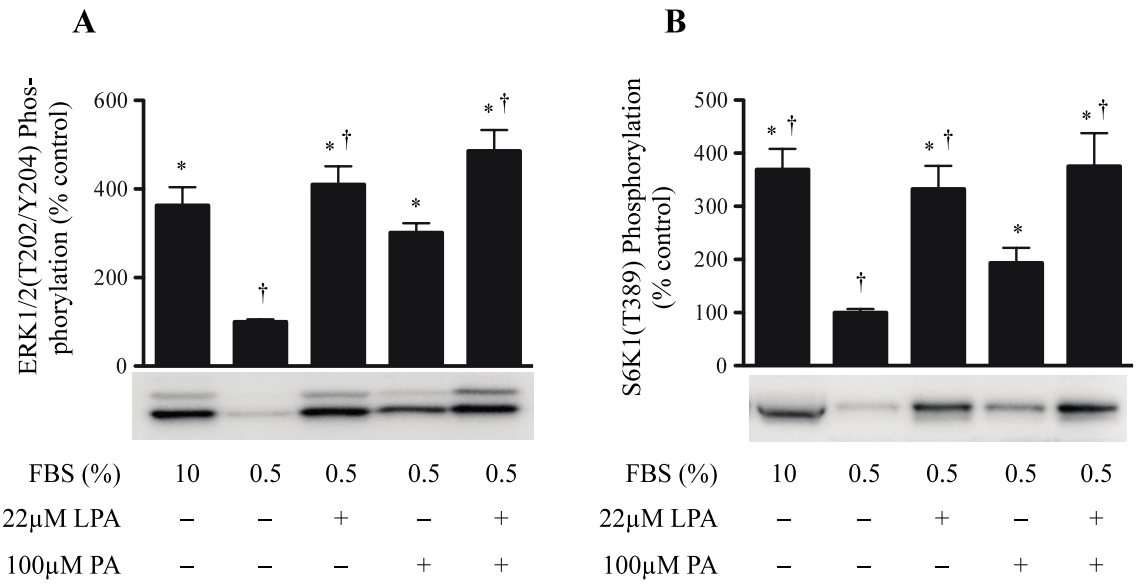

Figure 16. LPA and PA act through the same signaling pathway to activate mTORC1. Cells were incubated in medium containing 0.5% serum without leucine for 2h before addition of LPA and/or PA at the concentrations noted in the figure. (A) ERK1/2 phosphorylation on Thr202/Tyr204 was measured by Western blot analysis using an antibody specific for the phosphorylated form of the protein and (B) S6K1 phosphorylation was assessed as described in the legend to Fig. 13. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. Representative blots are shown in each panel. * $p<0.0005$ vs. cells incubated in medium containing 0.5% serum without leucine. † $p<0.05$ vs. cells treated with PA alone.

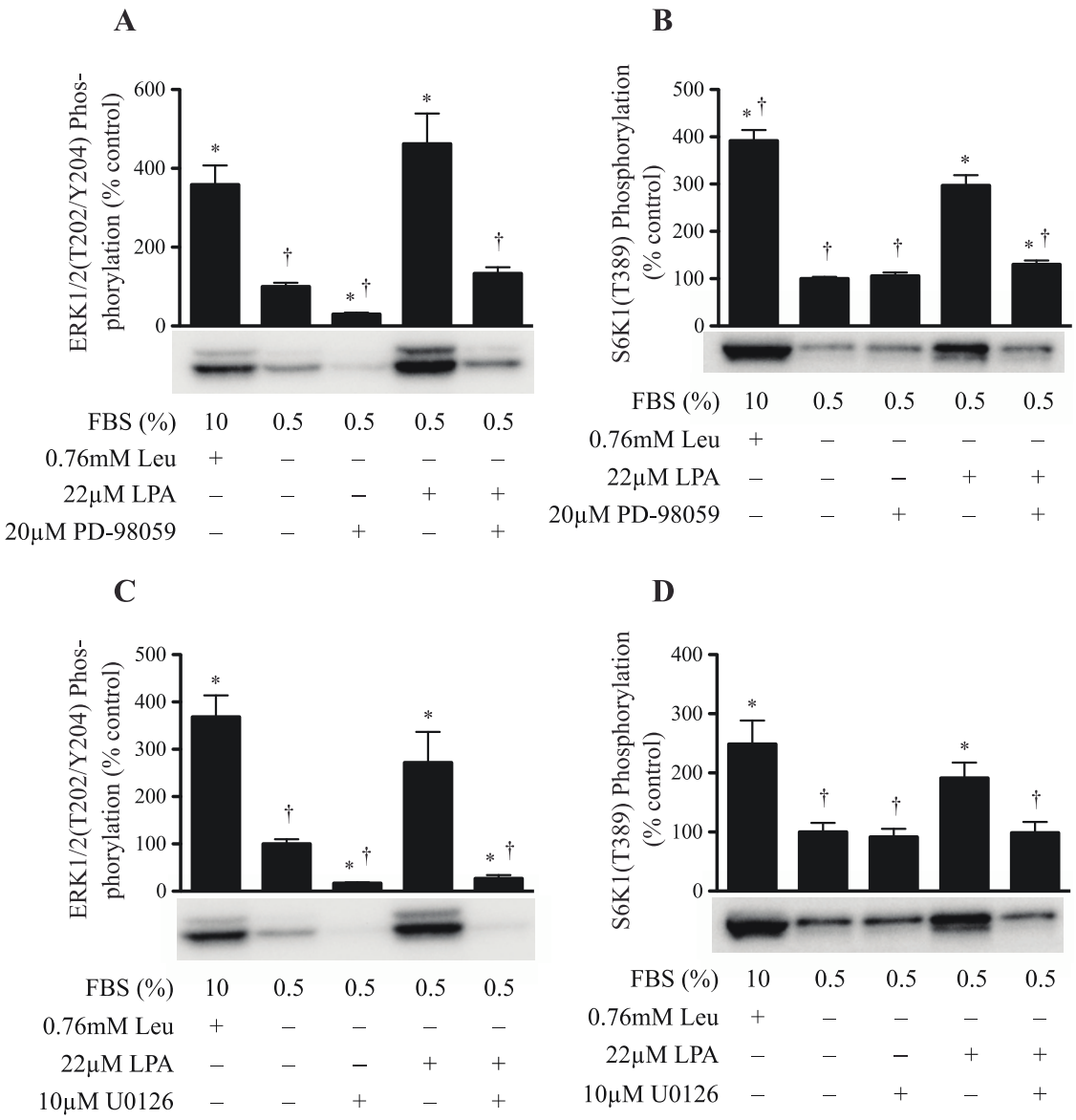

Figure 17. LPA, but not leucine, activates mTORC1 signaling through the MAP kinase pathway. Cells were incubated in medium containing 0.5% serum without leucine for 2h. Leucine, LPA, PD-98059, and/or U0126 was added to the medium and S6K1 phosphorylation on Thr389 and ERK1/2 phosphorylation on Thr202/Tyr204 were assessed as described in the legends to Fig. 13 and 16, respectively. (A and B) The results represent the mean ± SEM of five experiments; in each experiment, 2 samples per condition were independently analyzed. (C and D) The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. Representative blots are shown in each panel. * $p<0.02$ vs. cells treated in medium containing 0.5% serum without leucine. † $p<0.011$ vs. cells treated with LPA alone.

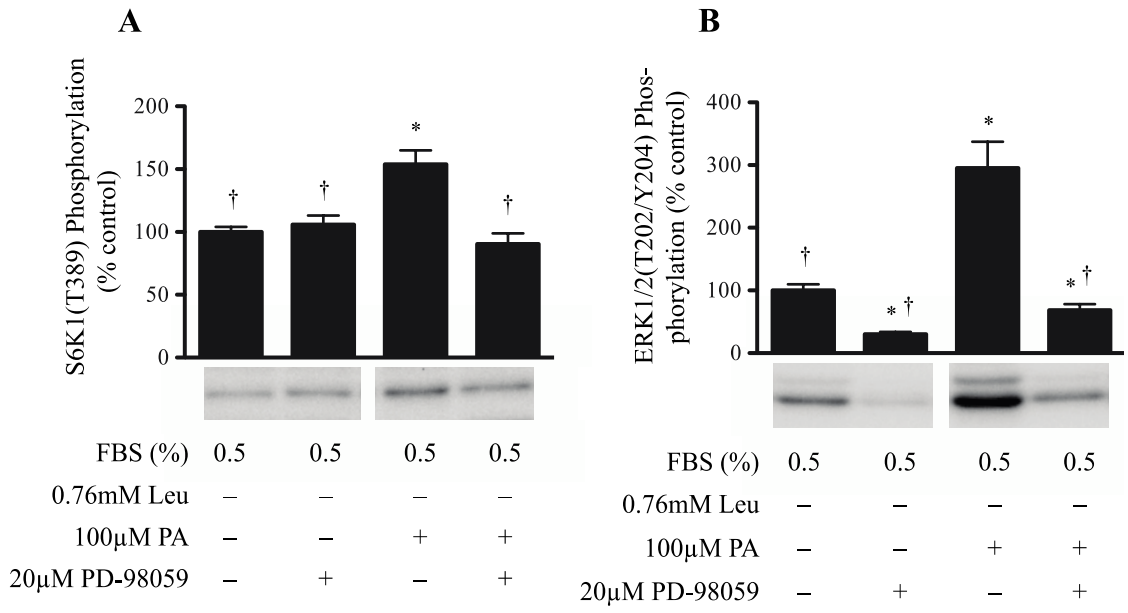

Figure 18. PA upregulates mTORC1 signaling through the MAP kinase pathway. Rat 2 cells were incubated in medium containing 0.5% serum without leucine for 2h. PA and/or PD-98059 was added to the medium and (A) S6K1 phosphorylation on Thr389 and (B) ERK1/2 phosphorylation on Thr202/Tyr204 were assessed as described in the legends to Fig. 13 and 16, respectively. Results represent the mean ± SEM of five experiments; in each experiment, 2 samples per condition were independently analyzed. Representative blots are shown. In the blots shown, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. * $p<0.05$ vs. cells incubated in medium containing 0.5% serum without leucine. † $p<0.003$ vs. cells treated with PA alone.

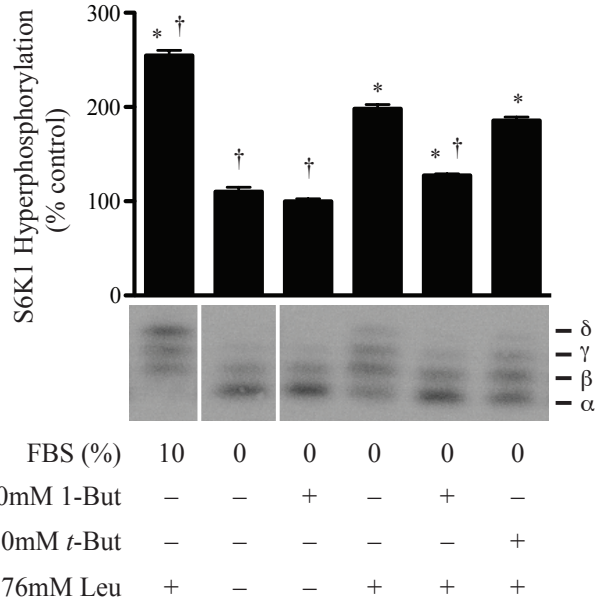

Figure 19. 1-Butanol, but not *tert*-butanol, attenuates leucine-mediated activation of mTORC1. Rat2 fibroblasts were maintained in DMEM medium containing 10% FBS, or deprived of serum and leucine for 2h. 1-Butanol, tert-butanol, and/or leucine were added to the medium at the concentrations noted 30min prior to harvest. S6K1 hyperphosphorylation was assessed as described in Fig. 13. A representative blot is shown. In the blot shown in the figure, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. * $p<0.004$ vs. cells deprived of leucine; † $p<0.0001$ vs. leucine alone.

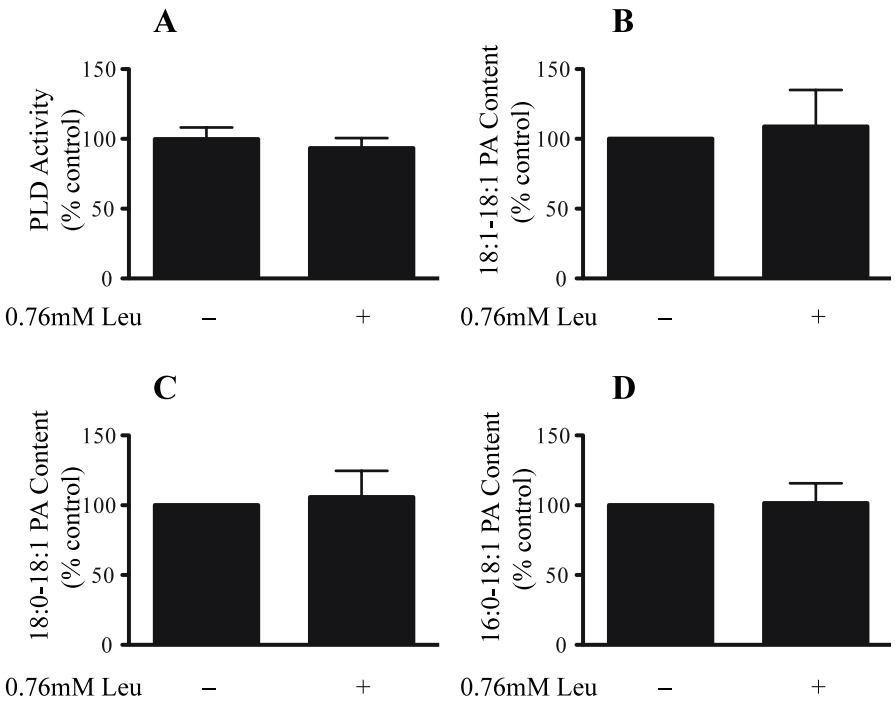

Figure 20. Leucine neither activates PLD nor increases PA content in leucine- and serum-deprived cells. Rat2 cells were incubated in medium lacking serum and leucine for 2h prior to addition of 0.76 mM leucine. Cells were harvested 30min later, and PLD activity and PA content were measured as described under Materials and Methods. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed.

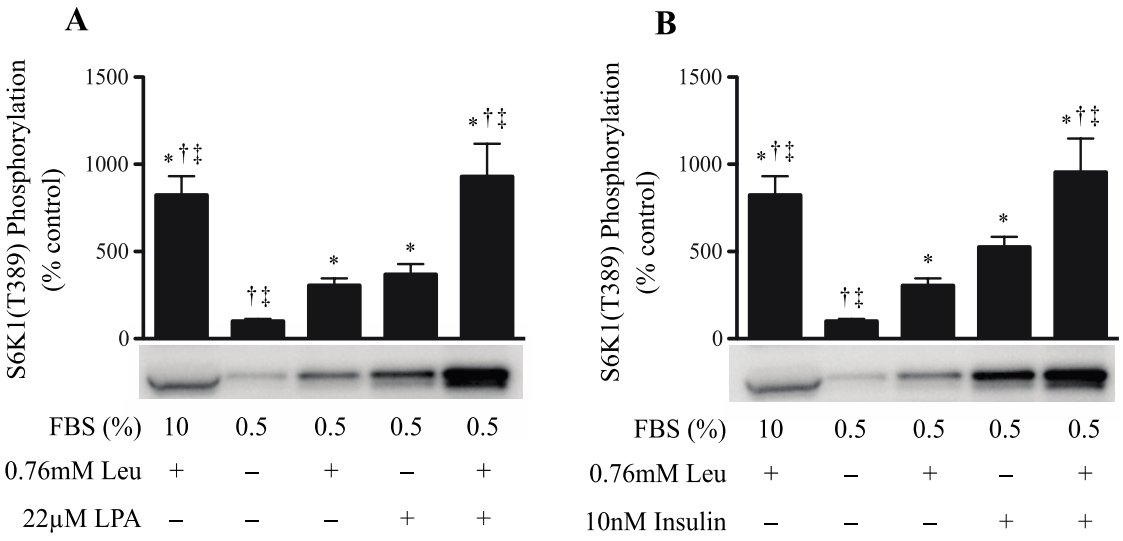

Figure 21. Leucine acts in an additive manner with LPA or insulin to activate mTORC1 signaling. Cells were incubated in medium containing 0.5% serum without leucine for 2h. (A) Leucine and/or LPA or (B) leucine and/or insulin were then added, and 2h later S6K1 phosphorylation was assessed as described in the legend to Fig. 13. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. Representative blots are shown in each panel. * $p<0.0005$ vs. cells incubated in medium containing 0.5% serum without leucine. † $p<0.005$ vs. cells treated with leucine alone. ‡ $p<0.05$ vs. cells treated with LPA alone or insulin alone.

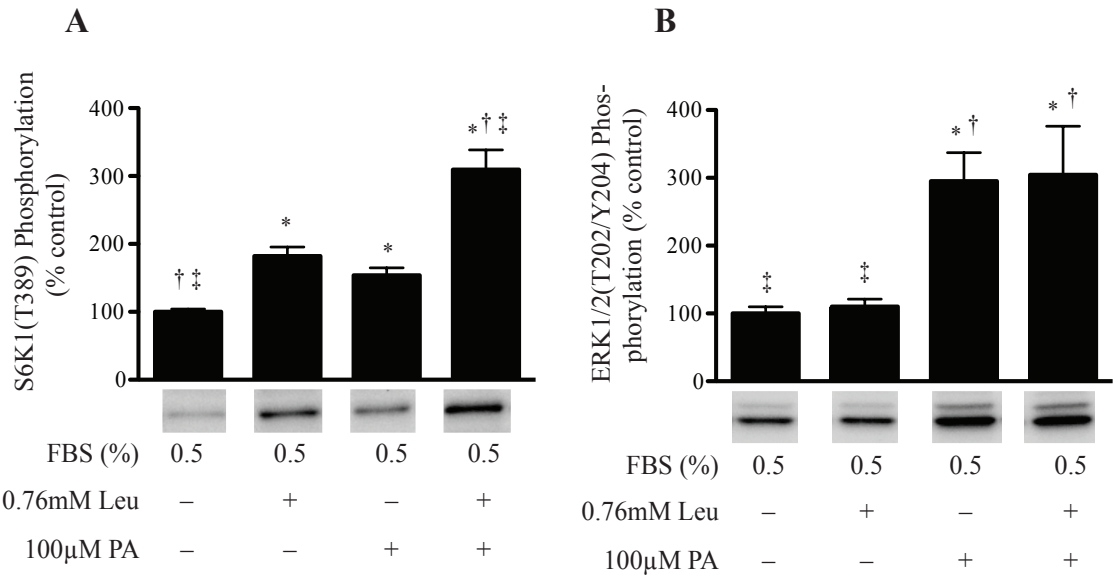

Figure 22. Leucine acts in an additive manner with PA to activate mTORC1 signaling. Rat2 cells were incubated in medium containing 0.5% serum without leucine for 2h. Leucine and/or PA was added to the medium and (A) S6K1 phosphorylation on Thr389 and (B) ERK1/2 phosphorylation on Thr202/Tyr204 were assessed as described in the legends to Fig. 13 and 16, respectively. Results represent the mean ± SEM of five experiments; in each experiment, 2 samples per condition were independently analyzed. Representative blots are shown. In the blots shown, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. * $p<0.015$ vs. cells deprived of leucine alone. † $p<0.02$ vs. cells treated with leucine alone. ‡ $p<0.0005$ vs. cells treated with PA alone.

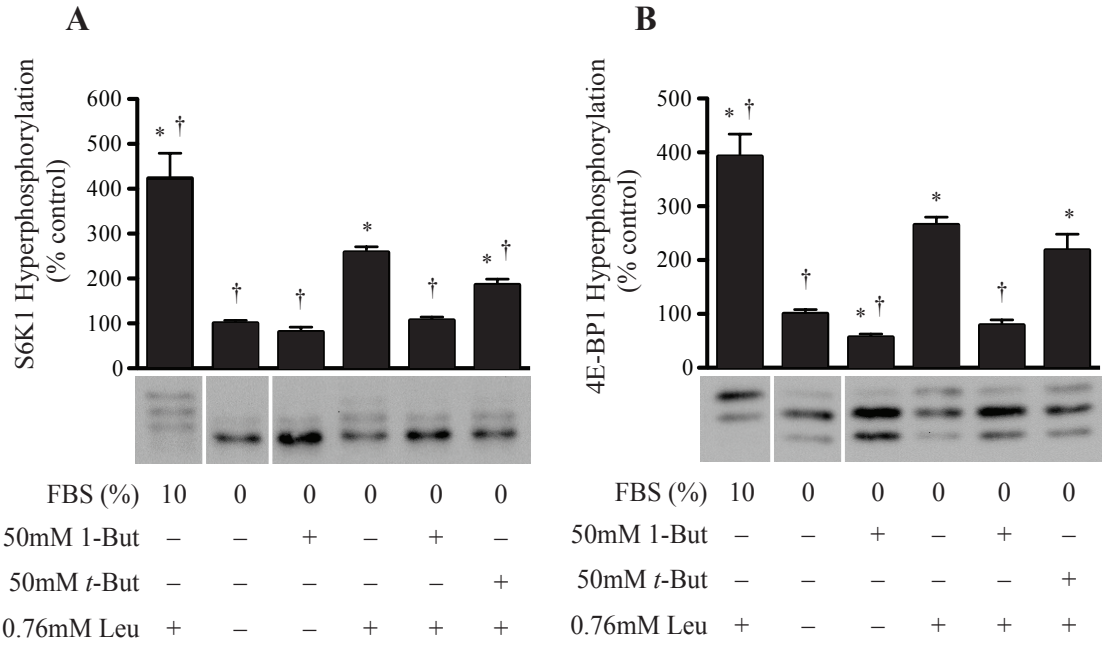

Figure 23. 1-Butanol, but not *tert*-butanol, attenuates leucine-mediated activation of mTORC1 in Rat2V25 fibroblasts. Cells were maintained in DMEM medium containing 10% FBS, or deprived of serum and leucine for 2h. 1-Butanol or tert-butanol were added to the medium at the concentrations noted 30min prior to harvest. (A) S6K1 and (B) 4E-BP1 hyperphosphorylation were assessed as seen in Fig. 13. Representative blots are shown. In the blots shown in the figure, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. (A) * $p<0.0001$ vs. cells deprived of serum and leucine; † $p<0.0015$ vs. leucine treatment. (B) * $p<0.002$ vs. cells deprived of serum and leucine; † $p<0.0001$ vs. leucine treatment.

