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PHOSPHATIDIC ACID INCREASES LEAN BODY MASS AND STRENGTH IN
RESISTANCE-TRAINED MEN

by

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B.S. University of Central Florida, 2010

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ABSTRACT

Phosphatidic Acid (PA) is a natural phospholipid compound derived from lecithin which is commonly found in egg yolk, grains, fish, soybeans, peanuts and yeast. It has been suggested that PA is involved in several intracellular processes associated with muscle hypertrophy. Specifically, PA has been reported to activate protein synthesis through the mammalian target of rapamycin (mTOR) signaling pathway and thereby may enhance the anabolic effects of resistance training. To our knowledge, no one has examined the effect of PA supplementation in humans while undergoing a progressive resistance training program.

To examine the effect of PA supplementation on lean soft tissue mass (LM) and strength after 8 weeks of resistance training.

Fourteen resistance-trained men (mean $\pm$ SD; age 22.7 $\pm$ 3.3 yrs; height: 1.78 $\pm$ 0.10m; weight: 89.3 $\pm$ 16.3 kg) volunteered to participate in this randomized, double-blind, placebo-controlled, repeated measures study. The participants were assigned to a PA group (750mg/day; Mediator®, ChemiNutra, MN, n=7) or placebo group (PL; rice flower; n=7), delivered in capsule form that was identical in size, shape and color. Participants were tested for 1RM strength in the bench press (BP) and squat (SQ) exercise.

LM was measured using dual-energy X-ray absorptiometry. After base line testing, the participants began supplementing PA or PLfor 8 weeks during a progressive resistance training program intended for muscular hypertrophy. Data was analyzed using magnitude-based inferences on mean changes for BP, SQ and LM. Furthermore, the magnitudes of the inter-relationships between changes in total training volume and LM were interpreted using Pearson correlation coefficients, which had uncertainty (90% confidence limits) of approximately $\pm$0.25.

In the PA group, the relationship between changes in training volume and LM was large(r=0.69, $\pm$0.27; 90%CL), however, in the PL group the relationship was small (r=0.21, $\pm$0.44; 90%CL).

PA supplementation was determined to be likely beneficial at improving SQ and LM over PL by 26% and 64%, respectively. The strong relationship between changes in total training volume and LM in the PA group suggest that greater training volume most likely lead to the greater changes in LM, however, no such relationship was found with PL group. For the BP data, the PA group resulted in a 42% greater increase in strength over PL, although the effect was considered unclear.

While more research is needed to elucidate mechanism of action; the current findings suggest that in experienced resistance trained men supplementing 750mg PA per day for 8 weeks may likely benefit greater changes in muscle mass and strength compared with resistance training only.

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LIST OF ACRONYMS/ABBREVIATIONS

ALSTM	Arms and legs soft tissue mass
BP	Bench Press
FRB	FKBP12 rapamycin binding domain
LM	Lean mass
LST	Lean soft tissue
MQ	Muscle quality
mTOR	Mammalian target of rapamycin
PA	Phosphatidic acid
PC	Phosphatidylcholine
PL	Phospholipid
PLD	Phospholipase D
SMI	Skeletal muscle index
SQ	Squat
S6K1	Serine 6 kinase protein 1
VL	Vastus lateralis

CHAPTER ONE: INTRODUCTION

Phosphatidic Acid or (PA) is a phospholipid metabolite that makes up a very small percentage of the total phospholipid pool. Phospholipids (PL) are a major component of all biological membrane systems, both structurally and dynamically. PL's are comprised of a membrane barrier that separate the cell's internals from the outside world. They also serve as communicators between cells able to transfer biochemical messages into the cell. PA is a lipid secondary messenger in mitogenic signaling pathways that plays a role in several intracellular processes. PA is also involved in regulating multiple signaling proteins and protein kinases. Protein metabolism in cells including both protein synthesis and degradation is crucial for muscular growth. Phosphatidic Acid has been shown to activate mTOR (mammalian target of rapamycin), a serine threonine kinase that integrates metabolic signals from various factors including protein metabolism and cytoskeleton organization. Cellular growth and metabolism regulation is largely the responsibility of mTOR also called the mammalian target of rapamycin. Several studies have implicated mTOR as a necessary component at the cellular level for a hypertrophic response to resistance exercise. The implications for Phosphatidic Acid in the sports world for increasing lean muscle mass and its potential role in the medical field for rehabilitation are astounding. This represents a need to further investigate the efficacy of PA on muscle mass accruement and strength gains.