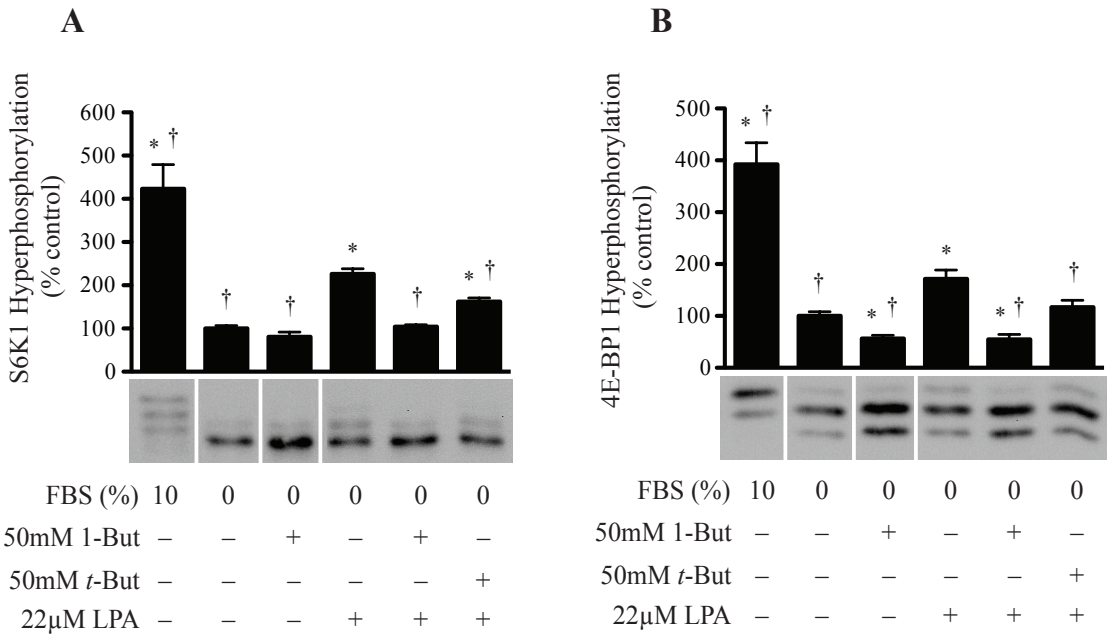

Figure 24. 1-Butanol, but not *tert*-butanol, attenuates LPA-mediated activation of mTORC1 in Rat2V25 fibroblasts. Cells were maintained in DMEM medium containing 10% FBS, or deprived of serum and leucine for 2h. 1-Butanol or tert-butanol were added to the medium at the concentrations noted 30min prior to harvest. (A) S6K1 and (B) 4E-BP1 hyperphosphorylation were assessed as seen in Fig. 13. Representative blots are shown. In the blots shown in the figure, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. (A) * $p<0.0001$ vs. cells deprived of serum and leucine; † $p<0.0006$ vs. LPA treatment. (B) * $p<0.003$ vs. cells deprived of serum and leucine; † $p<0.03$ vs. LPA treatment.

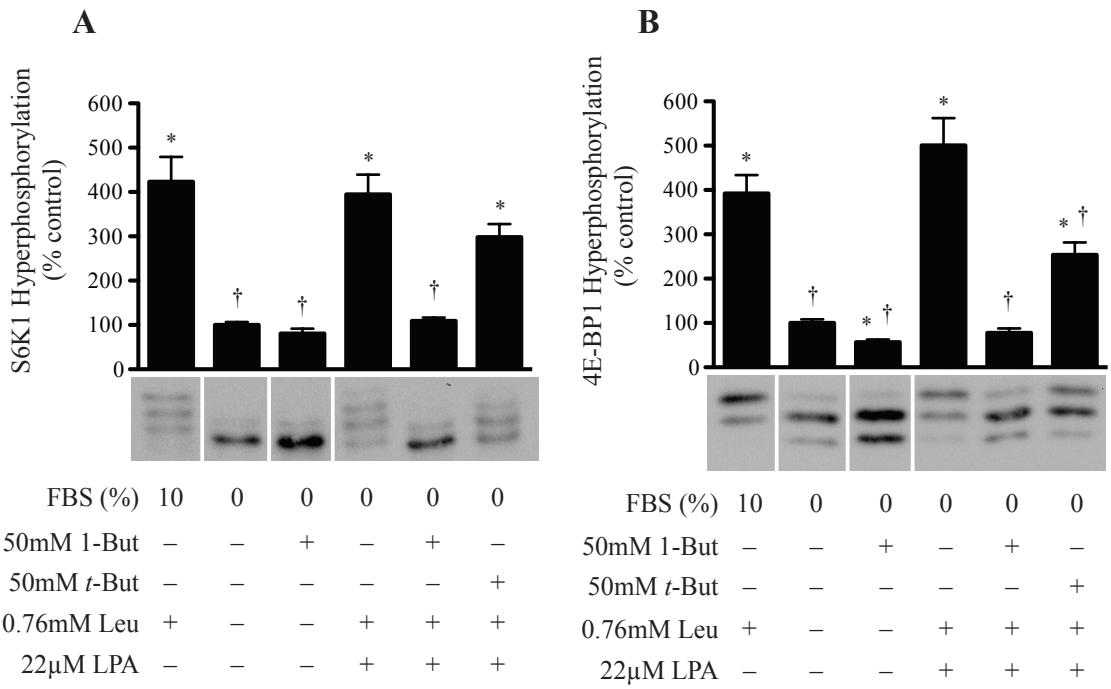

Figure 25. 1-Butanol, but not *tert*-butanol, attenuates the combined effect of leucine and LPA on the activation of mTORC1 in Rat2V25 fibroblasts. Cells were maintained in DMEM medium containing 10% FBS, or deprived of serum and leucine for 2h. 1-Butanol or tert-butanol were added to the medium at the concentrations noted 30min prior to harvest. (A) S6K1 and (B) 4E-BP1 hyperphosphorylation were assessed as seen in Fig. 13. Representative blots are shown. In the blots shown in the figure, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. (A) * $p<0.0001$ vs. cells deprived of serum and leucine; † $p<0.0001$ vs. leucine and LPA treatment. (B) * $p<0.0001$ vs. cells deprived of serum and leucine; † $p<0.003$ vs. leucine and LPA treatment.

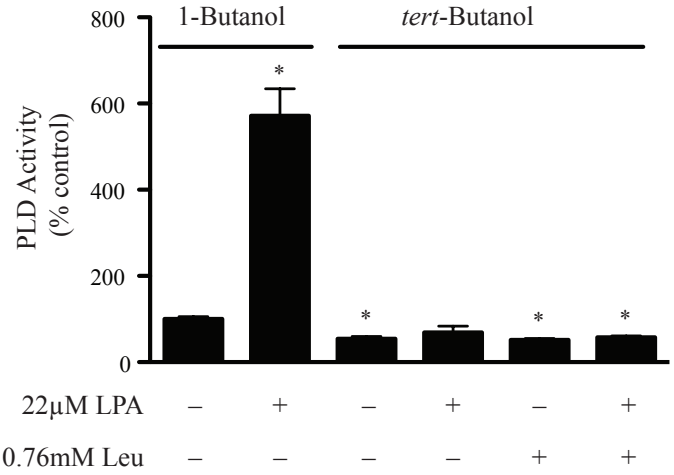

Figure 26. PLD does not utilize *tert*-butanol in the hydrolysis reaction that produces phosphatidylbutanol. Rat2 cells were deprived of serum and leucine for 2h before addition of 22 μM LPA and/or 0.76mM leucine, as seen in figures 12 and 20 respectively. PLD activity was measured in the presence of 1-butanol or tert-butanol, as described under Materials and Methods. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. * $p<0.0001$ vs. the deprived condition in the presence of 1-butanol.

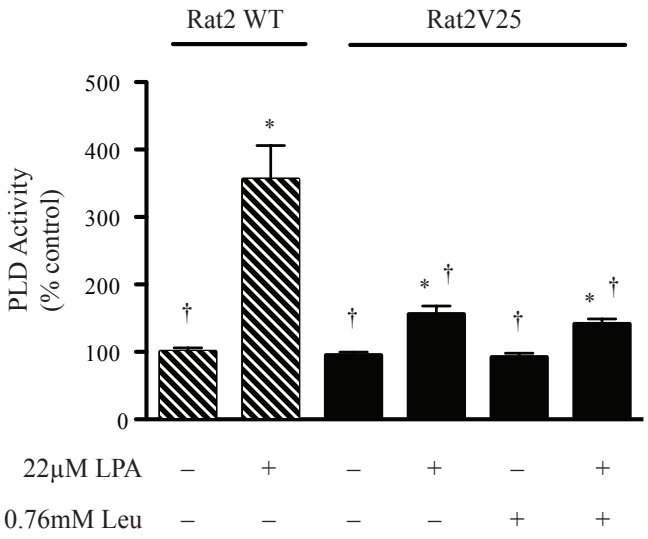

Figure 27. PLD activity is significantly reduced in Rat2V25 fibroblast cells. Wild type Rat2 cells (Rat2 WT) or Rat2V25 cells were deprived of serum and leucine for 2h before the addition of 22 μM LPA or 0.76mM leucine, as seen in figures 12 and 20 respectively. PLD activity was measured as described under Materials and Methods. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. * $p<0.0001$ vs. WT cell in the deprived condition; † $p<0.0015$ vs. WT cells treated with LPA.

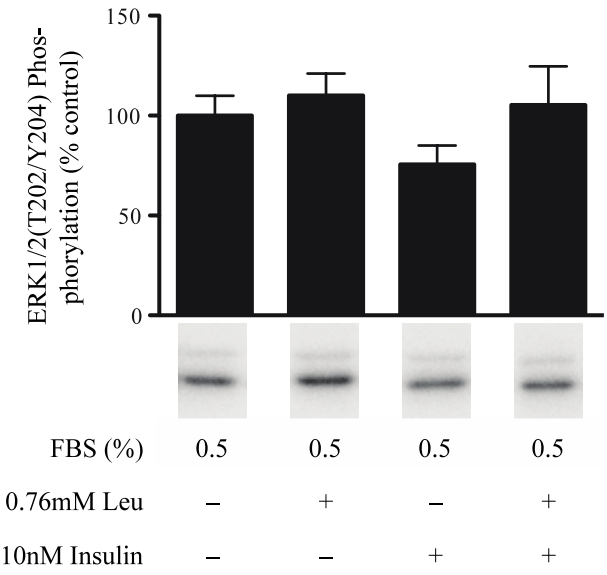

Figure 28. ERK1/2 phosphorylation is not increased after the addition of leucine or insulin to deprived cells. Rat2 cells were incubated in medium containing 0.5% serum without leucine for 2h before addition of either leucine or insulin. Two h later, ERK1/2 phosphorylation on T202/Y204 was assessed as described in the legend to Fig. 16. The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed.

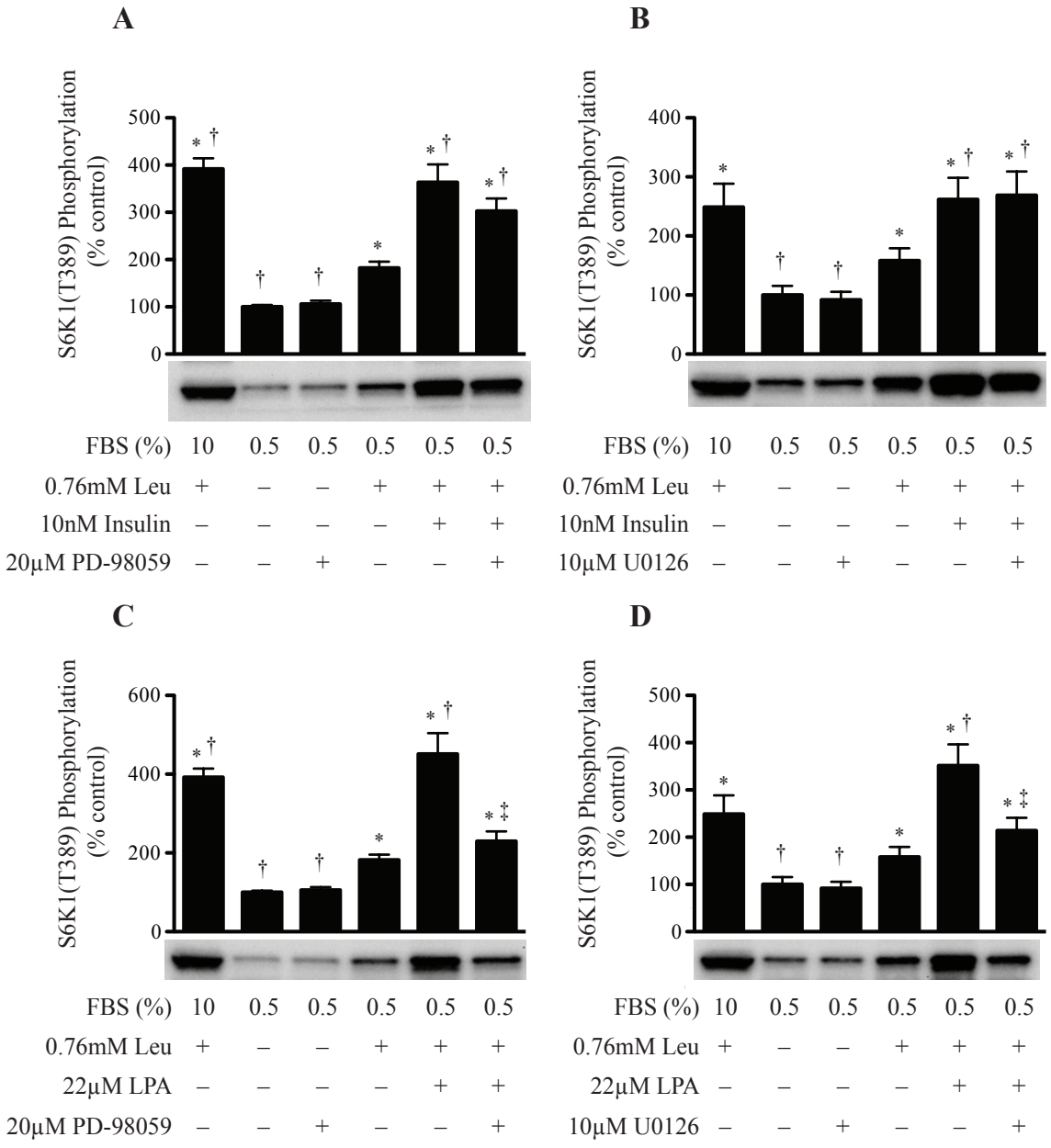

Figure 29. Inhibition of MEK attenuates LPA-induced, but not leucine- or insulin-induced, activation of mTORC1 signaling. Cells were incubated in medium containing 0.5% serum without leucine for 2h. Leucine, insulin, LPA, U0126, and/or PD-98059 was added to the medium and S6K1 phosphorylation was assessed as described in the legend to Fig. 13. (A and C) The results represent the mean ± SEM of five experiments; in each experiment, 2 samples per condition were independently analyzed. (B and D) The results represent the mean ± SEM of three experiments; in each experiment, 3 samples per condition were independently analyzed. Representative blots are shown in each panel. * $p<0.045$ vs. cells incubated in medium containing 0.5% serum without leucine. † $p<0.03$ vs. cells treated with leucine alone. ‡ $p<0.02$ vs. cells treated with leucine and LPA.

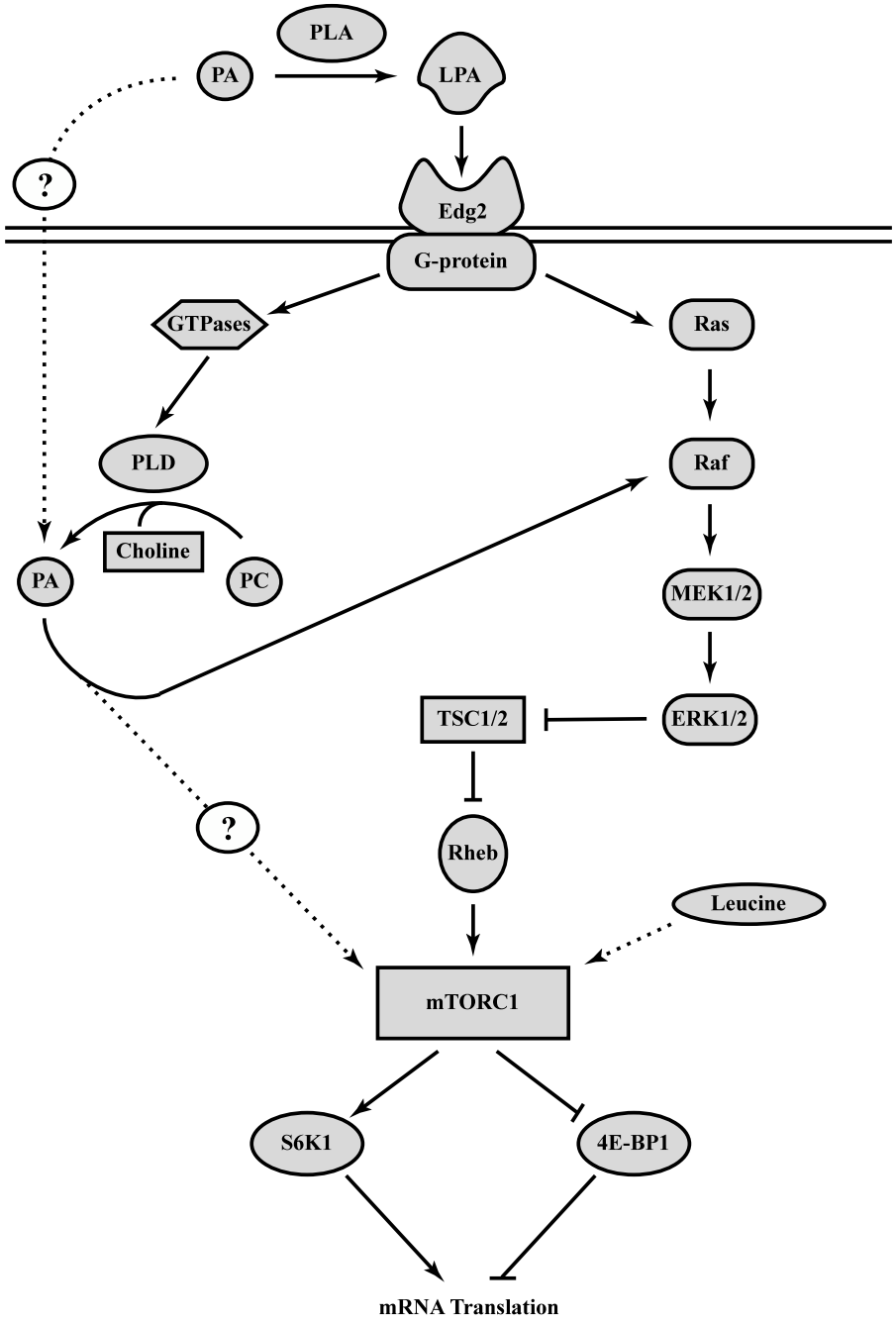

Figure 30. Model for the PA-mediated activation of mTORC1 signaling. PA is hydrolyzed by PLA and the resulting LPA activates the EDG-2 receptor. Activation of the EDG-2 receptor results in upregulated signaling through the MEK/ERK pathway via two distinct mechanisms. One mechanism involves a G-protein-mediated increase in PLD activity, leading to the hydrolysis of phosphatidylcholine (PC) to choline and PA. PA then binds to Raf, allowing for activation of the ERK cascade. In the second mechanism, G proteins act through Ras to activate the MEK-ERK pathway. Subsequently, ERK acts to inhibit TSC1/2, thereby increasing GTP loading on Rheb, and

thus upregulating mTORC1 activation. mTORC1 then phosphorylates downstream targets, such as S6K1 and 4E-BP1, ultimately leading to increased mRNA translation. Leucine also activates mTORC1, although the mechanism involved in the effect is not currently understood.

CHAPTER IV

THE ERK AND AKT SIGNALING PATHWAYS FUNCTION THROUGH PARALLEL MECHANISMS TO PROMOTE mTORC1 SIGNALING

4.1 Abstract

Signaling by mTORC1, which acts as an important regulator of growth, metabolism, and also regulates muscle hypertrophy, is regulated through multiple upstream signaling pathways, including those involving Akt and ERK. Previous studies have shown that, in part, Akt and ERK promote mTORC1 signaling through phosphorylation of the GAP, TSC2, that acts as an upstream inhibitor of mTORC1. In the present study we extend the earlier studies to show that activation of the Akt and ERK pathways acts in a synergistic manner to promote mTORC1 signaling. Moreover, we provide evidence that the Akt and ERK signaling pathways converge on TSC2, and that Akt phosphorylates residues on TSC2 distinct from those phosphorylated by ERK. The results also suggest that leucine-induced stimulation of mTORC1 signaling occurs through a mechanism distinct from TSC2, and the Akt and ERK signaling pathways. Overall, the results are consistent with a model in which Akt and ERK phosphorylate distinct sites on TSC2, leading to greater repression of its GAP activity, and consequently a magnified stimulation of mTORC1 signaling, compared to either input alone. The results further suggest that leucine acts through a mechanism distinct from TSC2 to stimulate mTORC1 signaling.

4.2 Introduction

One example of mTORC1 involvement in cell growth is skeletal muscle hypertrophy in which a role has been implicated for both the Akt and the MEK/ERK signaling pathways. In skeletal muscle, either exogenous expression of constitutively active Akt (caAkt) [37] or treatment with IGF-1 to activate Akt [161; 162] lead to increased fiber diameter. Similarly, expression of constitutively active MEK (caMEK) [163; 164] or treatment with clenbuterol to activate the MEK/ERK signaling pathway [59], promotes cardiac and skeletal muscle hypertrophy, respectively, whereas inhibition of ERK signaling abolishes clenbuterol-induced hypertrophy [59]. In addition, exogenous expression of MAPK phosphatase 1, which dephosphorylates and represses ERK signaling, decreases fiber size in skeletal muscle [58], further demonstrating the importance of ERK signaling in mediating the hypertrophic response. Both the Akt- and ERK-induced hypertrophy of skeletal muscle are blocked by rapamycin, demonstrating a critical role for mTORC1 in the response. Similar effects are observed in adult rat ventricular cardiomyocytes in which expression of caMEK1 activates mTORC1 [165]. Notably, the effect of caMEK1 on mTORC1 is inhibited by rapamycin [166], a result consistent with MEK acting through ERK and/or $p90^{RSK}$ to inhibit TSC1/2.

Although the studies cited above clearly show that signaling through either the Akt or the ERK signaling pathway leads to activation of mTORC1 and skeletal muscle hypertrophy, the relative contribution of the two pathways has not been examined. Moreover, the effect of simultaneous signaling inputs through both pathways on the activation state of

mTORC1 is as yet unexplored. Therefore, in the present study, we have employed a cell culture model that mimics skeletal muscle in regard to regulation of mTORC1 by hormones and nutrients to compare and contrast the effect of input signals through the Akt compared to the ERK pathway. We find that maximal input signals through either pathway activates mTORC1 to a similar extent, and that combined maximal input signals through both pathways leads to an even greater activation state of mTORC1 compared to either alone. Evidence for TSC2 being a point of convergence of the signaling inputs through the Akt and ERK pathways is provided by the observation that the activation of either pathway in TSC2 knock out cells no longer regulates mTORC1 signaling. Overall, the results support a model in which Akt signaling promotes phosphorylation of multiple residues on TSC2, including S939 and T1462, whereas ERK signaling acts through a mechanism distinct from phosphorylation of those residues. In this model, phosphorylation of both the Akt- and ERK-regulated sites leads to additive effects on the inhibition of TSC2, and subsequently a greater stimulation of mTORC1 signaling, compared to input signaling through either pathway alone.

4.3 Results

4.3.1 LPA and insulin treatments increase mTORC1 signaling in an additive fashion

In the present study, Rat2 fibroblasts were used as an experimental model because, unlike C2C12 and L6 myotubes, but similar to skeletal muscle in vivo, the activation state of

mTORC1 responds rapidly to the availability of hormones and nutrients at physiologically relevant concentrations [167]. Specifically, it is known that extended (18 hr) serum deprivation is required to reduce mTORC1 signaling to a basal level in C2C12 myotubes [85], which does not accurately mimic skeletal muscle *in vivo*. In contrast, in Rat2 fibroblasts, mTORC1 signaling is reduced to a basal level after 2hrs of deprivation [167]. Moreover, compared to muscle cell lines, Rat2 cells are relatively easy to transfect at high efficiency [167]. Indeed, in the present study, the transfection efficiency was 89±7% (n=3), as assessed by the proportion of cells expressing green fluorescent protein (GFP) after transfection with a plasmid encoding the protein. It was observed in Chapter III that LPA is a highly effective agent for activating the ERK signaling pathway in these cells. As illustrated in Fig. 31A, treatment with a concentration of LPA previously determined to be maximally effective in these cells produced a 7-fold increase in signaling through the ERK pathway, whereas a supraphysiological concentration of insulin had little effect, as assessed by changes in ERK1/2(T202/Y204) phosphorylation. When LPA and insulin treatments were combined, ERK signaling was not significantly different compared to LPA alone, indicating that the contribution of insulin was minimal (Fig. 31A). Additionally, while LPA treatment had only a minor effect on signaling through the Akt pathway, as assessed by Akt(S473) phosphorylation, insulin produced an ~2.5-fold increase (Fig. 31B). To test whether the ERK and Akt signaling pathways function together to activate mTORC1, cells were treated with maximally effective concentrations of LPA or insulin individually or in combination, and S6K1 phosphorylation on T389, a site directly phosphorylated by mTORC1 [168], was assessed. Individually, LPA and insulin activated mTORC1 by ~2-fold (Fig. 31C).

However, when combined the treatments activated mTORC1 ~4-fold (Fig. 31C). Thus, the ERK and Akt signaling pathways acted in an additive manner to promote mTORC1 signaling.

4.3.2 U0126 and Akt1/2 KI specifically inhibit MEK and Akt respectively

To assess the combined and individual roles of the ERK and Akt signaling pathways in the activation of mTORC1, the MEK inhibitor, U0126, and the Akt1/2 kinase inhibitor, Akt1/2 KI, were employed. As shown in Fig. 32A and 32B, respectively, U0126, but not Akt1/2 KI, prevented LPA-induced ERK1/2 phosphorylation whereas Akt1/2 KI, but not U0126, inhibited Akt phosphorylation. In confirmation of the results shown in Fig. 31B, combined treatment with LPA and insulin led to significantly greater phosphorylation of Akt on S473 compared to either agent alone (Fig. 32B). However, in cells treated with U0126, the combined effect was lost, and Akt(S473) phosphorylation in cells treated with U0126, LPA, and insulin was not different compared to cells treated with insulin alone. Thus, activation of MEK is largely responsible for the combined effect of LPA and insulin on Akt(S473) phosphorylation.

4.3.3 ERK and Akt activity increases mTORC1 signaling in an additive fashion

Having established the specificity of the inhibitors for the ERK and Akt signaling pathways, their respective effects on LPA- and insulin-induced activation of mTORC1

was assessed. As shown in Fig. 33A, in the presence of U0126, mTORC1 signaling in cells treated with both LPA and insulin was the same as in cells treated with insulin alone. Similarly, in the presence of Akt1/2 KI, the combined effect of LPA and insulin was abolished, and mTORC1 signaling was statistically identical to that observed in cells treated with LPA alone (Fig. 33B). Combined, the two inhibitors completely prevented the LPA- and insulin-induced activation of mTORC1 (Fig. 33C). Overall, the results shown in Figs. 31 and 33 demonstrate that the Akt and ERK signaling pathways are acting through parallel mechanisms to promote mTORC1 signaling. This conclusion is also supported by results from a recent report [169] showing that phosphorylation of ribosomal protein S6 on Ser240/244 was stimulated to a greater extent in HEK293 cells treated with a combination of insulin and PMA, a potent activator of the ERK signaling pathway, compared to treatment with insulin alone.

4.3.4 The expression of caMEK1 and caAkt stimulates mTORC1 signaling to the level observed in response to LPA and insulin treatments

To further confirm that the ERK and Akt signaling pathways were acting through parallel mechanisms to activate mTORC1, the effect of exogenous expression of constitutively active MEK1 (S218/S222D) and/or Akt (myr-HA-Akt) on S6K1 phosphorylation was assessed. First, to determine the effectiveness of the transfection of caMEK1 and caAkt, both which contained an HA tag, into Rat2 cells, the presence of the HA tag was examined through Western blot analysis. When pUSEamp activated MEK1 was expressed, the HA tag was detected at ~45-kD, which is the size of MEK1. The HA tag

was also found when pCMV5 myr-HA-Akt was expressed, appearing at ~60-kD, the size of Akt. When both constructs were present, the HA tag was seen at ~45-kD and ~60-kD, demonstrating that both constructs were successfully overexpressed (Fig. 34D). Upon examination of ERK1/2 signaling, it was found that the expression of caMEK1 led to a 4-fold increase in ERK1/2 phosphorylation, whereas expression of caAkt had little effect (Fig. 34A). In contrast, caMEK1 had essentially no effect on phosphorylation of PRAS40, a direct target of Akt [48], whereas caAkt significantly increased PRAS40 phosphorylation (Fig. 34B). Importantly, in cells expressing both kinase variants, no combined effect on either ERK or PRAS40 was observed. In cells individually expressing caMEK1 or caAkt, the activation state of mTORC1 was increased ~2.5-fold relative to cells transfected with a control plasmid (Fig. 34C). When caMEK1 and caAkt were expressed together, the activation state of mTORC1 was greater than when either construct was expressed alone (Fig. 34C), a result consistent with the observation that LPA and insulin treatments acted through parallel mechanisms to activate mTORC1.

4.3.5 ERK and Akt regulation of mTORC1 signaling requires TSC2

Although ERK and Akt phosphorylate, and thereby inactivate, TSC2, both kinases can also act downstream of TSC2 to modulate mTORC1 activity. For example, through activation of $p90^{RSK}$, ERK promotes phosphorylation of raptor on multiple sites, and exogenous expression of a raptor variant that cannot be phosphorylated by $p90^{RSK}$ attenuates ERK-induced mTORC1 signaling [170]. Moreover, ERK directly phosphorylates raptor on multiple residues, leading to the stimulation of mTORC1

signaling [169]. Similarly, although Akt phosphorylates, and thereby inactivates TSC2, it also phosphorylates mTOR [171] and the mTORC1 repressor, PRAS40 [172]. Thus, activation of ERK and/or Akt could potentially activate mTORC1 through both TSC2-dependent and –independent mechanisms. To assess the contribution of TSC2-independent mechanisms in the regulation of mTORC1, the effect of LPA and insulin was assessed in MEF cells lacking TSC2. Similar to the results from Rat2 cells, in WT MEFs LPA and insulin acted in an additive manner to activate mTORC1 (Fig. 35A). The combined inhibition of MEK and Akt by U0126 and Akt1/2 KI respectively, reduced S6K1(T389) phosphorylation to the control value, while inhibiting MEK or Akt individually led to only a partial reduction in S6K1(T389) phosphorylation (data not shown). However, in TSC2 KO cells, mTORC1 signaling was constitutively high, and neither LPA nor insulin treatment had any additional effect (Fig. 35B). This finding suggests that in the absence of TSC2, ERK signaling to raptor and Akt signaling to PRAS40 to control the activation state of mTORC1 is rendered ineffective.

4.3.6 The expression of caRheb significantly attenuates LPA- and insulin-mediated regulation of mTORC1 signaling

The increased mTORC1 signaling observed in TSC2 KO cells (Fig. 35B) is presumably due to increased levels of Rheb-GTP, which would effectively render mTORC1 insensitive to ERK and Akt signaling, provided that ERK and Akt signaling converge at TSC1/2. To test this, Rat2 fibroblasts were transfected with a pRK7 empty vector or Rheb (S16H), a mutant that preferentially binds GTP and therefore expresses constitutive

activity. These cells were then treated with LPA and/or insulin and ERK1/2, Akt, and mTORC1 signaling was examined. As expected, the expression of Rheb (S16H) did not alter the response of ERK1/2 to LPA or insulin, as ERK1/2 phosphorylation increased ~3-fold in response to LPA treatment in cells expressing either empty vector or Rheb (S16H), while insulin alone had no stimulatory effect (Fig. 36A), similar to what was shown previously (Fig. 31A). Akt signaling was also similar to what was previously observed (Fig. 31B), as phosphorylation of S473 on Akt due to insulin and/or LPA treatment in cells expressing Rheb (S16H) was similar to that observed in cells expressing empty vector, though it did appear slightly attenuated (Fig. 36B). This is likely because of negative feedback, as it is known that prolonged mTORC1 activation attenuates Akt signaling through the S6K1 mediated phosphorylation and inactivation of IRS-1 [173]. Importantly, mTORC1 signaling was altered in the presence of Rheb (S16H), though not in the presence of empty vector. LPA and insulin separately increased mTORC1 signaling in cells expressing the empty vector, and treating cells with both agonists increased mTORC1 signaling additively (Fig. 36C), as shown previously (Fig. 31C). Although mTORC1 signaling increased slightly in response to LPA and/or insulin treatment when Rheb (S16H) was expressed, the activation was marginal compared to cells that expressed the empty vector (Fig. 36C). Indeed, in cells expressing Rheb (S16H) the activation of mTORC1 approximated what was observed in cells lacking TSC2 (Fig. 35B), indicating the importance of the regulation of Rheb by TSC1/2. As neither the ERK nor Akt signaling pathways were significantly affected by the expression of Rheb (S16H), the data further supports that ERK and Akt increase

mTORC1 signaling through the inactivation of TSC1/2, resulting in increased concentrations of Rheb-GTP.

4.3.7 Akt activation increases phosphorylation of TSC2 on S939 and T1462, while ERK activation has no affect on the Akt specific residues

One mechanism through which the ERK and Akt signaling pathways might act in concert to repress TSC2 function and thus stimulate mTORC1 signaling is through phosphorylation of specific residues that act in a complimentary manner to repress the GAP activity of the protein. To assess this possibility, the effect of LPA and insulin treatment on phosphorylation of S939 and T1462, sites phosphorylated by Akt, was assessed. Insulin treatment increased phosphorylation of TSC2 on both S939 (Fig. 37A) and T1462 (Fig. 37B). In contrast, LPA alone had no effect on phosphorylation of either S939 or T1462, and LPA had no additional effect in insulin-treated cells, showing that LPA acts through a mechanism distinct from that of insulin.

4.3.8 The combination of leucine, insulin, and LPA maximally increase mTORC1 signaling

Amino acids are thought to stimulate mTORC1 signaling through a pathway parallel to TSC1/2 [64] that involves the Rag GTPases [174; 175]. If insulin and LPA are acting primarily through TSC2 to regulate mTORC1 signaling, then restoration of leucine to the culture medium would be expected to enhance the stimulation of mTORC1 signaling

beyond that observed with just insulin and LPA. Indeed, as shown in Fig. 38, leucine acted in an additive manner to stimulate mTORC1 signaling when combined with either insulin or LPA. Moreover, in cells treated with insulin, LPA, and leucine, mTORC1 signaling was significantly greater compared to cells treated with any two agents, confirming that insulin and LPA were acting through a pathway distinct from that activated by leucine.

4.4 Discussion

In the present study, insulin and LPA were used to selectively stimulate the Akt and ERK signaling pathways. Individually, insulin and LPA activated mTORC1 to a similar extent. However, together they acted in an additive manner, suggesting that they signal through parallel pathways to stimulate mTORC1 signaling. A caveat to this conclusion is that combined treatment with both insulin and LPA increased Akt phosphorylation to a greater extent compared to either agent alone, suggesting that MEK/ERK might act through the Akt pathway to stimulate mTORC1 signaling. If this caveat were correct, then inhibition of Akt should block LPA-induced stimulation of mTORC1 signaling. However, stimulation of mTORC1 signaling by a combination of insulin and LPA was only partially attenuated by inhibition Akt; inhibition of both the Akt and MEK/ERK pathways was required to completely block the stimulation of mTORC1 signaling. This finding provides support for the conclusion that the two pathways function in parallel to stimulate mTORC1 signaling. Results from studies utilizing constitutively active variants of Akt and MEK1 provided further support for this conclusion. Thus, exogenous

expression of caMEK1 had essentially no effect on Akt signaling, as assessed by phosphorylation of PRAS40, and co-expression of caMEK1 and caAkt did not increase Akt activation beyond that engendered by expression of caAkt alone. However, co-expression of the two constructs resulted in a significantly greater stimulation of mTORC1 signaling compared to that observed with expression of either one alone. Combined, the results strongly suggest that Akt and ERK act through parallel mechanisms to stimulate mTORC1 signaling.

A possible point of convergence of input stimuli through the Akt and ERK pathways is at the TSC1•TSC2 complex. The complex is a nexus through which a variety of positive and negative signals converge to control the activation state of mTORC1. For example, AMP-activated protein kinase (AMPK), which is activated in response to energy stress, phosphorylates, and thereby activates TSC2 [176]. Akt and ERK also phosphorylate TSC2, but in contrast to AMPK, phosphorylation by either Akt [177] or ERK [52] inhibits TSC2 function. Thus, phosphorylation of TSC2 can either activate or repress its function, depending on the sites that are modified. Interestingly, the sites phosphorylated by Akt and ERK are distinct [42; 53], providing a potential mechanism through which the two pathways might act in a cooperative manner to repress TSC2 function. In the present study, activation of Akt, but not MEK/ERK, increased the phosphorylation of TSC2 on S939 and T1462, two sites previously shown to be targeted by Akt [42]. Unfortunately, we were unable to detect TSC2 phosphorylated on S664, a site known to be phosphorylated by ERK [178], using two different antibodies specific for that residue. However, it is tempting to speculate that phosphorylation of TSC2 by ERK on a

residue(s) distinct from those phosphorylated by Akt mediates LPA-induced repression of TSC2, and that a combination of phosphorylation by the two kinases is required for maximal inhibition of TSC2 activity.

Further support for the conclusion that Akt and ERK act through TSC2 to regulate mTORC1 is provided by the finding that leucine restoration to deprived cells stimulated mTORC1 signaling in the presence of both insulin and LPA. Amino acids stimulate mTORC1 signaling through a TSC2-independent mechanism involving the Rag GTPases [175; 179]. Although the details are incompletely defined, a recent study suggests that amino acids activate mTORC1 by causing it to be recruited to a complex consisting of the Rag GTPases and a trimeric complex consisting of MAPK scaffold protein 1 (MP1), p14, and p18 that is referred to as Ragulator [180]. Based on the results of that study, a model has been proposed wherein association of mTORC1 with the Rag/Ragulator complex at the lysosomal membrane brings it into proximity to Rheb [181], resulting in partial activation of the kinase. However, full activation of mTORC1 requires both amino acid-induced recruitment of mTORC1 to Rheb, as well as increased GTP loading on Rheb, an event that is mediated by repression of TSC2. The results of the present study are consistent with such a model. Thus, leucine restoration to deprived cells resulted in partial activation of mTORC1, as did treatment with either insulin or LPA in the absence of leucine. Combined, treatment with leucine, insulin, and LPA caused a greater stimulation of mTORC1 signaling than did treatment with any pair.

The results of the present study suggest that efforts to increase and/or maintain muscle mass, e.g. in ageing or sepsis, through activation of mTORC1 may be enhanced by simultaneous activation of the Akt, MEK/ERK, and Rag pathways. Thus, activation of the Rag•Ragulator pathway by amino acids without simultaneously downregulating TSC2 activity, e.g. by increasing insulin concentrations, is relatively ineffective in activating mTORC1 in skeletal muscle. For example, in skeletal muscle of diabetic rats [182] or in rats administered diazoxide to block insulin secretion [183], the amino acid-induced stimulation of mTORC1 signaling is blunted compared to control animals. The results of the present study also suggest that activation of both the Akt and MEK/ERK signaling pathways leads to greater repression of TSC2 activity compared to activation of either alone. In this regard, both the Akt and the MEK/ERK signaling pathways have been implicated in muscle hypertrophy. In skeletal muscle, either exogenous expression of caAkt [184] or treatment with IGF-1 to activate Akt [185; 186] lead to increased fiber diameter. Similarly, expression of caMEK [187; 188] or treatment with clenbuterol to activate the MEK/ERK signaling pathway [189], promotes cardiac and skeletal muscle hypertrophy, respectively, whereas inhibition of ERK signaling abolishes clenbuterol-induced hypertrophy [190]. In addition, exogenous expression of MAPK phosphatase 1, which dephosphorylates and represses ERK signaling, decreases fiber size in skeletal muscle [190], further demonstrating the importance of ERK signaling in mediating the hypertrophic response. Both the Akt- and ERK-induced hypertrophy of skeletal muscle are blocked by rapamycin, demonstrating a critical role for mTORC1 in the response. Similar effects are observed in adult rat ventricular cardiomyocytes in which expression of caMEK1 activates mTORC1 [191]. Notably, the effect of caMEK1 on mTORC1

signaling is inhibited by rapamycin [192], a result consistent with MEK acting through ERK to inhibit TSC1/2. However, in contrast to the aforementioned studies, evidence supporting a direct role for ERK signaling in muscle hypertrophy under more physiological conditions, e.g. in response to exercise, is less clear. For example, muscle protein synthesis, ERK phosphorylation, and mTORC1 signaling are increased during recovery from resistance exercise [e.g. 60; 144; 193]. Whether or not ERK activation is directly involved in the stimulation of protein synthesis and mTORC1 signaling under such conditions is unclear.

Combined activation of the Akt and MEK/ERK pathways may also be important in the development of pathophysiological conditions such as cancer. For example, in anaplastic thyroid cancer, simultaneous genetic alterations in components of both pathways are observed with a frequency of 81% [194], and concurrent activation of the Akt and ERK pathways is also observed in other types of cancer [194; 195; 196]. A recent study [197], demonstrated that in tumor cell lines in which independent mutations occur in both the catalytic subunit of phosphoinositide-3 kinase (PI3K) and B-Raf, inhibition of either the Akt or ERK pathway alone is ineffective in repressing cell proliferation. Instead, simultaneous inhibition of both pathways is required to repress the proliferation of such cells, both in culture and in xenografts in mice. A similar requirement for combined inhibition of both pathways when both Akt and ERK are constitutively activated has been observed in earlier studies [198; 199; 200; 201; 202]. One interpretation of the results of these studies is that both pathways target a common downstream effector that mediates upregulation of cell proliferation, and that inhibition of both pathways is therefore

required to repress the function of the effector. Based on the results of the present study, the common effector may be the TSC1•TSC2 complex.

Overall, the results of the present study support a model in which activation of the Akt signaling pathway results in phosphorylation of TSC2 on specific residues, including S939 and T1462, leading to partial repression of its GAP activity. In contrast, activation of the MEK/ERK pathway has no effect on phosphorylation of either S939 or T1462, but instead leads to phosphorylation on separate residues, most likely S664. Combined, phosphorylation of the Akt- and MEK/ERK-directed sites on TSC2 results in a greater repression of GAP activity toward Rheb compared to phosphorylation of either alone. The results also provide further evidence supporting a model in which amino acids act in a pathway parallel to TSC2 to activate mTORC1.