CHAPTER TWO: LITERATURE REVIEW

mTOR

Sometimes referred to as the intracellular central kinase sensor, mTOR is a serine

threonine kinase that integrates metabolic signals from stress factors, energy status, hormonal

factors, and nutrient availability and regulates many physiological functions such as cell cycle,

gene transcription, cytoskeleton organization, and protein metabolism (Sarbassov, Ali, Sabatinin,

2005; Foster, D. 2007; Yang, X. et al, 2008; Schmelze & Hall, 2000; Guertin, Kim, Sabatini,

2004; Hahn & Myers, 2006). There are other names for mTOR such as FKBP rapamycin

associated protein (FRAP), rapamycin and FKBP target, and rapamycin target (Yang, X. et al,

2008). The mammalian target of rapamycin belongs to the phosphoinositol-3-kinase related

kinase family. Members of this family typically have over 2,500 amino acids (Hay & Sonenberg,

2004). With 2,549 amino acids and a molecular weight of 289 kDa, mTOR has several

structurally conserved domains.

mTOR Complex 1

There are two complexes of mTOR (mTORC1 and mTORC2). These two complexes

may be multimeric; however, the upstream regulatory aspects of mTORC2 have not yet been

defined (Wullscheleger, Loweith, Hall, 2006). Of the two complexes, mTORC1is the most

understood. The function of mTOR complex 1 is largely responsible for growth and cellular

metabolism regulation (Corradetti & Guan 2006; X. Yang. et al, 2008).

It is also in control of translation, ribosome biogenesis, transcription, autophagy, and

hypoxic adaptation (X. Bai & Jiang, Yu. 2009). It is comprised of 4 components, regulatory

associated protein TOR (also known as raptor, which is unique to mTORC1 and it acts as an adapter protein by presenting and binding substrates to mTOR), mLST8 (mammalian lethal with SEC 12 protein 8) also known as G protein beta subunit-like (GβL) it has been found to associate and stimulate mTOR kinase activity), PRAS40 (a suggested inhibitor of mTORC1) and mTOR (X. Bai. & Y. Jiang, 2009; Wullschleger et al, 2006; X. Yang. et al, 2008). Interestingly, mTOR complex 1 is sensitive to rapamycin (Corradetti & Guan 2006; X. Yang. et al, 2008). S6K1 (ribosomal protein S6 Kinase beta 1) and eIF4E-BP1, are the two best characterized downstream effectors of mTORC1. Both are important factors in the protein synthetic pathway (Hay & Sonenberg, 2004). In addition, both 4E-BP1 and S6K1 activation are stimulated by mitogens and mTOR is a crucial component for both responses (Fang, Vilella-Bach, Bachmann, Flanigan, Chen, 2001; Lim, Choi, Park, Lee, Ryu, Kim, Kim, Baek, 2003). Phosphorylation of the S6 protein is associated with the activation of protein synthesis through induced growth factors (Ballou, Jiang, Du, Frohman, Lin, 2003). P70S6K is the main kinase that phosphorylates S6 (Ballou, L. et al, 2003). It appears that full activation of S6K1 requires cooperation between 3-phosphoinositide-dependent protein kinase-1 (PDK1) and MTORC1. Eukaryotic elongation factor 2 kinase, insulin receptor substrate-1 (IRS-1), S6, and eIF4B, are all biological targets of S6K1 (Ruvisky and Meyuhas, 2006). 4E-BP1 is defined as a translation suppressor that prevents formation of a functional eIF4F complex when it is hypophosphorylated.

Interestingly, cells treated with growth factors results in multiple phosphorylation of 4E-BP1 leading to its dissociation from eIF4E which removes the block of translation (Ballou, L. et al, 2003). Activation of eIF4E-BP1 by mTORC1 and various protein kinases lead to increasing global protein synthesis efficiency from the formation of the eIF4F complex through the available eIF4F (Proud, 2007).