4.5 Chapter IV: Figures and legends

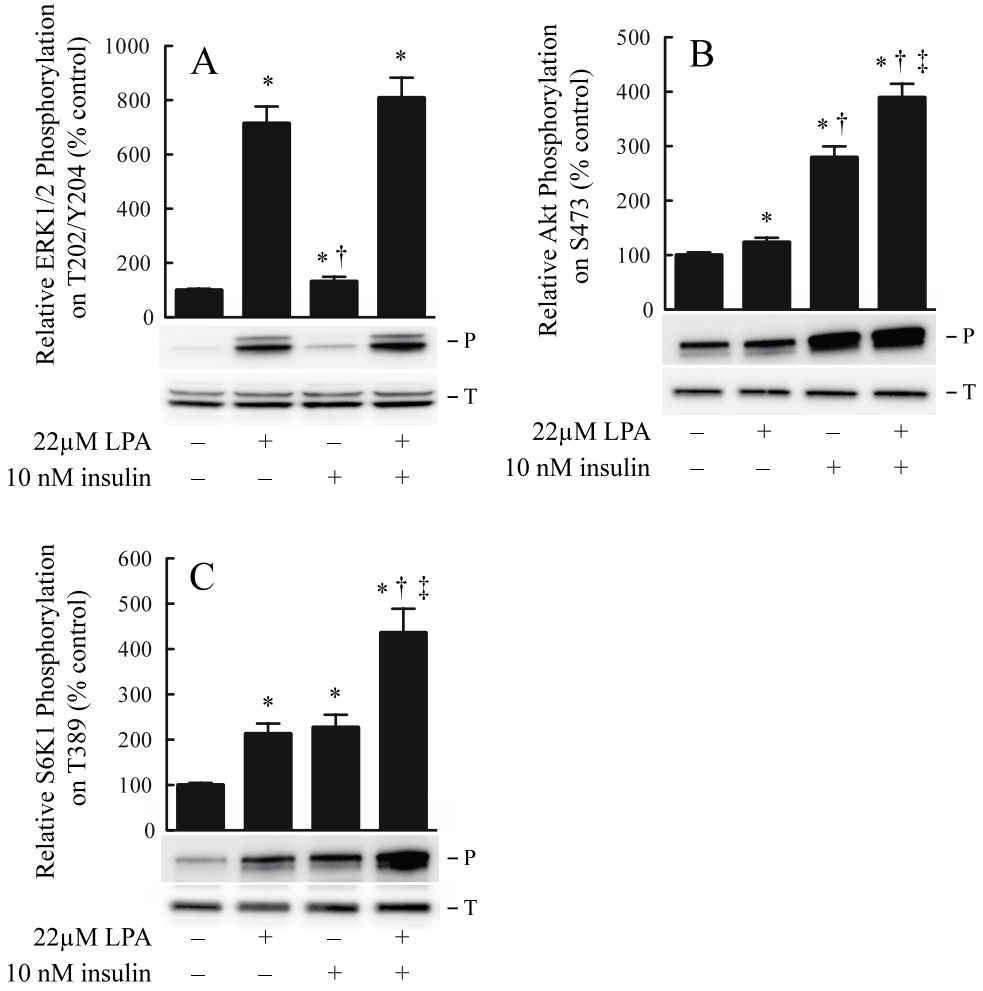

Figure 31. Effect of LPA and/or insulin on ERK1/2, Akt, and S6K1 phosphorylation in Rat2 fibroblasts. Cells were incubated for 2 h in medium containing 0.5% FBS and lacking leucine, and then LPA and/or insulin were added at final concentrations of 22 μM and 10 nM, respectively. Two h later, cells were harvested and homogenates were subjected to Western blot analysis for phosphorylation of (A) ERK1/2 on T202/Y204 (assessed as described in Fig. 16), (B) Akt on S473 (measured by Western blot analysis using an antibody specific for the phosphorylated form of the protein), and (C) S6K1 on T389 (assessed as described in Fig. 13). The results represent the mean ± SEM of (A and B) 6 or (C) 3 experiments. Within each experiment 3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. P, phosphorylated protein; T, the respective total protein. * $p<0.05$ vs. no additions; † $p<0.002$ vs. LPA; ‡ $p<0.005$ vs. insulin.

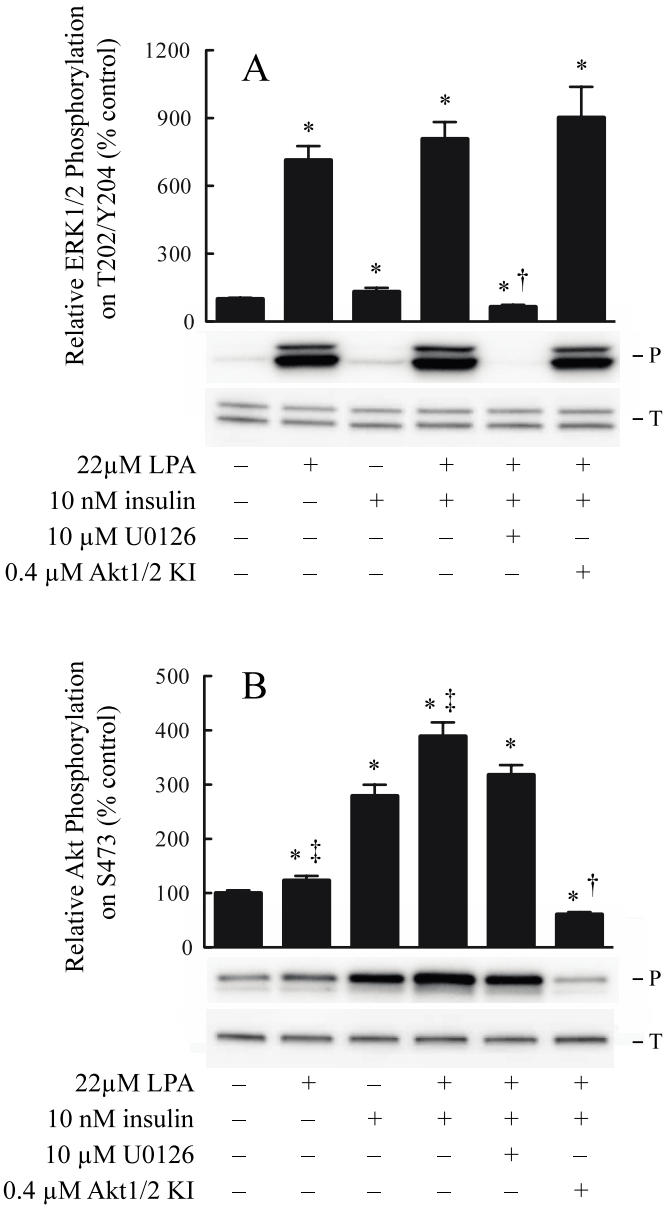

Figure 32. Selectivity of U0126 and Akt1/2 KI in Rat2 fibroblasts. Cells were incubated for 2.25 h in medium containing 0.5% FBS and lacking leucine. During the last 15 min, either U0126 or Akt1/2KI was added to the medium to final concentrations of 10 μM or 0.4 μM, respectively. LPA and/or insulin were then added at final concentrations of 22 μM and 10 nM, respectively, and 2 h later, cells were harvested and homogenates were subjected to Western blot analysis for phosphorylation of (A) ERK1/2 on T202/Y204 or (B) Akt on S473, assessed as described in Fig. 16 and Fig. 31 respectively. The results represent the mean ± SEM of 6 experiments. Within each experiment 3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. P, phosphorylated protein; T, the respective total protein. * $p<0.05$ vs. no additions; † $p<0.0001$ vs. LPA plus insulin; ‡ $p<0.005$ vs. insulin.

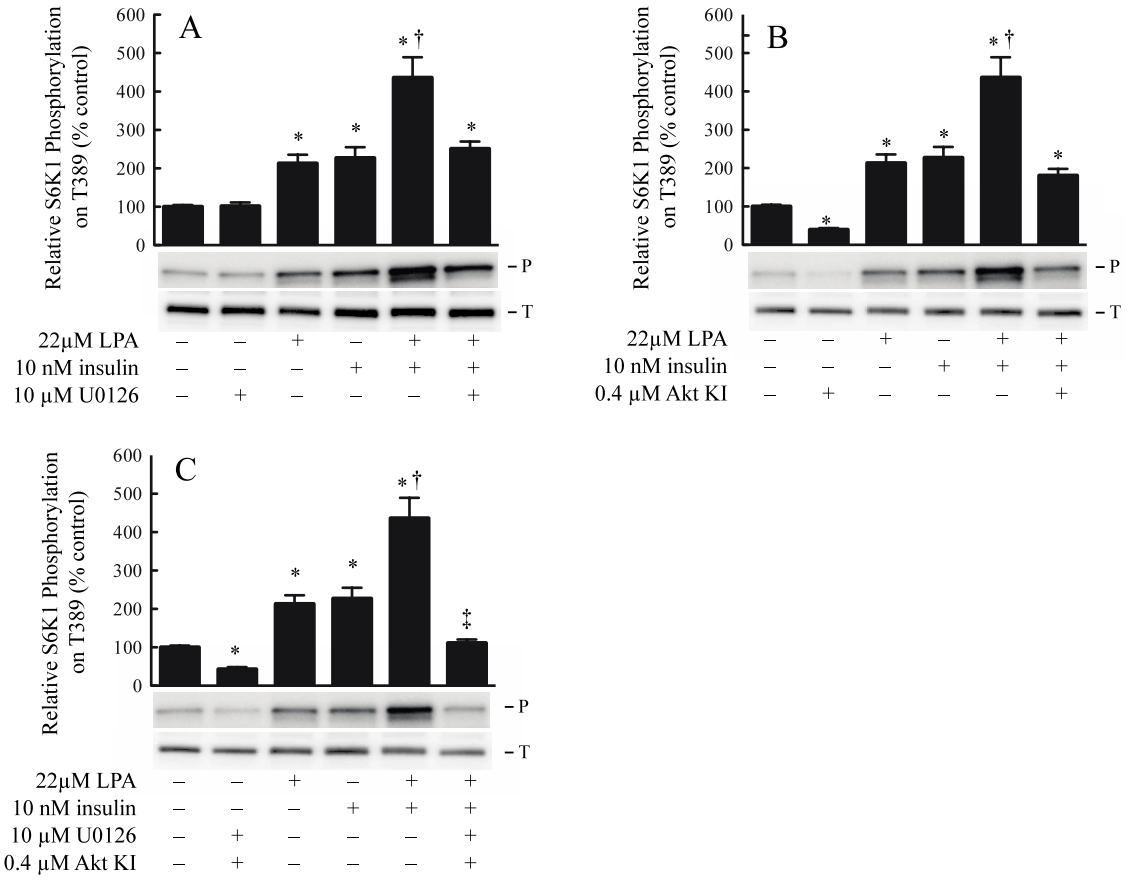

Figure 33. LPA and insulin act through the MEK/ERK and Akt signaling pathways to stimulate mTORC1 signaling in Rat2 fibroblasts. Cells were incubated with (A) U0126, (B) Akt KI, or (C) U1026 and Akt KI, as described in the legend to Fig. 32, and homogenates were subjected to Western blot analysis for phosphorylation of S6K1 on T389, assessed as described in Fig. 13. The results represent the mean ± SEM of 3 experiments. Within each experiment 3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. P, phosphorylated protein; T, the respective total protein. * $p<0.0005$ vs. no additions; † $p<0.005$ vs. LPA, insulin, or LPA plus insulin in the presence of U0126 and/or Akt KI; ‡ $p<0.002$ vs. LPA, insulin, or LPA plus insulin.

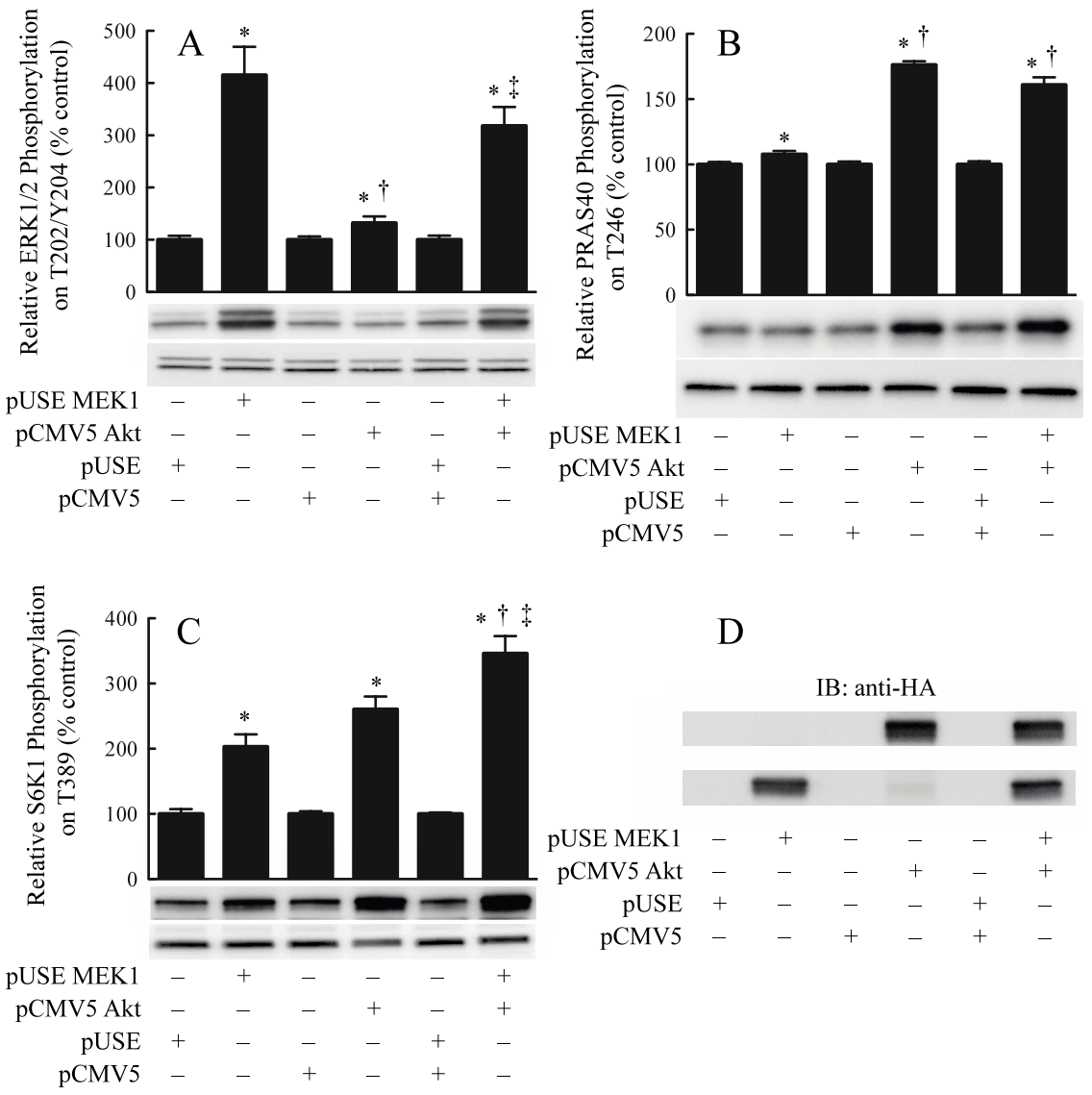

Figure 34. Effect of exogenous expression of constitutively active MEK1 and/or Akt1 on mTORC1 signaling in Rat2 fibroblasts. Cells were transfected with plasmids encoding constitutively active, HA-tagged MEK1 (pUSE MEK1) and/or Akt1 (pCMV5 Akt), or control plasmids (pUSE or pCMV5, respectively) and were harvested approximately 18 h later. Homogenates were analyzed for phosphorylation of (A) ERK1/2 on T202/Y204 (assessed as described in Fig. 16), (B) PRAS40 on T246 (measured by Western blot analysis using an antibody specific for the phosphorylated form of the protein), and (C) S6K1 on T389 (assessed as described in Fig. 13). The results represent the mean ± SEM of 3 experiments. Within each experiment 2-3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. The top blot in each panel depicts a blot for the phosphorylated protein, whereas the bottom blot depicts the respective total protein. * $p<0.05$ vs. cells transfected with the respective control plasmid(s); † $p<0.001$ vs cells transfected with pUSE MEK1; ‡ $p<0.005$ vs. cells transfected with pCMV5 Akt.

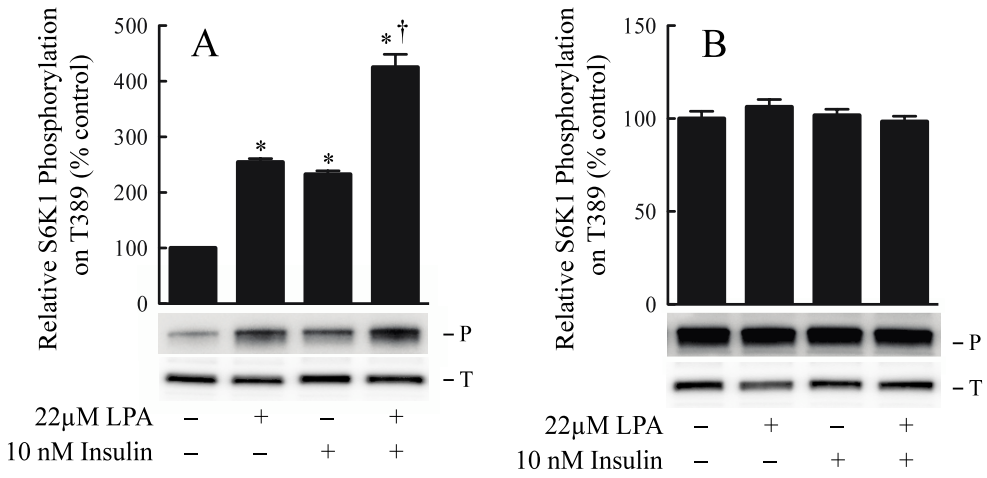

Figure 35. Effect of LPA and insulin on mTORC1 signaling in (A) wild-type or (B) TSC2 KO mouse embryo fibroblasts (MEF). Cells were incubated as described in the legend to Fig. 1, and homogenates were subjected to Western blot analysis for phosphorylation of S6K1 on T389, assessed as described in Fig. 13. The results represent the mean ± SEM of 3 or 4 experiments. Within each experiment 3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. P, phosphorylated S6K1; T, total S6K1. * $p<0.0001$ vs. no additions; † $p<0.0001$ vs. either LPA or insulin alone.

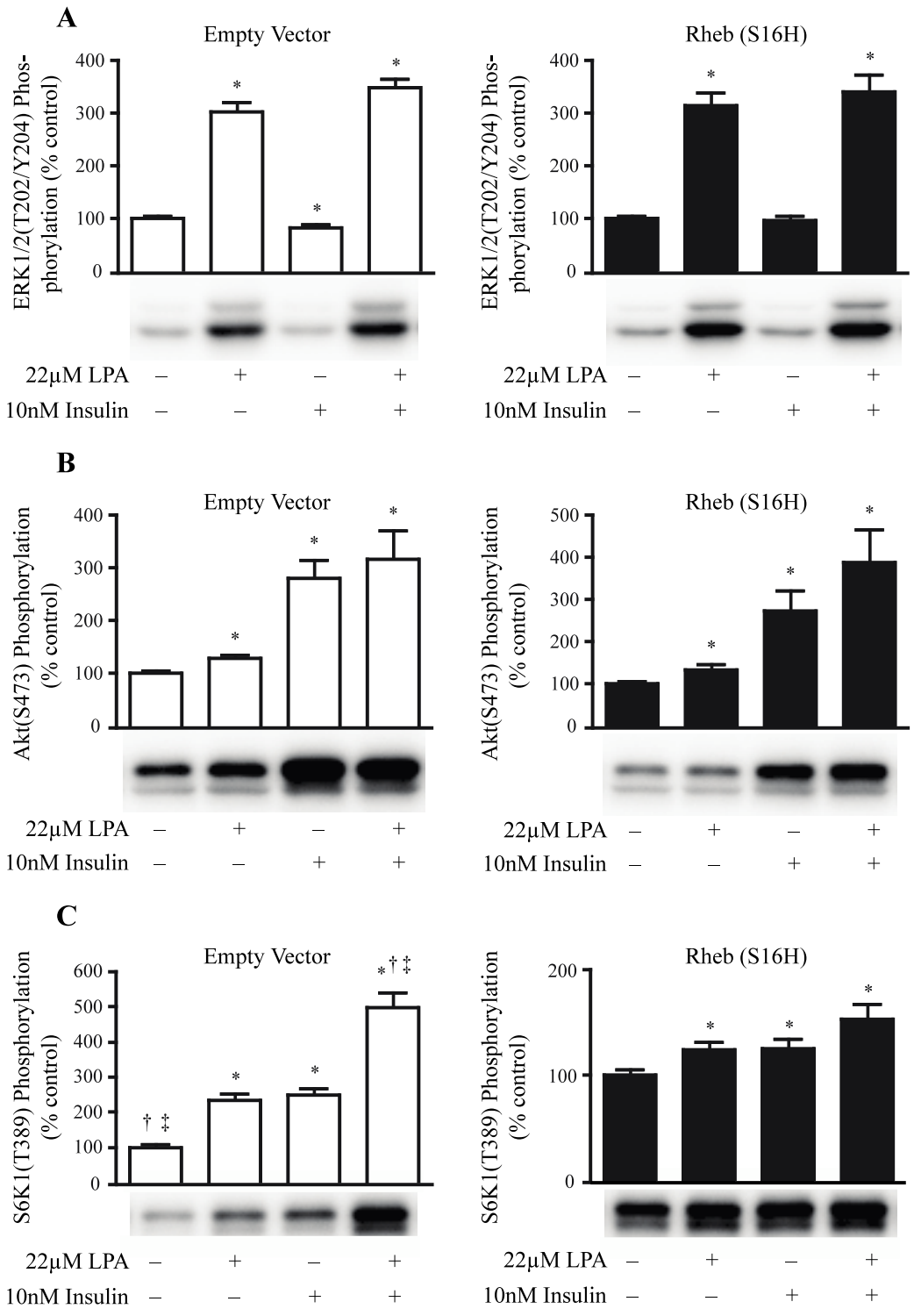

Figure 36. LPA- and insulin-mediated regulation of mTORC1 is attenuated due to the expression of Rheb that is preferentially bound to GTP. Rat2 fibroblasts were transfected with Rheb (S16H) and maintained in medium containing 10% FBS for 22h. Cells were then incubated in 0.5% FBS medium without leucine for 2h prior to the

addition of 0.76mM leucine and/or 22μM LPA for 2h. Representative blots are shown in each panel. Results represent the mean ± SEM of 3 experiments; in each experiment, 3 samples per condition were independently analyzed. (A) Phosphorylation of ERK1/2 on Thr202/Tyr204 was assessed as described in Fig. 16. * $p<0.015$ vs. cells incubated in 0.5% serum without leucine. (B) Phosphorylation of Akt on Ser473 was assessed as described in Fig. 31. * $p<0.025$ vs. cells incubated in 0.5% serum without leucine. (C) Phosphorylation of S6K1 on Thr389 was assessed as described in Fig. 13. * $p<0.05$ vs. cells incubated in 0.5% serum without leucine. † $p<0.0001$ vs. cells treated with LPA alone. ‡ $p<0.0001$ vs. cells treated with insulin alone.

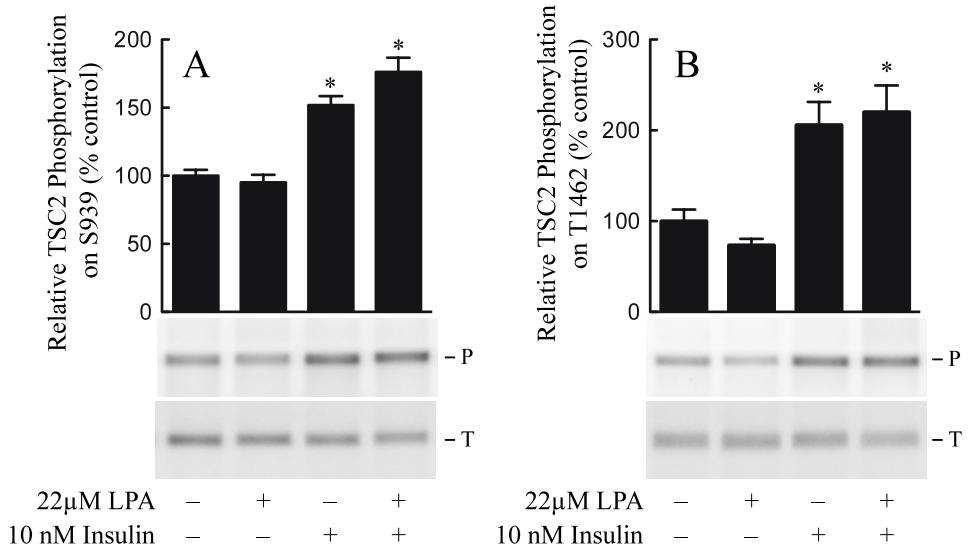

Figure 37. Effect of LPA and insulin on TSC2 phosphorylation on S939 and T1462 in Rat2 fibroblasts. Cells were incubated as described in the legend to Fig. 31, and homogenates were subjected to immunoprecipitation using an anti-TSC2 antibody followed by Western blot analysis of the immunoprecipitates for phosphorylation of TSC2 phosphorylation on (A) S939 or (B) T1462. The results represent the mean ± SEM of 3 experiments. Within each experiment 3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. P, phosphorylated TSC2; T, total TSC2. * $p<0.002$ vs. no additions or LPA alone.

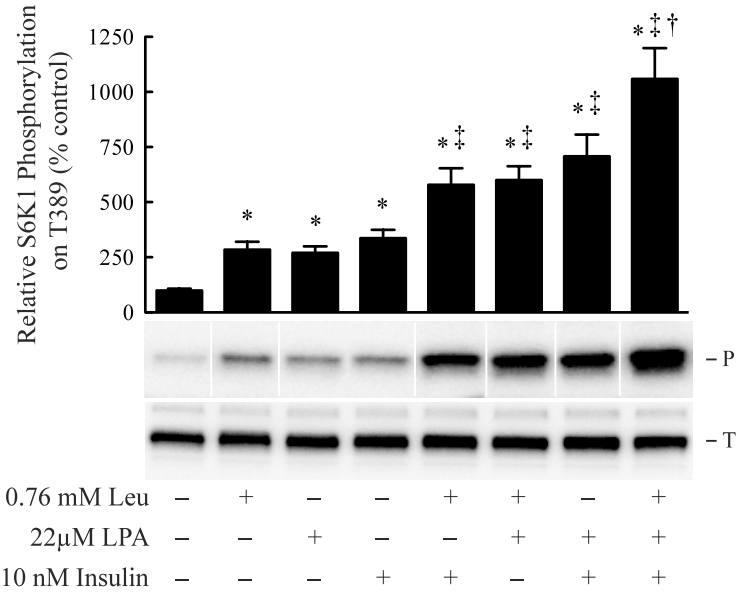

Figure 38. Effect of LPA, insulin, and/or leucine on mTORC1 signaling in Rat2 fibroblasts. Cells were incubated for 2 h in medium containing 0.5% FBS and lacking leucine, and then LPA, insulin, and/or leucine were added at final concentrations of 22 μM, 10 nM, and 0.76 mM, respectively. Two h later, cells were harvested and homogenates were subjected to Western blot analysis for phosphorylation of S6K1 on T389, as described in Fig. 13. The results represent the mean ± SEM of 6 or 7 experiments. Within each experiment 2-3 wells of cells were independently analyzed. Representative blots are shown beneath the graphs. In the blot shown in the figure, all samples were run on the same gel, but not in contiguous lanes. Noncontiguous lanes are separated by white lines. P, phosphorylated S6K1; T, total S6K1. * $p<0.002$ vs. no additions; ‡ $p<0.01$ vs. LPA, insulin, or leucine alone; † $p< 0.05$ vs. leucine plus insulin, LPA plus insulin, or leucine plus LPA.

CHAPTER V

CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

5.1 Conclusions/Discussion

5.1.1 The involvement of mTORC1, PA, and LPA in physiological and pathophysiological conditions

It is known that the mTORC1 signaling pathway is involved in various physiologic and pathophysiologic conditions, such as inflammatory processes [82], cancer [15], diabetes, and obesity [16]. LPA and PA have also been implicated in the induction of inflammatory responses. LPA mediated inflammation is partially inhibited by Ki16425 in corneal epithelial cells [132], and the knock down of two LPA receptors in human umbilical endothelial cells reduces the expression of genes involved in inflammation, such as interleukin-1β (IL-1β) [133]. IL-1β secretion is one of the results of PA signaling, and the cytokine serum levels in mice increase in response to PA treatment [82]. Additionally, rapamycin inhibits the PA-induced production of cytokines in Raw264.7 macrophage cells [82], clearly demonstrating the important role of PA-mediated mTORC1 signaling in inflammation.

Activated mTORC1 signaling is also common in human cancer, and LPA and PA are involved in certain types of cancer as well. The activity of PLD, the major source of PA, is high in breast and colon cancers, and its increased activity suppresses apoptosis and counteracts cellular senescence normally triggered by Raf signaling [203]. Further, studies have indicated that elevated PLD activity increases rapamycin resistance in MDA-MB-231 cells, a breast cancer cell line [104], which correlates with the observation

that increased intracellular levels of PA induces rapamycin resistance [84]. The involvement of LPA in cancer has been shown to both stimulate and inhibit cell growth in breast cancer cells [204]. Also, in prostate cancer, LPA increases cellular proliferation in a PLD dependent manner [205]. In other contexts, such as lung and colon cancer, LPA is known to increase metastasis [206; 207]. Further, the expression of EDG-2, -4, and -7 increases LPA mediated metastasis in different conditions, while siRNA mediated knock down of EDG-4, and -7 reduces LPA mediated cell proliferation in colorectal cancer cells [208]. The inhibition of EDG-2 with Ki16425 also reduces cell motility in cancer cells [209], and EDG-4 and -7 may be involved in metastasis in ovarian cancer [210; 211]. In addition to PA and LPA involvement in cancer pathophysiology, PLA_2 concentrations are increased in the serum of those with pancreatic cancer [120], presenting a possible link between PA, LPA, and PLA in certain cancers.

While the involvement of LPA and PA in cancer has been thoroughly studied and is a continued area of interest, a growing area of research has implicated LPA and PA in muscle hypertrophy. Studies have shown that muscle hypertrophy is regulated by mTORC1, as muscle growth is inhibited by rapamycin. LPA increases cardiomyocyte hypertrophy through the activation of ERK1/2 and Rho signaling [134], and increases contractility in smooth muscle cells [212]. In addition to LPA, PA is also implicated in cardiac hypertrophy, as the addition of PA to rat cardiomyocytes increases intracellular free Ca^{2+} [88; 90] which correlates with increased contractile force [88]. Most recently, it has been demonstrated that PA has a role in skeletal muscle hypertrophy and is produced in response to both mechanical stimulation [84] and eccentric contractions [85].

5.1.2 Summary of findings regarding PA-mediated mTORC1 signaling

Due to the involvement of lipids in the stated processes, particularly skeletal muscle hypertrophy, an understanding of LPA- and PA-mediated activation of mTORC1 will provide insight into important disease states, which will aid in the development of treatments for multiple conditions. Therefore, the present study examined PA-mediated activation of mTORC1 signaling. Rat2 fibroblasts were used in these studies, which serve as a useful model for skeletal muscle, as mTORC1 signaling is increased in response to physiological concentrations of hormones as well as nutrients [167]. Known skeletal muscle models, such as C2C12 myotubes, which are derived from mice, and L6 myotubes, derived from rats, were not used because they do not respond to physiologically relevant concentrations of nutrients [167]. Indeed, mTORC1 signaling in these skeletal muscle cell lines increases due to very small concentrations of hormones and nutrients, which would not increase mTORC1 signaling *in vivo*. Also, mTORC1 signaling in Rat2 fibroblasts is reduced to a basal level after 2hrs of serum and leucine deprivation, while longer deprivations are required to reduce mTORC1 signaling in muscle cell lines. The need for longer serum and leucine deprivation in these skeletal muscle cell lines does not adequately model the response of mTORC1 signaling observed in an *in vivo* setting. Because of these factors, Rat2 fibroblasts were used, and it was demonstrated that the addition of exogenous PA to cells in culture increased mTORC1 signaling through an LPA receptor based mechanism. Additionally, both PA- and LPA-mediated activation of mTORC1 occurred through the ERK1/2 signaling pathway. The induction of mTORC1 could be blocked through the use of two distinct MEK inhibitors,

PD-98059 and U0126, and in the presence of these inhibitors, neither PA nor LPA increased mTORC1 signaling. It was shown that the PA and LPA pathway that activated mTORC1 was parallel to the leucine pathway, which has not yet been elucidated.

5.1.3 An alternative mechanism by which PA increases the activation of mTORC1

Integrating the multiple inputs of mTORC1 signaling and understanding how their effects increase skeletal muscle hypertrophy is a difficult undertaking. However, there is strong evidence in the literature that mTORC1 is activated in response to the agonists mentioned, such as LPA [106], PA [84; 105], insulin [135; 149], and AAs [77; 78]. In fact, some of these agonists function through ERK and Akt, which are known to inactivate TSC1/2 [49; 52]. The current work has presented an alternative mechanism by which PA activates mTORC1 in which PA mediates mTORC1 signaling through the activation of the ERK pathway rather than the direct binding of the FRB domain on mTOR [80]. The current data support that extracellular PA is hydrolyzed by PLA to LPA, which then binds an EDG receptor, leading to increased mTORC1 activity through ERK1/2 signaling.

5.1.3.1 LPA, PA, mTORC1 and Skeletal Muscle Hypertrophy

Skeletal muscle is known to undergo hypertrophy in response to mTORC1 signaling [19; 23; 38; 162; 213]. Therefore, an understanding of how mTORC1 is regulated to induce

hypertrophy is fundamentally important. Since LPA is responsible for the increased mTORC1 signaling observed in this study, it is important to understand how LPA could induce the activation of mTORC1 in a physiological setting, and future studies are warranted. It has been mentioned that LPA circulates in human serum [127], is generated by platelets [128], and is involved in cardiac muscle hypertrophy [134]. Regarding skeletal muscle, it is known that certain EDG receptors are ubiquitously expressed [214], and that EDG-2 is found human skeletal muscle [215]. The present work suggests that both LPA and PA induce mTORC1 signaling through the EDG-2 receptor in Rat2 fibroblasts. While PA is typically localized to the plasma membrane and is largely intracellular it is possible that PA is generated extracellularly, as it is an important crossroad for phospholipid biosynthesis, and that it functions to increase mTORC1 signaling through the EDG-2 receptor. However, any extracellular PA in human serum would be quickly converted to LPA through the action of PLA. Therefore, increased mTORC1 signaling in skeletal muscle hypertrophy in response to lipids is likely more complicated than the activation of EDG-2 receptors.

Although the current work demonstrates that PA increases mTORC1 signaling through an EDG-2 receptor, which in turn leads to ERK activation, it does not discount a requirement for the binding of PA to mTOR for mTORC1 signaling. However, it may be that PA is crucial for mTOR signaling while not directly increasing mTOR activity. This is supported by the literature, as PA binding to the FRB domain does not increase mTOR kinase activity [80]. Studies done by Dr. David Foster's research group have demonstrated that PA is important for mTOR complex formation [105], as previously

mentioned. They showed that the inhibition of PLD-mediated PA production with 1-butanol enabled the activity of mTORC2, which is considered rapamycin insensitive, to be inhibited by rapamycin [105], and suggested that the role of PA in mTOR signaling may be explained through PA directly binding the FRB domain and stabilizing the mTOR complex.

5.1.3.2 Possible mechanisms whereby PA could increase mTORC1 signaling in muscle hypertrophy

Ultimately, the role of PA-mediated mTOR signaling requires further study, as does the role of PA in mTORC1 regulated muscle hypertrophy. It is known that both mechanical stimulation and eccentric contraction increase mTORC1 signaling [84; 85], and that both depend on the production of PA. If PA is functioning to increase mTORC1 activation through ERK1/2, as the current data demonstrates, then the extracellular increase of PA, and subsequently LPA, in response to mechanical stimulation should correlate with mTORC1 activation. This would be difficult to test, however, as any PA present would likely be hydrolyzed to LPA. Additionally, it is not known whether extracellular LPA content increases due to exercise, and further research is needed to elucidate whether LPA is involved with increased mTORC1 signaling in response to mechanical stimulation. While future work is required to determine whether mechanical stimulation increases extracellular levels of PA, which could then activate mTORC1, there is another possible explanation for the seemingly different results in the present work compared with the current literature. Knowing that PA production and mTORC1 signaling increase

due to mechanical stretch [84], and that the increase is PLD dependent, demonstrates that PA is likely produced in a variety of physiological contexts, such as exercise and natural body movement that would activate stretch receptors. It would be incorrect to assume that the production of PA alone would correlate with muscle hypertrophy, as muscle growth does not occur due to merely muscle stretch. However, mTORC1 activation and muscle hypertrophy does occur in response to resistance exercise [143]. Therefore, while PA production in response to mechanical stretch could be crucial for mTOR complex formation, it is not directly responsible for mTORC1 activation according to the current study. It could be that once the mTOR complex is stable that it is then activated through another mechanism, such as ERK signaling. This is supported by the present study as well. Further, other groups have shown that MEK1/2 and ERK1/2 are activated in response to exercise, and the activation is dependent on the intensity of the exercise [60]. Additionally, by blocking PLD-mediated production of PA, mTOR complex formation would be attenuated, and without efficient mTOR complex formation there would be no increase in mTOR activity and no subsequent increase in muscle hypertrophy. However, in the presence of PA, mTOR complexes could form, which could be activated in response to the corresponding increase in ERK1/2 signaling due to exercise.

Therefore, an alternative hypothesis is that PA functions to stabilize the mTORC1 complex, which, in concert with ERK activation, increases mTORC1 signaling. In this manner skeletal muscle hypertrophy due to resistance exercise may occur in the following manner: during a resistance exercise bout PLD activity in skeletal muscle would be increased in response to mechanical activation, which would produce PA,

allowing for the stabilization of mTORC1. Additionally, the exercise would activate the ERK cascade, which would subsequently inactivate the TSC1/2 complex, allowing for the activation of mTORC1, and ultimately increased protein synthesis. The present work may represent a model to examine this effect in a cell culture system. Specifically, LPA treatment elicited three effects in Rat2 fibroblasts as it increased ERK1/2 phosphorylation (Fig. 16A), PLD activity, and PA content (Fig. 12). As previously stated, the combination of these effects may be responsible for the subsequent activation of mTORC1 signaling. In support of this hypothesis, it was demonstrated that 1-butanol partially inhibited S6K1 (Fig. 13A) and 4E-BP1 (Fig. 13B) hyperphosphorylation in response to LPA treatment. Since the predominant form of PA produced in response to LPA treatment was 18:1-18:1 (Fig. 12B), and the LPA used was 18:1, it is probable that some of the PA produced was due to LPAAT activity, which would provide an alternative source of PA. Should the reduction of mTORC1 signaling be due to decreased PA content, then it would be expected that S6K1 and 4E-BP1 phosphorylation would be slightly reduced in response to the reduction in PA produced from PLD. This reduction, however, would not be total because of the production of PA from LPAAT, which would allow for a stable mTOR complex and subsequent activation of mTORC1 through the ERK pathway. Additional support for this hypothesis is that 1-butanol had a greater inhibitory effect on leucine-mediated mTORC1 signaling (Fig. 19) than on LPA-mediated mTORC1 signaling (Fig. 13A), even though leucine did not increase PLD activity or PA content (Fig. 20). In this case, 1-butanol reduced the amount of PA produced from PLD and there was no additional PA produced from LPAAT, as no LPA was present. This could explain why mTORC1 signaling was inhibited to a greater

extent, as mTOR complex formation would have been disrupted due to the reduced levels of intracellular PA.

5.1.4 The ERK- and Akt-mediated regulation of mTORC1 signaling

The fact that ERK signaling is an important regulator of muscle growth provides additional support for the hypothesis that PA is involved in the regulation of muscle hypertrophy through an ERK dependent mechanism. In addition to ERK, Akt is also involved in muscle hypertrophy, and the inhibition of either ERK or Akt reduces cell growth [38; 57]. Both pathways are regulated through specific mechanisms, although they do respond to similar agonists. For instance, ERK and Akt are both activated by IGF-1 [57; 216]. However, the pathways respond differently to other agonists. Specifically, insulin increases Akt signaling in multiple cell types while ERK activation in response to insulin seems more cell specific as insulin stimulates ERK in fetal rat astrocytes [217] but has little if any effect on ERK activation in Rat2 fibroblasts, as seen in the current studies. Additionally, while ERK mediated activation of mTORC1 requires the presence of PA for Raf translocation to the site of active Ras, resulting in the full activation of ERK signaling [61; 62], it is known that Akt does not require PA to translocate to the cell membrane to elicit its effects on mTORC1. It is of particular interest that MEK1/2 and ERK1/2 signaling is increased in response to exercise [60], and that mTORC1 signaling is increased for an extended period of time due to eccentric contractions [85]. Increased mTORC1 activity in response to eccentric contraction appears to be independent of Akt, as Akt signaling under these conditions is transient,

whereas PA levels are increased for a longer and sustained period [85], thus indicating that Akt does not respond to exercise in the same way that ERK does.

5.1.4.1 Summary of findings regarding the ERK- and Akt-mediated stimulation of mTORC1 signaling

This work demonstrated that ERK and Akt signaling increase mTORC1 signaling through parallel pathways, which was shown through the treatment of Rat2 cells with ERK and Akt agonists, LPA and insulin respectively. While LPA and insulin increased mTORC1 signaling, when added in concert mTORC1 signaling increased additively. The inhibition of either MEK1/2 or Akt partially blocked mTORC1 signaling in response to LPA and insulin, while the inhibition of both pathways completely blocked mTORC1 signaling. The overexpression of caMEK1 and/or caAkt produced similar results with mTORC1 signaling increasing due to either caMEK1 or caAkt, and increasing more due to the combination of the two constructs. Finally, the increased activation of mTORC1 was most likely due to the inactivation of TSC2 by ERK and Akt. In support of this, neither LPA nor insulin regulated mTORC1 signaling in TSC2 knock out cells, nor did they regulate mTORC1 signaling when a constitutively active Rheb (caRheb) was expressed (Fig. 36).

5.2 TSC1/2 Inhibition by ERK and Akt

While the current studies clearly demonstrate that ERK and Akt activation increases mTORC1 signaling in an additive fashion, it is not known from the available data how ERK and Akt inhibit the TSC1/2 complex. It is possible that Akt and ERK1/2 phosphorylate TSC2 in a cooperative manner (Fig. 39). Knowing that 14-3-3 binding to TSC2 is an important step in the activation of mTORC1, it is possible that this binding partially inhibits the GAP activity of TSC2. Additionally, 14-3-3 binding to TSC2 may increase the ERK1/2 mediated phosphorylation of TSC2, which could then inhibit the TSC1/2 complex to a greater extent. In this manner, Akt and ERK1/2 may function cooperatively to inhibit TSC1/2, as suggested in Figure 35. Further studies must be done to determine the mechanism of the additive activation of mTORC1 due to TSC1/2 inhibition. While the postulated mechanism may occur, it is also possible that the inactivation of the TSC1/2 complex by ERK and Akt may depend on subcellular localization of the individual proteins instead of, or in addition to, cooperative phosphorylation of TSC2. It has been stated that Akt may inactivate the TSC1/2 complex by the phosphorylation of TSC2 and the subsequent dissociation of TSC1 and TSC2. However, should Akt inactivate the complex primarily in this way, then the additive effect of ERK activity should not increase mTORC1 activity. Indeed, at maximal activation of Akt, mTORC1 should not be further stimulated. Similarly, the dissociation of the TSC1/2 complex in response to ERK phosphorylation of TSC2 would seemingly render mTORC1 signaling insensitive to additional stimuli. However, should the ERK and Akt components of the pathway be isolated from each other, it would account for

increased mTORC1 signaling in response to both stimuli. Interestingly, it is known that MAPK scaffolding activity can localize to the surface of endosomes, suggesting that in certain circumstances that ERK signaling may be partitioned to endosomes [218]. Indeed, in previous studies groups have shown that the scaffold protein MEK1 partner (MP1) and p14, a conserved protein that is found in late endosomes, are responsible for the regulation of ERK signaling on endosomes [219]. It was found that p14 and MP1 colocalized with ERK1/2, and importantly the overexpression of both ectopic p14 and MP1 was necessary to enhance ERK1/2 signaling, as neither led to a significant increase in ERK1/2 signaling when expressed alone [219]. As it is known that Akt signaling takes place when Akt is localized to the plasma membrane, it is tempting to speculate that the TSC1/2 complex is inactivated by Akt and ERK individually in separate subcellular locations. In this manner, insulin would stimulate Akt, which would then phosphorylate and inactivate the TSC1/2 complex. In the presence of agonists that stimulate ERK1/2 activity, such as LPA or PA, ERK1/2 would phosphorylate and inactivate the TSC1/2 complex as well, although in a different subcellular location. In this manner the activation of both Akt and ERK would lead to increased amounts of inactive TSC1/2 complexes, which would allow for increased mTORC1 signaling.