mTOR Complex 2

The 2 known primary functions of mTORC2 are in the cell cycle-dependent organization of the actin cytoskeleton and regulation of Akt (Wullschleger et al, 2006; X. Bai, & Y. Jiang, 2009). Rictor (rapamycin – insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase-interacting protein 1), PRR5 (proline-rich repeat protein-5), mLST8 (only component shared by both mTORC1 and mTORC2), and mTOR all combine to make up mTORC2 (Wullschleger et al, 2006). mTOR and mLST8 are shared by mTORC1 and mTORC2 (X. Bai, & Y. Jiang 2009). The binding aspects of mTORC2 are believed to be protein observed with rictor and proline-rich protein 5, in addition to stress-activated protein kinase-interacting protein 1(Pearce, Huang, Boudeau, Pawloski, Wullschleger, Deak, Ilbrahim, Gourlay, Manguson, Alessi, 2007; Woo, Kim, Jun, Kim, Haar, Lee, hegg, Bandhakavi, Griffin, Kim, 2007; Wullschleger et al, 2006; Jacinto, Facchinettil, Liu, Soto, Wei, Jung, Huang, Qin, Su, 2006). Unlike mTORC1, mTORC2 is insensitive to rapamycin and its signaling mechanism must be further studied. Protein kinase B (PKB), Akt activation by mTORC2 has been demonstrated.

Phosphatidic Acid

Phosphatidic Acid or (PA) is a phospholipid metabolite that makes up a very small percentage of the total phospholipid pool (Singer, Brown, Sternweis, 1997; Mcdermott, Wakelam, Morris, 2004; Andresen, Rizzo, Shome, Romero, 2002). Phospholipids (PL) are a major component of all biological membrane systems, both structurally and dynamically (Hanahan, DJ. & Nelson, DR. 1984; Fang, Y. et al, 2001). As the smallest of the phospholipids, PA makes up less than 5% of that of Phosphatidylcholine (PC), the most abundant membrane

phospholipid (Lim, H. et al 2003). PA is a lipid secondary messenger in mitogenic signaling pathways that plays a role in several intracellular processes (Singer, WD. et al, 1997; McDermott, M. et al, 2004; Andresen, BT. et al, 2002). It is also involved in regulating multiple signaling proteins and protein kinases including protein-tyrosine phosphatase, phosphoinositide 4-kinase, and sphingosine kinase (Lim, H. et al, 2003). PA is very transient as it is a target for lipid phosphate phosphatases and is involved in phospholipid synthesis (English, Cui, Siddiqui, 1996; Jager, R. et al, 2007). The regulation of PA by certain pathways has not yet been completely defined (Hornberger et al, 2006).

PA Derived from PLD

At the cellular level, PA is most commonly generated by the hydrolysis of membrane PC by phospholipase D or (PLD) to PA and Choline (McDermott, M. et al, 2004). PLD activation is stimulated by mitogen and growth factor (X. Bai, & Y. Jiang, 2009).

PA activation is dependent on PLD activity under normal conditions (Hong, Oh, Lee, Kim, Kim, Dong-Uk, Hur, Lee, Kim, Hwang, Park, 2001). There are two isoforms of PLD in mammalian cells, PLD 1 and PLD 2. Abundance of either one has been shown to stimulate mTORC1 activity. Suppression of either one will reduce mitogen-stimulated mTORC1 activity (Hornberger, TA. et al, 2006; Fang, Y. et al, 2006; Ha, Kim, Kim, Kim, Lee, Lee, Kim, Jang, Suh, Ryu, 2006; Lehman, Ledford, Fulvio, Frondorf, Mcphail, Gomez-Cambroner, 2007; Sun, Fang, Yoon, Shang, Roccio, Zwarkruis, Armstrong, Brown, Chen, 2008). Protein kinase C and other small GTPases, including Rac 1, RhoA, Arf, and Cdc42 are responsible for regulation of PLD1 activity in cells (Exton, JH. 2008). PLD1 can also be activated by Rheb (Ras-homology enriched in brain) which binds to it in a GTP-dependent way (Sun, Y. et al, 2008). Inhibition of

Rheb through TSC2 over-expression or a drop in siRNA will decrease activation of mitogen-stimulation PLD1 (Xiaochun, Bai. & Yu, Jiang. 2009). Decreased levels of PLD1 and thus less generation of PA will stop mTORC1 stimulated activation by Rheb which demonstrates that the effect of Rheb on mTORC1 activation requires PLD1-dependent PA production (Sun, Y. et al, 2008; Xiaochun, Bai. & Yu, Jiang. 2009). PLD2 may also activate mTORC1 due to an interaction between raptor and PLD2 (Ha, SH. et al, 2006). PA can also be synthesized by lysophosphtidic acid (LPA) by the LPA acyltranferase and by diacylglycerol (DAG) by the DAG kinase (DGK) (Exton, JH. 2002). PA can also be generated through de novo synthesis (brand new creation).