5.3 Future studies examining the phosphorylation of TSC2 on specific sites by ERK and Akt

When examining both the current literature and the data presented in the context of muscle hypertrophy, it is seen that there is a complex interplay between various signaling

pathways, particularly ERK and Akt. The combined effect of ERK and Akt signaling on mTORC1 activity is greater than either input alone. While the current results strongly suggest that ERK and Akt activate mTORC1 through the phosphorylation and inactivation of TSC2, further work is needed to establish this. Our current hypothesis is that the activation of ERK signaling would increase phosphorylation at S664 whereas Akt activation would have no effect on S664. Should this be validated, it would provide convincing evidence that TSC1/2 mediated inactivation of mTORC1 signaling is modulated differently in response to signaling pathways upstream of TSC2. Additionally, site directed mutations against the ERK and/or Akt specific sites on TSC2 would yield valuable information regarding mTORC1 signaling in response to LPA or insulin treatments. We predict that LPA would be unable to activate mTORC1 due to a S664A mutation on TSC2, although insulin would still be able to regulate mTORC1 signaling. Conversely, insulin would be unable to regulate mTORC1 due the mutation of T1462, S939, and the additional Akt sites, while LPA-induced mTORC1 signaling would be unaffected. Mutations in both the Akt and ERK specific sites on TSC2 would ablate mTORC1 activation in response to either LPA or insulin. It is predicted that the results from this postulated experiment would correlate with the MEK and Akt inhibitor data presented (Fig. 33). Namely, that the inhibition of MEK blocked LPA-mediated signaling to mTORC1 while the inhibition of Akt blocked insulin-mediated signaling to mTORC1, and the presence of both inhibitors abolished the regulation of mTORC1 in response to either agonist. Another valuable test would be to express phosphomimetic mutants of TSC2 where each Akt and ERK site was mutated to glutamate or aspartate and show that mTORC1 signaling was no longer regulated by LPA and insulin and that it

would no longer be repressed by the inhibition of MEK or Akt. Finally, it will be important to examine the subcellular localization of ERK1/2, Akt, TSC1, TSC2, as well as Rheb in response to ERK1/2 and Akt agonists in each of the stated conditions. It is also possible that Akt and ERK1/2 phosphorylate TSC2 in a cooperative manner. Should this be the case, examining TSC1/2 activity in a cell-free system in the presence of constitutively active Akt and constitutively active ERK1/2, both individually as well as combined would yield important information. In this case, TSC1/2 activity should decrease additively in the presence Akt and ERK1/2 combined. In this way, it could be verified whether Akt and ERK1/2 phosphorylate TSC2 in a cooperative fashion. While that may be the case, it does not preclude the possibility that the subcellular localization of Akt and ERK1/2 are also involved with the specific activation of mTORC1 through the inactivation of TSC1/2. Additionally, if the ERK1/2 regulated activation of mTORC1 is occurring in endosomes, then siRNA mediated knockdown of p14 should ablate the effect of activated ERK1/2 on mTORC1 signaling, though Akt mediated activation of mTORC1 should remain unaffected.

5.4 The activation of mTORC1 in response to multiple regulatory inputs

While it has been shown that lipids and hormones regulate the activation of mTORC1, through the activation of ERK and Akt respectively, in a whole body system mTORC1 signaling is regulated simultaneously by multiple inputs, rather than just one or two isolated inputs alone. Unfortunately, studies do not typically examine mTORC1 signaling in response to multiple inputs in concert. However, the current work has shown

that the combination of LPA, insulin, and leucine tend to increase mTORC1 signaling beyond that observed when any two agonists are combined (Fig. 38), which supports previous work that demonstrates that AAs signal to mTORC1 in a TSC1/2 independent manner [30; 68; 69]. Having shown that mTORC1 signaling is greater due to the combination of leucine, LPA, and insulin treatments (Fig. 38), it will be important to determine how each of these pathways function to increase mTORC1 activation. LPA and insulin appear to work through ERK and Akt respectively, while the leucine mediated increase in mTORC1 signaling has not yet been fully elucidated. It is known that Rag proteins are important in AA-mediated mTORC1 signaling [68], and groups have shown that when mTORC1 is activated by AAs that mTORC1 localizes to lysosomes [68; 220]. In fact, it has recently been demonstrated that Rag proteins are located at the lysosomal surface and that mTORC1 is targeted to that surface where it is then activated by Rheb-GTP [69]. This targeting is the result of the Rag proteins interacting with a complex of three proteins termed "Ragulator." With the help of Ragulator, the Rag proteins bind to mTORC1 in response to the presence of AAs and target it to the lysosomal surface. Targeting mTORC1 to the surface of the lysosome via molecular techniques made mTORC1 insensitive to AAs [69]. However, when Rheb was knocked down through the use of RNAi, the forced targeting of mTORC1 to the lysosome had no effect on mTORC1 signaling, demonstrating that the activation of mTORC1 was dependent on Rheb-GTP [69]. Knowing this, the importance of ERK and Akt mediated phosphorylation of TSC2 resulting in the inactivation of TSC1/2 is reinforced, since it would function as an important regulator of Rheb-GTP levels. Interestingly, two of the proteins that make up Ragulator are p14 and MP1, further

supporting a convergence of ERK1/2 and AA mediated mTORC1 activation in endosomes.

Therefore, this provides a possible explanation for the results demonstrating that the combination of LPA and insulin treatments increase mTORC1 signaling to a greater level than when either LPA or insulin alone are combined with leucine (Fig. 38). The current data may indicate that the deactivation of TSC1/2 increases Rheb-GTP levels, which is primarily responsible for mTORC1 activation. The addition of leucine would increase signaling slightly, as any mTORC1 not already associated with Rheb-GTP would be transported to the lysosomal surface, increasing mTORC1 activation further. While the data collected supports this hypothesis, further research is required to understand this process. For instance, performing a TSC1/2 activity assay that directly measures the levels of Rheb-GTP in response to LPA and insulin treatments would yield valuable data. Additionally, the targeting of mTORC1 to the location of Rheb-GTP while expressing caAkt and caMEK1 should approximate the maximal levels of mTORC1 signaling observed in the presence of simultaneous leucine, LPA, and insulin treatments.

The current study primarily focused on multiple mTORC1 stimulatory signals that function through the TSC1/2 complex. Further research is needed to elucidate the combined effect of stimulatory and inhibitory inputs. Importantly, it is known that AMPK inhibits mTORC1 signaling through the phosphorylation of TSC2. In addition to AMPK, it has been shown that the protein REDD1 (regulated in development and DNA damage response 1) inhibits mTORC1 signaling is response to hypoxia and ER stress

[221; 222]. In response to ER stress, global cap-dependent protein synthesis is inhibited, although paradoxically the synthesis of various transcription factors, namely ATF4, is upregulated. ATF4 has been shown to be both necessary and sufficient for the upregulation of REDD1 due to ER stress [222]. REDD1 inhibits mTORC1 signaling through the binding of 14-3-3 proteins and sequestering them from TSC2, which allows for the formation of TSC1/2. This inhibition occurs in spite of TSC2 being phosphorylated by Akt, demonstrating that REDD1 competitively binds 14-3-3 proteins, as well as suggesting that phosphorylation of TSC2 by Akt is not sufficient to activate mTORC1 in the absence of 14-3-3 proteins [221]. In hypoxic conditions global protein synthesis is energetically costly, which is why REDD1 mediated inhibition of mTORC1 through the TSC1/2 complex is functionally important. Therefore, in an exercise model where both ATP and oxygen content is significantly reduced, both AMPK as well as REDD1 are likely functioning together to reduce mTORC1 signaling. However, the combined effect of these pathways has not been tested. Inflammation has also been linked to mTORC1 activation through the activation of IKKβ and the subsequent phosphorylation of TSC1 at S487 and S511, which inhibits the TSC1/2 complex and increases mTORC1 signaling [223; 224]. Additionally, it is not known whether increased mTORC1 signaling in response to ERK and Akt activation would overcome the inhibition of mTORC1 that occurs at TSC1/2 in response to AMPK and REDD1, nor is it known how IKKβ works in conjunction with these pathways. The combined effect of these signals would yield useful information regarding skeletal muscle hypertrophy, as TSC1/2 seems to serve as a central hub in mTORC1 signaling, as well as mTORC1 inhibition [158] (Fig. 40). This balance of stimulatory and inhibitory signals through

TSC1/2 is functionally important, as it has been demonstrated that the absence of mTORC1 inhibition results in cancer, while the absence of mTORC1 stimulation results in muscle atrophy.

5.5 Primary contributions of the present work

While the activation of mTORC1 has been studied in response to individual agonists, this work is novel since it examines the effect of the various combinations of leucine, PA, LPA, and insulin. Indeed, mTORC1 signaling *in vivo* is complex, and multiple agonists and signaling pathways function simultaneously to regulate the activation of mTORC1. By solely studying one input to mTORC1 activation, a full appreciation for the complexity of the signaling pathways cannot be obtained. More importantly, it would be a disservice to science and the general public to assume that individual pathways signal to mTORC1 in a mutually exclusive fashion. Particularly, in disease states, multiple signaling pathways can be activated that modulate mTORC1 activity at the same time. Therefore, an understanding of mTORC1 signaling in response to multiple inputs will be therapeutically beneficial and additional research examining the interplay of both positive and negative signals to mTORC1 is warranted. Any research of this type would further the current understanding of mTORC1 regulation and possibly lead to treatments for conditions characterized by the dysregulation of mTORC1 signaling, particularly muscle atrophy.

5.6 Chapter V: Figures and legends

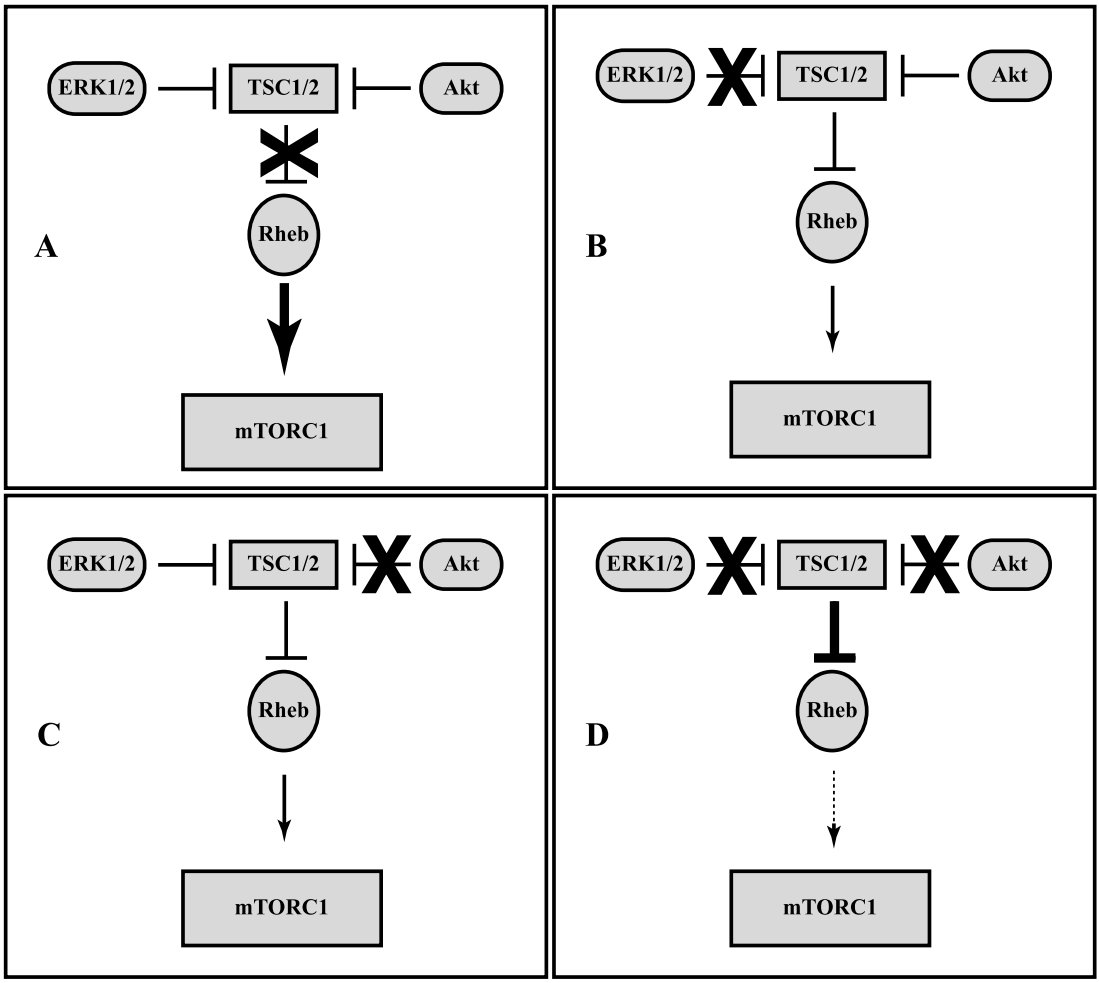

Figure 39. Model for the activation of mTORC1 mediated by the ERK and Akt signaling pathways. (A) The Akt and ERK signaling pathways promote phosphorylation of distinct sites on TSC2 leading to inhibition of its GAP activity toward Rheb, leaving Rheb in a GTP bound state, and activation of mTORC1. Phosphorylation of both sets of sites acts in concert to repress TSC1/2 function and activate mTORC1. In the absence of either ERK (B) or Akt (C) signaling, the activation state of mTORC1 is reduced, though not ablated. (D) When both ERK and Akt signaling are minimal, TSC1/2 function is dramatically enhanced, resulting in almost complete inactivation of mTORC1.

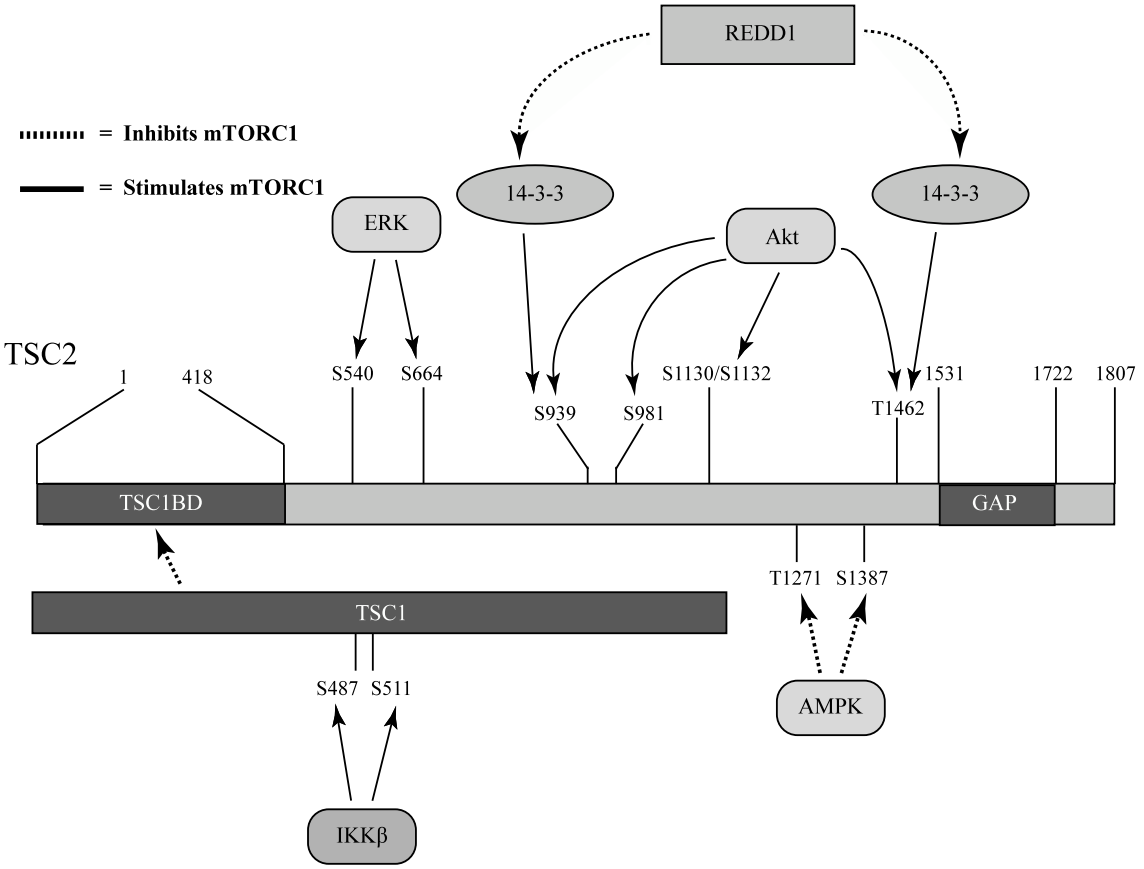

Figure 40. Map of TSC2 regulatory phosphorylation sites. Both ERK and Akt phosphorylation of TSC2 increase mTORC1 activation. The ERK1/2 mediated phosphorylation of S664 has been shown to result in the dissociation of the TSC1/2 complex, increasing mTORC1 signaling. The phosphorylation of the Akt sites on TSC2 similarly increase mTORC1 signaling, though 14-3-3 proteins are involved. Specifically, once phosphorylated, S939 and T1462 act as docking sites on TSC2 for 14-3-3. Once bound, the GAP activity of the TSC1/2 complex is significantly reduced. REDD1 functions to inhibit mTORC1 signaling by competitively binding to 14-3-3 proteins, allowing the formation of the TSC1/2 complex. AMPK phosphorylates TSC2 due to an increase of cellular AMP concentrations, resulting in the activation of the TSC1/2 complex, and the subsequent inhibition of mTORC1. IKKβ phosphorylates TSC1 at S487 and S511 in response to inflammation, resulting in the inhibition of TSC1/2 and the subsequent activation of mTORC1 signaling. The combined effect of the stimulatory and inhibitory signals to the TSC1/2 complex on mTORC1 signaling is currently unknown, although this study has shown that mTORC1 signaling is increased in the presence of multiple mTORC1 agonists, specifically Akt and ERK1/2. Dashed lines represent events that increase TSC1/2 activity, namely TSC1 binding, phosphorylation of the AMPK sites, and the binding of 14-3-3 by REDD1.

REFERENCES

[1] E.J. Brown, M.W. Albers, T.B. Shin, K. Ichikawa, C.T. Keith, W.S. Lane, and S.L. Schreiber, A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 369 (1994) 756-8.

[2] M.I. Chiu, H. Katz, and V. Berlin, RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. Proc Natl Acad Sci U S A 91 (1994) 12574-8.

[3] S.B. Helliwell, P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M.N. Hall, TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. Mol Biol Cell 5 (1994) 105-18.

[4] J. Heitman, N.R. Movva, and M.N. Hall, Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 253 (1991) 905-9.

[5] S.N. Sehgal, Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem 31 (1998) 335-40.

[6] J. Choi, J. Chen, S.L. Schreiber, and J. Clardy, Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science 273 (1996) 239-42.

[7] M. Laplante, and D.M. Sabatini, mTOR signaling at a glance. J Cell Sci 122 (2009) 3589-3594.

[8] X.M. Ma, and J. Blenis, Molecular mechanisms of mTOR-mediated translational control. Nature Reviews Molecular Cell Biology 10 (2009) 307-318.

[9] K. Hara, Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, and K. Yonezawa, Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110 (2002) 177-89.

[10] D.H. Kim, D.D. Sarbassov, S.M. Ali, R.R. Latek, K.V. Guntur, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini, GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell 11 (2003) 895-904.

[11] D.D. Sarbassov, S.M. Ali, D.H. Kim, D.A. Guertin, R.R. Latek, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini, Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14 (2004) 1296-302.

[12] D.D. Sarbassov, S.M. Ali, S. Sengupta, J.-H. Sheen, P.P. Hsu, A.F. Bagley, A.L. Markhard, and D.M. Sabatini, Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Molecular Cell 22 (2006) 159-68.

[13] N. Hay, and N. Sonenberg, Upstream and downstream of mTOR. Genes Dev 18 (2004) 1926-45.

[14] C.A. Hoeffer, and E. Klann, mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci 33 67-75.

[15] D.A. Guertin, and D.M. Sabatini, Defining the role of mTOR in cancer. Cancer Cell 12 (2007) 9-22.

[16] S.G. Dann, A. Selvaraj, and G. Thomas, mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends Mol Med 13 (2007) 252-9.

[17] G. Terzis, G. Georgiadis, G. Stratakos, I. Vogiatzis, S. Kavouras, P. Manta, H. Mascher, and E. Blomstrand, Resistance exercise-induced increase in muscle mass correlates with p70S6 kinase phosphorylation in human subjects. Eur J Appl Physiol 102 (2008) 145-52.

[18] P.A. Tesch, J.T. Trieschmann, and A. Ekberg, Hypertrophy of chronically unloaded muscle subjected to resistance exercise. J Appl Physiol 96 (2004) 1451-8.

[19] A. Chale-Rush, E.P. Morris, T.L. Kendall, N.E. Brooks, and R.A. Fielding, Effects of chronic overload on muscle hypertrophy and mTOR signaling in young adult and aged rats. J Gerontol A Biol Sci Med Sci 64 (2009) 1232-9.

[20] G.R. Adams, and S.A. McCue, Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. J Appl Physiol 84 (1998) 1716-22.

[21] R.T. Hinkle, K.M. Hodge, D.B. Cody, R.J. Sheldon, B.K. Kobilka, and R.J. Isfort, Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the beta2-adrenergic receptor. Muscle Nerve 25 (2002) 729-34.

[22] D.J. Glass, Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. Nat Cell Biol 5 (2003) 87-90.

[23] W.O. Kline, F.J. Panaro, H. Yang, and S.C. Bodine, Rapamycin inhibits the growth and muscle-sparing effects of clenbuterol. J Appl Physiol 102 (2007) 740-7.

[24] T.A. Hornberger, T.J. McLoughlin, J.K. Leszczynski, D.D. Armstrong, R.R. Jameson, P.E. Bowen, E.-S. Hwang, H. Hou, M.E. Moustafa, B.A. Carlson, D.L. Hatfield, A.M. Diamond, and K.A. Esser, Selenoprotein-deficient transgenic mice exhibit enhanced exercise-Induced muscle growth. Journal of Nutrition 133 (2003) 3091-3097.

[25] K. Yamagata, L.K. Sanders, W.E. Kaufmann, W. Yee, C.A. Barnes, D. Nathans, and P.F. Worley, rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. J Biol Chem 269 (1994) 16333-9.

[26] X. Long, Y. Lin, S. Ortiz-Vega, K. Yonezawa, and J. Avruch, Rheb binds and regulates the mTOR kinase. Curr Biol 15 (2005) 702-13.

[27] K. Inoki, Y. Li, T. Xu, and K.-L. Guan, Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes and Development 17 (2003) 1829-1834.

[28] Y. Li, K. Inoki, and K.-L. Guan, Biochemical and functional characterization of small GTPase Rheb and TSC2 GAP activity. Molecular & Cellular Biology 24 (2004) 7965-7975.

[29] A.R. Tee, D.C. Fingar, B.D. Manning, D.J. Kwiatkowski, L.C. Cantley, and J. Blenis, Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proceedings of the National Academy of Sciences USA 99 (2002) 13571-13576.

[30] H. Zhang, G. Cicchetti, H. Onda, H.B. Koon, K. Asrican, N. Bajraszewski, F. Vazquez, C.L. Carpenter, and D.J. Kwiatkowski, Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. J Clin Invest 112 (2003) 1223-33.

[31] J.P. Cheadle, M.P. Reeve, J.R. Sampson, and D.J. Kwiatkowski, Molecular genetic advances in tuberous sclerosis. Hum Genet 107 (2000) 97-114.

[32] L.S. Harrington, G.M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N.R. Leslie, S. Cheng, P.R. Shepherd, I. Gout, C.P. Downes, and R.F. Lamb, The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 166 (2004) 213-23.