PA and mTOR

Evidence suggests that resistance training actions (concentric & eccentric muscle contractions) are transduced into growth-promoting pathways inside the cell within the muscles worked, which stimulates the production of PA synthesis by PLD (O'Neil et al, 2009; Hornberger et al, 2006; Rasmussen, B. 2009). Phosphatidic Acid directly binds to a site on mTOR called the FKBP12 rapamycin binding domain (FRB), (Fang, Y. et al, 2001; Xiaochun, Bai. & Yu, Jiang. 2009). It has been established that residue Arg2109 must be present in the FRB domain for binding between PA and mTOR to occur. PA is in competition with rapamycin to bind to the FRB domain site. Interactions between PA and mTOR are reduced if mutations occur at the binding site (Fang, Y. et al, 2001). Thus, mTOR signaling will be repressed (Xiaochun, Bai. & Yu, Jiang. 2009). Activation of mTOR from the binding of PA is not completely understood. There are two mechanisms that have been proposed that may mediate the effect of PA on mTORC1. It is possible that PA may be able to reduce the interaction between

mTOR and FKBP38 (an endogenous inhibitor of mTOR) in vitro and in cells (X. Bai, & Y. Jiang, 2009; Sun, Y. & Chen, J. 2008; Avruch, Long, Ortiz-Vega, Rapley, Papageorgiou, Dai, 2009). Thus, PA might stimulate mTORC1 by mitigating the suppression of FKBP38 (Xiaochun, Bai. & Yu, Jiang. 2009). However, it has been shown that inhibition of PA generation prevented the association of raptor with mTOR (Toschi, A. et al, 2009). This shows that the binding of PA might be necessary for mTORC1 stability (Xiaochun, Bai. & Yu, Jiang).

Due to FKBP38's role in mTORC1 regulation, it is possible that the interaction of raptor with mTOR could be altered upon reduction of PA and increased binding of mTOR with FKBP38 (Xiaochun, Bai. & Yu, Jiang). When PA binds to the protein mTOR via the FRB domain it activates the ribosomal protein kinase S6 or (p70s6K) through a complex network of signaling molecules (Avruch, Belham, Weng, Hara, Yonezawa, 2001). P70s6K is a key ribosome for the translation phase of protein synthesis (Fang, Y. 2003). Protein kinase Serine 6 catalyzes the phosphorylation of the S6 protein which is a component of the eukaryotic ribosomal 40 subunit (Lehman, N. et al, 2007). Importantly, p70s6k has an impact on protein translation and formation of new ribosome (Lehman, N. et al, 2007).
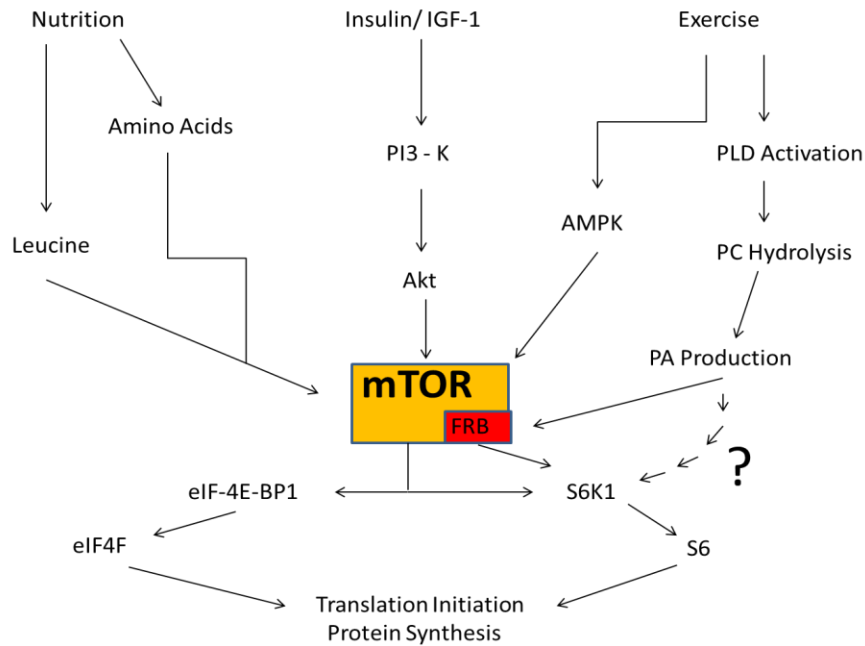
Figure 1: Schematic of mTOR pathways leading to protein synthesis