[33] A. Sofer, K. Lei, C.M. Johannessen, and L.W. Ellisen, Regulation of mTOR and cell growth in response to energy stress by REDD1. Mol Cell Biol 25 (2005) 5834-45.

[34] J.I. Baum, S.R. Kimball, and L.S. Jefferson, Glucagon acts in a dominant manner to repress insulin-induced mammalian target of rapamycin complex 1 signaling in perfused rat liver. Am J Physiol Endocrinol Metab 297 (2009) E410-5.

[35] S.R. Kimball, A.N. Do, L. Kutzler, D.R. Cavener, and L.S. Jefferson, Rapid turnover of the mTOR complex 1 (mTORC1) repressor REDD1 and activation of mTORC1 signaling following inhibition of protein synthesis. J Biol Chem 283 (2008) 3465-75.

[36] J. Brugarolas, K. Lei, R.L. Hurley, B.D. Manning, J.H. Reiling, E. Hafen, L.A. Witters, L.W. Ellisen, and W.G. Kaelin, Jr., Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev 18 (2004) 2893-904.

[37] G. Pallafacchina, E. Calabria, A.L. Serrano, J.M. Kalhovde, and S. Schiaffino, A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proc Natl Acad Sci U S A 99 (2002) 9213-8.

[38] S.C. Bodine, T.N. Stitt, M. Gonzalez, W.O. Kline, G.L. Stover, R. Bauerlein, E. Zlotchenko, A. Scrimgeour, J.C. Lawrence, D.J. Glass, and G.D. Yancopoulos, Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3 (2001) 1014-9.

[39] E.S. Kandel, and N. Hay, The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 253 (1999) 210-29.

[40] S. Persad, S. Attwell, V. Gray, N. Mawji, J.T. Deng, D. Leung, J. Yan, J. Sanghera, M.P. Walsh, and S. Dedhar, Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. J Biol Chem 276 (2001) 27462-9.

[41] D.D. Sarbassov, D.A. Guertin, S.M. Ali, and D.M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307 (2005) 1098-101.

[42] H.H. Zhang, J. Huang, K. Duvel, B. Boback, S. Wu, R.M. Squillace, C.L. Wu, and B.D. Manning, Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway. PLoS One 4 (2009) e6189.

[43] L. Wang, J.C. Lawrence, Jr., T.W. Sturgill, and T.E. Harris, Mammalian target of rapamycin complex 1 (mTORC1) activity is associated with phosphorylation of raptor by mTOR. J Biol Chem 284 (2009) 14693-7.

[44] S.R. von Manteuffel, A.C. Gingras, X.F. Ming, N. Sonenberg, and G. Thomas, 4E-BP1 phosphorylation is mediated by the FRAP-p70s6k pathway and is independent of mitogen-activated protein kinase. Proc Natl Acad Sci U S A 93 (1996) 4076-80.

[45] F. Tremblay, A. Gagnon, A. Veilleux, A. Sorisky, and A. Marette, Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology 146 (2005) 1328-37.

[46] P. Gual, Y. Le Marchand-Brustel, and J.F. Tanti, Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 87 (2005) 99-109.

[47] M.P. Czech, PIP2 and PIP3: complex roles at the cell surface. Cell 100 (2000) 603-6.

[48] E. Vander Haar, S.I. Lee, S. Bandhakavi, T.J. Griffin, and D.H. Kim, Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol 9 (2007) 316-23.

[49] S.L. Cai, A.R. Tee, J.D. Short, J.M. Bergeron, J. Kim, J. Shen, R. Guo, C.L. Johnson, K. Kiguchi, and C.L. Walker, Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Cell Biol 173 (2006) 279-89.

[50] S.D. Shumway, Y. Li, and Y. Xiong, 14-3-3beta binds to and negatively regulates the tuberous sclerosis complex 2 (TSC2) tumor suppressor gene product, tuberin. J Biol Chem 278 (2003) 2089-92.

[51] A. Hahn-Windgassen, V. Nogueira, C.C. Chen, J.E. Skeen, N. Sonenberg, and N. Hay, Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. J Biol Chem 280 (2005) 32081-9.

[52] L. Ma, Z. Chen, H. Erdjument-Bromage, P. Tempst, and P.P. Pandolfi, Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 121 (2005) 179-93.

[53] L. Ma, J. Teruya-Feldstein, P. Bonner, R. Bernardi, D.N. Franz, D. Witte, C. Cordon-Cardo, and P.P. Pandolfi, Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer. Cancer Res 67 (2007) 7106-12.

[54] R. Hoshino, Y. Chatani, T. Yamori, T. Tsuruo, H. Oka, O. Yoshida, Y. Shimada, S. Ari-i, H. Wada, J. Fujimoto, and M. Kohno, Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 18 (1999) 813-22.

[55] B. Govindarajan, M.C. Mizesko, M.S. Miller, H. Onda, M. Nunnelley, K. Casper, D. Brat, C. Cohen, and J.L. Arbiser, Tuberous sclerosis-associated neoplasms express activated p42/44 mitogen-activated protein (MAP) kinase, and inhibition of MAP kinase signaling results in decreased in vivo tumor growth. Clin Cancer Res 9 (2003) 3469-75.

[56] G. Thiel, M. Ekici, and O.G. Rossler, Regulation of cellular proliferation, differentiation and cell death by activated Raf. Cell Commun Signal 7 (2009) 8.

[57] F. Haddad, and G.R. Adams, Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles. J Appl Physiol 96 (2004) 203-10.

[58] H. Shi, J.M. Scheffler, C. Zeng, J.M. Pleitner, K.M. Hannon, A.L. Grant, and D.E. Gerrard, Mitogen-activated protein kinase signaling is necessary for the maintenance of skeletal muscle mass. Am J Physiol Cell Physiol 296 (2009) C1040-8.

[59] H. Shi, C. Zeng, A. Ricome, K.M. Hannon, A.L. Grant, and D.E. Gerrard, Extracellular signal-regulated kinase pathway is differentially involved in beta-agonist-induced hypertrophy in slow and fast muscles. Am J Physiol Cell Physiol 292 (2007) C1681-9.

[60] U. Widegren, C. Wretman, A. Lionikas, G. Hedin, and J. Henriksson, Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. Pflugers Arch 441 (2000) 317-22.

[61] M.A. Rizzo, K. Shome, C. Vasudevan, D.B. Stolz, T.C. Sung, M.A. Frohman, S.C. Watkins, and G. Romero, Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. J Biol Chem 274 (1999) 1131-9.

[62] M.A. Rizzo, K. Shome, S.C. Watkins, and G. Romero, The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. J Biol Chem 275 (2000) 23911-8.

[63] M. Miyazaki, J.J. McCarthy, M.J. Fedele, and K.A. Esser, Early activation of mTORC1 signalling in response to mechanical overload is independent of phosphoinositide 3-kinase/Akt signalling. J Physiol 589 1831-46.

[64] E.M. Smith, S.G. Finn, A.R. Tee, G.J. Browne, and C.G. Proud, The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. J Biol Chem 280 (2005) 18717-27.

[65] G.M. Findlay, L. Yan, J. Procter, V. Mieulet, and R.F. Lamb, A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. Biochem J 403 (2007) 13-20.

[66] T. Nobukuni, M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, M.P. Byfield, J.M. Backer, F. Natt, J.L. Bos, F.J. Zwartkruis, and G. Thomas, Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci U S A 102 (2005) 14238-43.

[67] E. Kim, P. Goraksha-Hicks, L. Li, T.P. Neufeld, and K.L. Guan, Regulation of TORC1 by Rag GTPases in nutrient response. Nat Cell Biol 10 (2008) 935-45.

[68] Y. Sancak, T.R. Peterson, Y.D. Shaul, R.A. Lindquist, C.C. Thoreen, L. Bar-Peled, and D.M. Sabatini, The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320 (2008) 1496-501.

[69] Y. Sancak, L. Bar-Peled, R. Zoncu, A.L. Markhard, S. Nada, and D.M. Sabatini, Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141 290-303.

[70] D. Ma, X. Bai, S. Guo, and Y. Jiang, The switch I region of Rheb Is critical for its interaction with FKBP38. Journal of Biological Chemistry 283 (2008) 25963-25970.

[71] X. Bai, D. Ma, A. Liu, X. Shen, Q.J. Wang, Y. Liu, and Y. Jiang, Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. Science 318 (2007) 977-80.

[72] T. Maehama, M. Tanaka, H. Nishina, M. Murakami, Y. Kanaho, and K. Hanada, RalA functions as an indispensable signal mediator for the nutrient-sensing system. Journal of Biological Chemistry 283 (2008) 35053-35059.

[73] T. Sato, A. Nakashima, L. Guo, and F. Tamanoi, Specific activation of mTORC1 by Rheb G-protein in vitro involves enhanced recruitment of its substrate protein. Journal of Biological Chemistry 284 (2009) 12783-12791.

[74] K. Uhlenbrock, M. Weiwad, R. Wetzker, G. Fischer, A. Wittinghofer, and I. Rubio, Reassessment of the role of FKBP38 in the Rheb/mTORC1 pathway. FEBS Letters 583 (2009) 965-970.

[75] X. Wang, B.D. Fonseca, H. Tang, R. Liu, A. Elia, M.J. Clemens, U.-A. Bommer, and C.G. Proud, Re-evaluating the roles of proposed modulators of mammalian target of rapamycin complex 1 (mTORC1) signaling. Journal of Biological Chemistry 283 (2008) 30482-30492.

[76] A. Suryawan, A.S. Jeyapalan, R.A. Orellana, F.A. Wilson, H.V. Nguyen, and T.A. Davis, Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. Am J Physiol Endocrinol Metab 295 (2008) E868-75.

[77] J.C. Anthony, F. Yoshizawa, T.G. Anthony, T.C. Vary, L.S. Jefferson, and S.R. Kimball, Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr 130 (2000) 2413-9.

[78] S.J. Crozier, S.R. Kimball, S.W. Emmert, J.C. Anthony, and L.S. Jefferson, Oral leucine administration stimulates protein synthesis in rat skeletal muscle. J Nutr 135 (2005) 376-82.

[79] X. Wang, S.P. Devaiah, W. Zhang, and R. Welti, Signaling functions of phosphatidic acid. Prog Lipid Res 45 (2006) 250-78.

[80] Y. Fang, M. Vilella-Bach, R. Bachmann, A. Flanigan, and J. Chen, Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294 (2001) 1942-5.

[81] Y. Xu, Y. Fang, J. Chen, and G.D. Prestwich, Activation of mTOR signaling by novel fluoromethylene phosphonate analogues of phosphatidic acid. Bioorg Med Chem Lett 14 (2004) 1461-4.

[82] H.K. Lim, Y.A. Choi, W. Park, T. Lee, S.H. Ryu, S.Y. Kim, J.R. Kim, J.H. Kim, and S.H. Baek, Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. J Biol Chem 278 (2003) 45117-27.

[83] D.A. Foster, Phosphatidic acid signaling to mTOR: signals for the survival of human cancer cells. Biochim Biophys Acta 1791 (2009) 949-55.

[84] T.A. Hornberger, W.K. Chu, Y.W. Mak, J.W. Hsiung, S.A. Huang, and S. Chien, The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proc Natl Acad Sci U S A 103 (2006) 4741-6.

[85] T.K. O'Neil, L.R. Duffy, J.W. Frey, and T.A. Hornberger, The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of mammalian target of rapamycin following eccentric contractions. J Physiol 587 (2009) 3691-701.

[86] C. Testerink, and T. Munnik, Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trends Plant Sci 10 (2005) 368-75.

[87] Z.W. Lee, S.M. Kweon, B.C. Kim, S.H. Leem, I. Shin, J.H. Kim, and K.S. Ha, Phosphatidic acid-induced elevation of intracellular Ca2+ is mediated by RhoA and H2O2 in Rat-2 fibroblasts. J Biol Chem 273 (1998) 12710-5.

[88] Y.J. Xu, V. Panagia, Q. Shao, X. Wang, and N.S. Dhalla, Phosphatidic acid increases intracellular free Ca2+ and cardiac contractile force. Am J Physiol 271 (1996) H651-9.

[89] Y.J. Xu, L. Yau, L.P. Yu, V. Elimban, P. Zahradka, and N.S. Dhalla, Stimulation of protein synthesis by phosphatidic acid in rat cardiomyocytes. Biochem Pharmacol 52 (1996) 1735-40.

[90] N.S. Dhalla, Y.J. Xu, S.S. Sheu, P.S. Tappia, and V. Panagia, Phosphatidic acid: a potential signal transducer for cardiac hypertrophy. J Mol Cell Cardiol 29 (1997) 2865-71.

[91] D. Hollenback, L. Bonham, L. Law, E. Rossnagle, L. Romero, H. Carew, C.K. Tompkins, D.W. Leung, J.W. Singer, and T. White, Substrate specificity of lysophosphatidic acid acyltransferase beta -- evidence from membrane and whole cell assays. J Lipid Res 47 (2006) 593-604.

[92] D. Li, L. Yu, H. Wu, Y. Shan, J. Guo, Y. Dang, Y. Wei, and S. Zhao, Cloning and identification of the human LPAAT-zeta gene, a novel member of the lysophosphatidic acid acyltransferase family. J Hum Genet 48 (2003) 438-42.

[93] F. Hong, D. Hollenback, J.W. Singer, and P. Klein, Diamino-C,N-diarylpyridine positional isomers as inhibitors of lysophosphatidic acid acyltransferase-beta. Bioorg Med Chem Lett 15 (2005) 4703-7.

[94] M. Coon, A. Ball, J. Pound, S. Ap, D. Hollenback, T. White, J. Tulinsky, L. Bonham, D.K. Morrison, R. Finney, and J.W. Singer, Inhibition of lysophosphatidic acid acyltransferase beta disrupts proliferative and survival signals in normal cells and induces apoptosis of tumor cells. Mol Cancer Ther 2 (2003) 1067-78.

[95] W. Tang, J. Yuan, X. Chen, X. Gu, K. Luo, J. Li, B. Wan, Y. Wang, and L. Yu, Identification of a novel human lysophosphatidic acid acyltransferase, LPAAT-theta, which activates mTOR pathway. J Biochem Mol Biol 39 (2006) 626-35.

[96] G.M. Carman, and G.S. Han, Phosphatidic acid phosphatase, a key enzyme in the regulation of lipid synthesis. J Biol Chem 284 (2009) 2593-7.

[97] A. Avila-Flores, T. Santos, E. Rincon, and I. Merida, Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. J Biol Chem 280 (2005) 10091-9.

[98] D.Y. Noh, S.J. Ahn, R.A. Lee, I.A. Park, J.H. Kim, P.G. Suh, S.H. Ryu, K.H. Lee, and J.S. Han, Overexpression of phospholipase D1 in human breast cancer tissues. Cancer Lett 161 (2000) 207-14.

[99] Y. Yamada, N. Hamajima, T. Kato, H. Iwata, Y. Yamamura, M. Shinoda, M. Suyama, T. Mitsudomi, K. Tajima, S. Kusakabe, H. Yoshida, Y. Banno, Y. Akao, M. Tanaka, and Y. Nozawa, Association of a polymorphism of the phospholipase D2 gene with the prevalence of colorectal cancer. J Mol Med 81 (2003) 126-31.

[100] M.A. Frohman, and A.J. Morris, Phospholipase D structure and regulation. Chem Phys Lipids 98 (1999) 127-40.

[101] G. Du, P. Huang, B.T. Liang, and M.A. Frohman, Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. Mol Biol Cell 15 (2004) 1024-30.

[102] F.D. Brown, N. Thompson, K.M. Saqib, J.M. Clark, D. Powner, N.T. Thompson, R. Solari, and M.J. Wakelam, Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr Biol 8 (1998) 835-8.

[103] V.A. Sciorra, S.A. Rudge, J. Wang, S. McLaughlin, J. Engebrecht, and A.J. Morris, Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. J Cell Biol 159 (2002) 1039-49.

[104] Y. Chen, Y. Zheng, and D.A. Foster, Phospholipase D confers rapamycin resistance in human breast cancer cells. Oncogene 22 (2003) 3937-42.

[105] A. Toschi, E. Lee, L. Xu, A. Garcia, N. Gadir, and D.A. Foster, Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. Mol Cell Biol 29 (2009) 1411-20.

[106] Y. Kam, and J.H. Exton, Role of phospholipase D1 in the regulation of mTOR activity by lysophosphatidic acid. FASEB J 18 (2004) 311-9.

[107] M. Schmidt, M. Voss, M. Thiel, B. Bauer, A. Grannass, E. Tapp, R.H. Cool, J. de Gunzburg, C. von Eichel-Streiber, and K.H. Jakobs, Specific inhibition of phorbol ester-stimulated phospholipase D by Clostridium sordellii lethal toxin and Clostridium difficile toxin B-1470 in HEK-293 cells. Restoration by Ral GTPases. J Biol Chem 273 (1998) 7413-22.

[108] K. Shome, Y. Nie, and G. Romero, ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. J Biol Chem 273 (1998) 30836-41.

[109] W.D. Singer, H.A. Brown, and P.C. Sternweis, Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. Annu Rev Biochem 66 (1997) 475-509.

[110] E. Meacci, V. Vasta, J.P. Moorman, D.A. Bobak, P. Bruni, J. Moss, and M. Vaughan, Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A549 cells. J Biol Chem 274 (1999) 18605-12.

[111] K. Shome, C. Vasudevan, and G. Romero, ARF proteins mediate insulin-dependent activation of phospholipase D. Curr Biol 7 (1997) 387-96.

[112] K. Ohguchi, Y. Banno, S. Nakashima, and Y. Nozawa, Regulation of membrane-bound phospholipase D by protein kinase C in HL60 cells. Synergistic action of small GTP-binding protein RhoA. J Biol Chem 271 (1996) 4366-72.

[113] J.S. Chen, and J.H. Exton, Regulation of phospholipase D2 activity by protein kinase C alpha. J Biol Chem 279 (2004) 22076-83.

[114] C.H. Yu, S.Y. Liu, and V. Panagia, The transphosphatidylation activity of phospholipase D. Molecular and Cellular Biochemistry 157 (1996) 101-105.

[115] T. Hu, and J.H. Exton, 1-Butanol interferes with phospholipase D1 and protein kinase Calpha association and inhibits phospholipase D1 basal activity. Biochem Biophys Res Commun 327 (2005) 1047-51.

[116] V. Veverka, T. Crabbe, I. Bird, G. Lennie, F.W. Muskett, R.J. Taylor, and M.D. Carr, Structural characterization of the interaction of mTOR with phosphatidic acid and a novel class of inhibitor: compelling evidence for a central role of the FRB domain in small molecule-mediated regulation of mTOR. Oncogene 27 (2008) 585-95.

[117] H.N. Higgs, and J.A. Glomset, Identification of a phosphatidic acid-preferring phospholipase A1 from bovine brain and testis. Proc Natl Acad Sci U S A 91 (1994) 9574-8.

[118] Y. Snitko, E.T. Yoon, and W. Cho, High specificity of human secretory class II phospholipase A2 for phosphatidic acid. Biochem J 321 (Pt 3) (1997) 737-41.

[119] J. Aoki, A. Taira, Y. Takanezawa, Y. Kishi, K. Hama, T. Kishimoto, K. Mizuno, K. Saku, R. Taguchi, and H. Arai, Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J Biol Chem 277 (2002) 48737-44.

[120] T.J. Nevalainen, Serum phospholipases A2 in inflammatory diseases. Clin Chem 39 (1993) 2453-9.

[121] R.S. Koduri, S.F. Baker, Y. Snitko, S.K. Han, W. Cho, D.C. Wilton, and M.H. Gelb, Action of human group IIa secreted phospholipase A2 on cell membranes. Vesicle but not heparinoid binding determines rate of fatty acid release by exogenously added enzyme. J Biol Chem 273 (1998) 32142-53.

[122] J. Pfeilschifter, C. Schalkwijk, V.A. Briner, and H. van den Bosch, Cytokine-stimulated secretion of group II phospholipase A2 by rat mesangial cells. Its contribution to arachidonic acid release and prostaglandin synthesis by cultured rat glomerular cells. J Clin Invest 92 (1993) 2516-23.

[123] T.I. Torkhovskaya, O.M. Ipatova, T.S. Zakharova, M.M. Kochetova, and E.M. Khalilov, Lysophospholipid receptors in cell signaling. Biochemistry (Mosc) 72 (2007) 125-31.

[124] W.H. Moolenaar, Bioactive lysophospholipids and their G protein-coupled receptors. Exp Cell Res 253 (1999) 230-8.

[125] N. Fukushima, X. Ye, and J. Chun, Neurobiology of lysophosphatidic acid signaling. Neuroscientist 8 (2002) 540-50.

[126] K. Hinokio, S. Yamano, K. Nakagawa, M. Iraharaa, M. Kamada, A. Tokumura, and T. Aono, Lysophosphatidic acid stimulates nuclear and cytoplasmic maturation of golden hamster immature oocytes in vitro via cumulus cells. Life Sci 70 (2002) 759-67.

[127] J.T. Swarthout, and H.W. Walling, Lysophosphatidic acid: receptors, signaling and survival. Cell Mol Life Sci 57 (2000) 1978-85.

[128] J.M. Gerrard, and P. Robinson, Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. Biochim Biophys Acta 1001 (1989) 282-5.

[129] H. Sun, J. Ren, Q. Zhu, F.Z. Kong, L. Wu, and B.R. Pan, Effects of lysophosphatidic acid on human colon cancer cells and its mechanisms of action. World J Gastroenterol 15 (2009) 4547-55.

[130] H. Zhang, A. Bialkowska, R. Rusovici, S. Chanchevalap, H. Shim, J.P. Katz, V.W. Yang, and C.C. Yun, Lysophosphatidic acid facilitates proliferation of colon cancer cells via induction of Kruppel-like factor 5. J Biol Chem 282 (2007) 15541-9.

[131] D. Bian, S. Su, C. Mahanivong, R.K. Cheng, Q. Han, Z.K. Pan, P. Sun, and S. Huang, Lysophosphatidic Acid Stimulates Ovarian Cancer Cell Migration via a Ras-MEK Kinase 1 Pathway. Cancer Res 64 (2004) 4209-17.

[132] Z. Zhang, Z. Liu, and K.E. Meier, Lysophosphatidic acid as a mediator for proinflammatory agonists in a human corneal epithelial cell line. Am J Physiol Cell Physiol 291 (2006) C1089-98.

[133] C.I. Lin, C.N. Chen, P.W. Lin, K.J. Chang, F.J. Hsieh, and H. Lee, Lysophosphatidic acid regulates inflammation-related genes in human endothelial cells through LPA1 and LPA3. Biochem Biophys Res Commun 363 (2007) 1001-8.