Studies and Reviews on PA

Jie Chen and colleagues were one of the first to show that exogenously provided PA caused the activation of mTOR substrate S6 Kinase. They observed that extracellular concentrations of 100 μM PA caused the activation of S6K1 and 4E-BP1 phosphorylation human embryonic kidney (HEK) 293 cells that were serum-starved (Fang, Y. et al, 2001). This activation was terminated upon interaction with rapamycin, suggesting the involvement of mTOR. Rapamycin causes rapid dephosphorylation of these downstream effectors (S6K1 and 4E-BP1) through the PI3K and MAPK- regulated pathways (Tee, Fingar, Manning, Kwiatkowski, Cantley, Blenis, 2003). Also, the stimulatory effect of PA was not detected in amino acid deprived cells which indicates that amino acids give a permissive signal to PA that will allow its action.

In addition, HEK293 cells treated with 0.3% of 1-butanol ceased serum-stimulated PA production. The production of PA was also effected by 2-butanol alcohol as well. The addition of 1-butanol almost totally inhibited serum-stimulated S6K1 activation while 2-butanol had a slightly lesser effect. Interestingly, 4E-BP1 serum stimulated phosphorylation was also ceased by 1-butanol while 2-butanol had a marginal impact (Fang, Y. eta l, 2001). Protein kinase Akt and extracellular signal regulated kinases (ERK1, ERK2) serum-stimulated activation was not affected by butanol in a replicated situations, proving the effect of butanol on the production of PA and the involvement of PA in the rapamaycin sensitive pathway (Fang, Y. et al, 2001; Foster, D 2007). They also showed that a concentration as low as 10% of PA was sufficient to bind to the FRB domain site. This finding was significant because it showed a specific affinity for PA as no other phospholipids tested (PC, phosphatidlyserine, phosphatidyhlethanolamine, and phosphatidylinositol) bound to the FRB site. Incubation with the FKBP12-rapamaycin complex completely terminated the binding of PA with FRB. On the other hand, rapamycin-resistant FRB mutant protein (S20351) demonstrated the binding of PA immune to rapamycin (Fang, Y. et al, 2001). Their findings displayed that PA may regulate mTOR through the FRB domain (Foster, D. 2007).

The PI3K and mTOR pathway are essential for the mitogenic activation of 4E-BP1 and S6K1. The activity of PI3K was unaffected by PA which shows that the PI3K pathway is not likely to be affected by PA signaling (Fang, Y. et al, 2001). To ensure this, Jie Chen and colleagues used mutation of S6K1 which was sensitive to wortmannin but resistant to rapamycin.

Through further testing they found that PA signaling to S6K1 only goes through mTOR and not the PI3K pathway. However, it appears that PI3K may still be important for downstream responses to PA (Fang, Y. et al, 2001). Their observations had several important points. One of

which is PA interaction directly with mTOR through a mitogenic pathway upstream of S6K1 and 4E-BP1. In addition, the inhibitory characteristics of rapamycin may stem from the competition between it and PA for binding to the FRB domain site on mTOR which prevents it from activating downstream effectors while still allowing the catalytic activity of mTOR to continue (Fang, Y. et al, 2001). PA also activated mTOR in macrophages in an Akt-dependent way (Lim, H et al, 2003). Their results reported that exogenous PA added to a cell culture medium incorporates rapidly into cellular membranes and thus impacts functions of cells (Lim, H et al, 2003). They also demonstrated that PA could stimulate cytokines synthesis in vivo by providing PA to C57BL/6 mice and measuring serum cytokines by ELISA. The mice were injected intraperitoneally with PA and compared to the animals receiving saline control. A dose of PA (0.5-2mg) to mice was enough to stimulate the detection of TNF-α, IL-1β, and IL-6 serum. This suggests that PA activates systemic pro-inflammatory cytokines in vivo through systemic exposure (Lim, H. et al, 2003). In addition, their findings indicate that PA specifically acts a mediator to stimulate pro-inflammatory cytokines, as well as NO and PGE_2 synthesis in macrophages (Lim, H. et al, 2003). They concluded that PA had a strong impact on Akt activity of Raw264.7 cells indicating that the Akt/PI3K pathway is probably affected by PA signaling and is cell type –specific (Lim, H. et al, 2003).