[134] R. Hilal-Dandan, C.K. Means, A.B. Gustafsson, M.R. Morissette, J.W. Adams, L.L. Brunton, and J. Heller Brown, Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho. J Mol Cell Cardiol 36 (2004) 481-93.

[135] J.S. Greiwe, G. Kwon, M.L. McDaniel, and C.F. Semenkovich, Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am J Physiol Endocrinol Metab 281 (2001) E466-71.

[136] M. Balage, S. Sinaud, M. Prod'homme, D. Dardevet, T.C. Vary, S.R. Kimball, L.S. Jefferson, and J. Grizard, Amino acids and insulin are both required to regulate assembly of the eIF4E. eIF4G complex in rat skeletal muscle. Am J Physiol Endocrinol Metab 281 (2001) E565-74.

[137] Y. Sun, Y. Fang, M.S. Yoon, C. Zhang, M. Roccio, F.J. Zwartkruis, M. Armstrong, H.A. Brown, and J. Chen, Phospholipase D1 is an effector of Rheb in the mTOR pathway. Proc Natl Acad Sci U S A 105 (2008) 8286-91.

[138] Y. Kam, and J.H. Exton, Phospholipase D activity is required for actin stress fiber formation in fibroblasts. Mol Cell Biol 21 (2001) 4055-66.

[139] D.S. Wijesinghe, J.C. Allegood, L.B. Gentile, T.E. Fox, M. Kester, and C.E. Chalfant, Use of high pressure liquid chromatography, electrospray ionization-tandem mass spectrometry for the analysis of ceramide-1-phosphate levels. J Lipid Res (2009).

[140] P.T. Ivanova, S.B. Milne, D.S. Myers, and H.A. Brown, Lipidomics: a mass spectrometry based systems level analysis of cellular lipids. Curr Opin Chem Biol (2009).

[141] T.A. Gautsch, J.C. Anthony, S.R. Kimball, G.L. Paul, D.K. Layman, and L.S. Jefferson, Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. Am J Physiol Cell Physiol 274 (1998) C406-414.

[142] S.R. Kimball, C.V. Jurasinski, J.C. Lawrence, Jr, and L.S. Jefferson, Insulin stimulates protein synthesis in skeletal muscle by enhancing the association of eIF-4E and eIF-4G. Am J Physiol Cell Physiol 272 (1997) C754-759.

[143] D.R. Bolster, N. Kubica, S.J. Crozier, D.L. Williamson, P.A. Farrell, S.R. Kimball, and L.S. Jefferson, Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. J Physiol 553 (2003) 213-20.

[144] M.J. Drummond, C.S. Fry, E.L. Glynn, H.C. Dreyer, S. Dhanani, K.L. Timmerman, E. Volpi, and B.B. Rasmussen, Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. The Journal of Physiology 587 (2009) 1535-1546.

[145] J. Xu, L.M. Love, I. Singh, Q.X. Zhang, J. Dewald, D.A. Wang, D.J. Fischer, G. Tigyi, L.G. Berthiaume, D.W. Waggoner, and D.N. Brindley, Lipid phosphate phosphatase-1 and Ca2+ control lysophosphatidate signaling through EDG-2 receptors. J Biol Chem 275 (2000) 27520-30.

[146] Y. Fang, I.H. Park, A.L. Wu, G. Du, P. Huang, M.A. Frohman, S.J. Walker, H.A. Brown, and J. Chen, PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr Biol 13 (2003) 2037-44.

[147] J.H. Hong, S.O. Oh, M. Lee, Y.R. Kim, D.U. Kim, G.M. Hur, J.H. Lee, K. Lim, B.D. Hwang, and S.K. Park, Enhancement of lysophosphatidic acid-induced ERK phosphorylation by phospholipase D1 via the formation of phosphatidic acid. Biochem Biophys Res Commun 281 (2001) 1337-42.

[148] H. Ohta, K. Sato, N. Murata, A. Damirin, E. Malchinkhuu, J. Kon, T. Kimura, M. Tobo, Y. Yamazaki, T. Watanabe, M. Yagi, M. Sato, R. Suzuki, H. Murooka, T. Sakai, T. Nishitoba, D.S. Im, H. Nochi, K. Tamoto, H. Tomura, and F. Okajima, Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. Mol Pharmacol 64 (2003) 994-1005.

[149] A. Garami, F.J. Zwartkruis, T. Nobukuni, M. Joaquin, M. Roccio, H. Stocker, S.C. Kozma, E. Hafen, J.L. Bos, and G. Thomas, Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell 11 (2003) 1457-66.

[150] Y. Zhao, H. Ehara, Y. Akao, M. Shamoto, Y. Nakagawa, Y. Banno, T. Deguchi, N. Ohishi, K. Yagi, and Y. Nozawa, Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. Biochem Biophys Res Commun 278 (2000) 140-3.

[151] S.B. Bocckino, P.B. Wilson, and J.H. Exton, Phosphatidate-dependent protein phosphorylation. Proc Natl Acad Sci U S A 88 (1991) 6210-3.

[152] Y.J. Xu, M.W. Botsford, V. Panagia, and N.S. Dhalla, Responses of heart function and intracellular free Ca2+ to phosphatidic acid in chronic diabetes. Can J Cardiol 12 (1996) 1092-8.

[153] N. Lehman, B. Ledford, M. Di Fulvio, K. Frondorf, L.C. McPhail, and J. Gomez-Cambronero, Phospholipase D2-derived phosphatidic acid binds to and activates ribosomal p70 S6 kinase independently of mTOR. FASEB J 21 (2007) 1075-87.

[154] S. Ghosh, J.C. Strum, V.A. Sciorra, L. Daniel, and R.M. Bell, Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. J Biol Chem 271 (1996) 8472-80.

[155] A.R. Tee, R. Anjum, and J. Blenis, Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin. J Biol Chem 278 (2003) 37288-96.

[156] P.P. Roux, B.A. Ballif, R. Anjum, S.P. Gygi, and J. Blenis, Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A 101 (2004) 13489-94.

[157] B.A. Ballif, P.P. Roux, S.A. Gerber, J.P. MacKeigan, J. Blenis, and S.P. Gygi, Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors. Proc Natl Acad Sci U S A 102 (2005) 667-72.

[158] J. Huang, and B.D. Manning, The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J 412 (2008) 179-90.

[159] J. Chen, and Y. Fang, A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. Biochem Pharmacol 64 (2002) 1071-7.

[160] R. Novotna, P. De Vito, L. Currado, P. Luly, and P.M. Baldini, Involvement of phospholipids in the mechanism of insulin action in HEPG2 cells. Physiol Res 52 (2003) 447-54.

[161] T. Yoshida, L. Semprun-Prieto, S. Sukhanov, and P. Delafontaine, IGF-1 prevents ANG II-induced skeletal muscle atrophy via Akt- and Foxo-dependent inhibition of the ubiquitin ligase atrogin-1 expression. Am J Physiol Heart Circ Physiol 298 H1565-70.

[162] C. Rommel, S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulos, and D.J. Glass, Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3 (2001) 1009-13.

[163] T. Ueyama, S. Kawashima, T. Sakoda, Y. Rikitake, T. Ishida, M. Kawai, T. Yamashita, S. Ishido, H. Hotta, and M. Yokoyama, Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. J Mol Cell Cardiol 32 (2000) 947-60.

[164] O.F. Bueno, L.J. De Windt, K.M. Tymitz, S.A. Witt, T.R. Kimball, R. Klevitsky, T.E. Hewett, S.P. Jones, D.J. Lefer, C.F. Peng, R.N. Kitsis, and J.D. Molkentin, The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J 19 (2000) 6341-50.

[165] L. Wang, and C.G. Proud, Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. Circ Res 91 (2002) 821-9.

[166] T.P. Herbert, A.R. Tee, and C.G. Proud, The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E-BP1 at multiple sites. J Biol Chem 277 (2002) 11591-6.

[167] N. Kubica, J.L. Crispino, J.W. Gallagher, S.R. Kimball, and L.S. Jefferson, Activation of the mammalian target of rapamycin complex 1 is both necessary and sufficient to stimulate eukaryotic initiation factor 2Bvarepsilon mRNA translation and protein synthesis. Int J Biochem Cell Biol 40 (2008) 2522-33.

[168] P.E. Burnett, R.K. Barrow, N.A. Cohen, S.H. Snyder, and D.M. Sabatini, RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci U S A 95 (1998) 1432-7.

[169] A. Carrière, Y. Romeo, H.A. Acosta-Jaquez, J. Moreau, E. Bonneil, P. Thibault, D.C. Fingar, and P.P. Roux, ERK1/2 phosphorylate raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). Journal of Biological Chemistry 286 (2011) 567-577.

[170] A. Carriere, M. Cargnello, L.A. Julien, H. Gao, E. Bonneil, P. Thibault, and P.P. Roux, Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr Biol 18 (2008) 1269-77.

[171] B.T. Nave, D.M. Ouwens, D.J. Withers, D.R. Alessi, and P.R. Shepherd, Mammalian target of rapamycin is a direct target for protein kinase B: Identification of a convergence point for opposing effects of insulin and amino acid deficiency on protein translation. Biochemical Journal 344 (1999) 427-431.

[172] E.V. Haar, S.-i. Lee, S. Bandhakavi, T.J. Griffin, and D.-H. Kim, Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nature Cell Biology 9 (2007) 316-323.

[173] F. Tremblay, S. Brule, S. Hee Um, Y. Li, K. Masuda, M. Roden, X.J. Sun, M. Krebs, R.D. Polakiewicz, G. Thomas, and A. Marette, Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A 104 (2007) 14056-61.

[174] E. Kim, and K.-L. Guan, RAG GTPases in nutrient-mediated TOR signaling pathway. Cell Cycle 8 (2009) 1014-8.

[175] Y. Sancak, T.R. Peterson, Y.D. Shaul, R.A. Lindquist, C.C. Thoreen, L. Bar-Peled, and D.M. Sabatini, The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science (2008) 1157535.

[176] K. Inoki, T. Zhu, and K.L. Guan, TSC2 mediates cellular energy response to control cell growth and survival. Cell 115 (2003) 577-90.

[177] C.J. Potter, L.G. Pedraza, and T. Xu, Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol 4 (2002) 658-65.

[178] L. Ma, Z. Chen, H. Erdjument-Bromage, P. Tempst, and P.P. Pandolfi, Phosphorylation and functional inactivation of TSC2 by Erk: Implications for tuberous sclerosis and cancer pathogenesis. Cell 121 (2005) 179-193.

[179] S.H. Kim, K.A. Roth, A.R. Moser, and J.I. Gordon, Transgenic mouse models that explore the multistep hypothesis of intestinal neoplasia. Journal of Cell Biology 123 (1993) 877-93.

[180] Y. Sancak, L. Bar-Peled, R. Zoncu, A.L. Markhard, S. Nada, and D.M. Sabatini, Ragulator-Rag complex targets mTORC1 to the lysosomal surface and Is necessary for its activation by amino acids. Cell 141 (2010) 290-303.

[181] R.T. Abraham, Lysosomal Rag-ulation of mTOR complex 1 activity. Cell Metabolism 11 (2010) 341-342.

[182] J.C. Anthony, A.K. Reiter, T.G. Anthony, S.J. Crozier, C.H. Lang, D.A. MacLean, S.R. Kimball, and L.S. Jefferson, Orally administered leucine enhances protein synthesis in skeletal muscle of diabetic rats in the absence of increases in 4E-BP1 or S6K1 phosphorylation. Diabetes 51 (2002) 928-936.

[183] M. Balage, S. Sinaud, M. Prod'Homme, D. Dardevet, T.C. Vary, S.R. Kimball, L.S. Jefferson, and J. Grizard, Amino acids and insulin are both required to regulate assembly of the eIF4E•eIF4G complex in rat skeletal muscle. American Journal of Physiology 281 (2001) E565-E574.

[184] G. Pallafacchina, E. Calabria, A. Serrano, J.M. Kalhovde, and S. Schiaffino, A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proceedings of the National Academy of Sciences USA 99 (2002) 9213-9218.

[185] C. Rommel, S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulous, and D.J. Glass, Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nature Cell Biology 3 (2001) 1009-1013.

[186] T. Yoshida, L. Semprun-Prieto, S. Sukhanov, and P. Delafontaine, IGF-1 prevents ANG II-induced skeletal muscle atrophy via Akt- and Foxo-dependent inhibition of the ubiquitin ligase atrogin-1 expression. Am J Physiol Heart Circ Physiol 298 (2010) H1565-1570.

[187] O.F. Bueno, L.J. De Windt, K.M. Tymitz, S.A. Witt, T.R. Kimball, R. Klevitsky, T.E. Hewett, S.P. Jones, D.J. Lefer, C.F. Peng, R.N. Kitsis, and J.D. Molkentin, The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO Journal 19 (2000) 6341-50.

[188] T. Ueyama, S. Kawashima, T. Sakoda, Y. Rikitake, T. Ishida, M. Kawai, T. Yamashita, S. Ishido, H. Hotta, and M. Yokoyama, Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. Journal of Molecular and Cellular Cardiology 32 (2000) 947-60.

[189] H. Shi, C. Zeng, A. Ricome, K.M. Hannon, A.L. Grant, and D.E. Gerrard, Extracellular signal-regulated kinase pathway is differentially involved in beta-agonist-induced hypertrophy in slow and fast muscles. American Journal of Physiology 292 (2007) C1681-1689.

[190] H. Shi, J.M. Scheffler, C. Zeng, J.M. Pleitner, K.M. Hannon, A.L. Grant, and D.E. Gerrard, Mitogen-activated protein kinase signaling is necessary for the maintenance of skeletal muscle mass. American Journal of Physiology 296 (2009) C1040-8.

[191] L. Wang, and C.G. Proud, Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. Circulation Research 91 (2002) 821-9.

[192] T.P. Herbert, A.R. Tee, and C.G. Proud, The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E-BP1 at multiple sites. The Journal of Biological Chemistry 277 (2002) 11591-6.

[193] N.A. Burd, D.W. West, A.W. Staples, P.J. Atherton, J.M. Baker, D.R. Moore, A.M. Holwerda, G. Parise, M.J. Rennie, S.K. Baker, and S.M. Phillips, Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men. PLoS ONE 5 (2010) e12033.

[194] Z. Liu, P. Hou, M. Ji, H. Guan, K. Studeman, K. Jensen, V. Vasko, A.K. El-Naggar, and M. Xing, Highly prevalent genetic alterations in receptor tyrosine kinases and phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pPathways in anaplastic and follicular thyroid cancers. J Clin Endocrinol Metab 93 (2008) 3106-3116.

[195] L. Simi, N. Pratesi, M. Vignoli, R. Sestini, F. Cianchi, R. Valanzano, S. Nobili, E. Mini, M. Pazzagli, and C. Orlando, High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. American Journal of Clinical Pathology 130 (2008) 247-253.

[196] H. Tsao, V. Goel, H. Wu, G. Yang, and F.G. Haluska, Genetic interaction between NRAS and BRAF mutations and PTEN//MMAC1 inactivation in melanoma. J Investig Dermatol 122 (2004) 337-341.

[197] Q.-B. She, E. Halilovic, Q. Ye, W. Zhen, S. Shirasawa, T. Sasazuki, D.B. Solit, and N. Rosen, 4E-BP1 is a key effector of the oncogenic activation of the Akt and ERK signaling pathways that integrates their function in tumors. Cancer Cell 18 (2010) 39-51.

[198] J.A. Engelman, L. Chen, X. Tan, K. Crosby, A.R. Guimaraes, R. Upadhyay, M. Maira, K. McNamara, S.A. Perera, Y. Song, L.R. Chirieac, R. Kaur, A. Lightbown, J. Simendinger, T. Li, R.F. Padera, C. Garcia-Echeverria, R. Weissleder, U. Mahmood, L.C. Cantley, and K.-K. Wong, Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nature Medicine 14 (2008) 1351-1356.

[199] K.P. Hoeflich, C. O'Brien, Z. Boyd, G. Cavet, S. Guerrero, K. Jung, T. Januario, H. Savage, E. Punnoose, T. Truong, W. Zhou, L. Berry, L. Murray, L. Amler, M. Belvin, L.S. Friedman, and M.R. Lackner, In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clinical Cancer Research 15 (2009) 4649-4664.

[200] Q.-B. She, D.B. Solit, Q. Ye, K.E. O'Reilly, J. Lobo, and N. Rosen, The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell 8 (2005) 287-297.

[201] S. Wee, Z. Jagani, K.X. Xiang, A. Loo, M. Dorsch, Y.-M. Yao, W.R. Sellers, C. Lengauer, and F. Stegmeier, PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Research 69 (2009) 4286-4293.

[202] K. Yu, L. Toral-Barza, C. Shi, W.-G. Zhang, and A. Zask, Response and determinants of cancer cell susceptibility to PI3K inhibitors: combined targeting of PI3K and Mek1 as an effective anticancer strategy. Cancer Biology & Therapy 7 (2008) 307-15.

[203] T. Joseph, A. Bryant, P. Frankel, R. Wooden, E. Kerkhoff, U.R. Rapp, and D.A. Foster, Phospholipase D overcomes cell cycle arrest induced by high-intensity Raf signaling. Oncogene 21 (2002) 3651-8.

[204] W. Imagawa, G.K. Bandyopadhyay, and S. Nandi, Analysis of the proliferative response to lysophosphatidic acid in primary cultures of mammary epithelium: differences between normal and tumor cells. Exp Cell Res 216 (1995) 178-86.

[205] C. Qi, J.H. Park, T.C. Gibbs, D.W. Shirley, C.D. Bradshaw, K.M. Ella, and K.E. Meier, Lysophosphatidic acid stimulates phospholipase D activity and cell proliferation in PC-3 human prostate cancer cells. J Cell Physiol 174 (1998) 261-72.

[206] T. Kusama, M. Mukai, M. Ayaki, F. Imamura, M. Tatsuta, Y. Matsumoto, H. Nakamura, and M. Inoue, Inhibition of lysophosphatidic acid-induced RhoA activation and tumor cell invasion by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. Int J Oncol 23 (2003) 1173-8.

[207] D. Shida, J. Kitayama, H. Yamaguchi, Y. Okaji, N.H. Tsuno, T. Watanabe, Y. Takuwa, and H. Nagawa, Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. Cancer Res 63 (2003) 1706-11.

[208] M. Yang, W.W. Zhong, N. Srivastava, A. Slavin, J. Yang, T. Hoey, and S. An, G protein-coupled lysophosphatidic acid receptors stimulate proliferation of colon cancer cells through the {beta}-catenin pathway. Proc Natl Acad Sci U S A 102 (2005) 6027-32.

[209] K. Hama, J. Aoki, M. Fukaya, Y. Kishi, T. Sakai, R. Suzuki, H. Ohta, T. Yamori, M. Watanabe, J. Chun, and H. Arai, Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1. J Biol Chem 279 (2004) 17634-9.

[210] S. Sengupta, Y.J. Xiao, and Y. Xu, A novel laminin-induced LPA autocrine loop in the migration of ovarian cancer cells. FASEB J 17 (2003) 1570-2.

[211] J. Xu, Y.J. Lai, W.C. Lin, and F.T. Lin, TRIP6 enhances lysophosphatidic acid-induced cell migration by interacting with the lysophosphatidic acid 2 receptor. J Biol Chem 279 (2004) 10459-68.

[212] M.L. Toews, E.E. Ustinova, and H.D. Schultz, Lysophosphatidic acid enhances contractility of isolated airway smooth muscle. J Appl Physiol 83 (1997) 1216-22.

[213] M.S. Aoki, E.H. Miyabara, A.G. Soares, E.T. Saito, and A.S. Moriscot, mTOR pathway inhibition attenuates skeletal muscle growth induced by stretching. Cell Tissue Res 324 (2006) 149-56.

[214] Y. Takuwa, N. Takuwa, and N. Sugimoto, The Edg family G protein-coupled receptors for lysophospholipids: their signaling properties and biological activities. J Biochem 131 (2002) 767-71.

[215] S. An, T. Bleu, O.G. Hallmark, and E.J. Goetzl, Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J Biol Chem 273 (1998) 7906-10.

[216] T.N. Stitt, D. Drujan, B.A. Clarke, F. Panaro, Y. Timofeyva, W.O. Kline, M. Gonzalez, G.D. Yancopoulos, and D.J. Glass, The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14 (2004) 395-403.

[217] R. Schechter, and T. Yanovitch, Insulin effects on extracellular signal regulated kinase cascade in fetal rat astrocyte cell cultures. Neurosci Lett 268 (1999) 1-4.

[218] W. Wunderlich, I. Fialka, D. Teis, A. Alpi, A. Pfeifer, R.G. Parton, F. Lottspeich, and L.A. Huber, A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J Cell Biol 152 (2001) 765-76.

[219] D. Teis, W. Wunderlich, and L.A. Huber, Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev Cell 3 (2002) 803-14.

[220] J.P. Luzio, P.R. Pryor, and N.A. Bright, Lysosomes: fusion and function. Nat Rev Mol Cell Biol 8 (2007) 622-32.

[221] M.P. DeYoung, P. Horak, A. Sofer, D. Sgroi, and L.W. Ellisen, Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev 22 (2008) 239-51.

[222] M.L. Whitney, L.S. Jefferson, and S.R. Kimball, ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. Biochem Biophys Res Commun 379 (2009) 451-5.

[223] D.F. Lee, H.P. Kuo, C.T. Chen, J.M. Hsu, C.K. Chou, Y. Wei, H.L. Sun, L.Y. Li, B. Ping, W.C. Huang, X. He, J.Y. Hung, C.C. Lai, Q. Ding, J.L. Su, J.Y. Yang, A.A. Sahin, G.N. Hortobagyi, F.J. Tsai, C.H. Tsai, and M.C. Hung, IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. Cell 130 (2007) 440-55.

[224] D.F. Lee, H.P. Kuo, C.T. Chen, Y. Wei, C.K. Chou, J.Y. Hung, C.J. Yen, and M.C. Hung, IKKbeta suppression of TSC1 function links the mTOR pathway with insulin resistance. Int J Mol Med 22 (2008) 633-8.

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Honors and Awards:

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Certifications, Workshops & Courses

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Publications

J.N. Winter, T.E. Fox, M. Kester, L.S. Jefferson, and S.R. Kimball, Phosphatidic acid mediates activation of mTORC1 through the ERK signaling pathway. *Am J Physiol Cell Physiol* 299 (2010) C335-44.

J.N. Winter, L.S. Jefferson, and S.R. Kimball, ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling. *Am J Physiol Cell Physiol* 300 (2011) C1172-80.