In 2004, Xu and colleagues also showed that increased levels of S6 Kinase activity occurred due to mTOR activation by phosphonate analogues of PA (Xu, Y et al, 2004). Their reports showed that shorter stimulation periods yielded increased S6K1 activity for the PA analogues, suggesting that PA is required for the early stage of mTOR activation (Xu, Y. et al, 2004). While the entry mechanism of PA into cell and its intracellular concentration are not completely clear, Xu and colleagues provided four important points that indicate PLD1 and PA

are important mediators for mTOR signaling. First, there was a correlation between cellular PA levels and mTOR signaling. Second, PA binds in vitro to a domain called FRB in mTOR. Third, mitogens activate the mTOR target S6K1. Lastly, mitogenic activation requires the presence of PLD1. In addition, other PA metabolites including DAG and LPA do not activate mTOR signaling (Fang, Y. & Chen, J. unpublished observations; Xu, Y. et al, 2004).A study conducted by Hornberger in 2006 supported these studies and also reported some interesting points. They determined that an increase in PA concentration was sufficient enough for the activation of mTOR signaling. PA phosphatase inhibitor propranolol was used to incubate mouse extensor digitorum longus (EDL) muscles. This process induced higher concentrations of PA and p70S6K phosphorylation (Hornberger, T. et al, 2006). Upon incubation with rapamycin, phosphorylation of p70S6K ceased in the control and propranolol treated muscles (Hornberger, T. et al, 2006). Mechanical loading has a direct impact in the regulation of skeletal muscle mass (Hornberger, T. et al, 2006) To test the role of PLD activity and PA accumulation in mechanical stimulation, Hornberger and colleagues used an ex vivo model to mechanically stimulate EDL muscle.

The source of mechanical stimulation used was an intermittent passive stretch which has been demonstrated to activate p70S6K through mTOR-dependent signaling. Their results showed that for 15 minutes after mechanical stimulation was started, an increase in PLD activity was seen and immediately following that an increase in PA was evident for 90 minutes. Interestingly, there was a correlation between mechanical activation of mTOR signaling to p70S6K and the mechanically induced rise in PA concentration (Hornberger, T. et al, 2006). This suggests that resistance training or a source of mechanical stimulation causes PLD to activate and thus the accumulation of PA which leads to mTOR signaling. Further testing with rapamycin and PA showed that enhanced binding of PA to the FRB domain on mTOR results in mechanical

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activation of mTOR signaling. They concluded that inhibition of PLD stopped the accumulation of PA which in turn inhibited mTOR signaling (Hornberger, T. et al, 2006). These studies demonstrated that exogenously supplied PA activated mTOR . However, Nicholas Lehman and colleagues published a study in 2007 with evidence that suggests PA will bind to and activate p70S6K even with the absence of mTOR (Lehman, N. et al, 2007). In testing, p70S6k was placed in wells on a microtiter plate to see if it would bind to immobilized lipids. The other portion of the test was taking a look at p70S6K binding ability to LUVs. The first test showed that PA bound to p70S6K in a saturable manner occurring at about 1nM PA. Other phospholipids (PC, PE, PI) did not bind to p70S6K. The binding of PA to p70S6k was dependent on how much p70S6K was in the wells. The saturation took place at about 40ng protein/well, and wasn't changed y Ca^{2+} absence or presence. These findings indicated that PA bound to p70S6K specifically.

The second portion of the test involving the LUV confirmed that p70S6K binds to PA in a preferred manner. Previous studies have shown that one of the upstream activators of p70S6K is mTOR (Lehman, N. et al, 2007). Lehman and colleagues also examined the possibility that PA could bind to p70S6K without mTOR. To do this they silenced mTOR gene expression with siRNA. This significantly reduced the level of mTOR protein expression. Thus, mTOR was diminished. Their results showed that even in the absence of mTOR, p70S6k activation can still occur through PLD-derived PA binding (Lehman, N. et al, 2007). In 2009, Hornberger and colleagues reported that there is substantial evidence supporting the requirement of PA synthesis from PLD in the activation of mTORC1 from resistance exercise independently of PI3K-PKB signaling(O'Neil, T. et al, 2009). Previous work from this group had been done on passive stretch protocols on mice. This particular study introduced an eccentric exercise model in rodent

mice. They showed that in their ex vivo model PA concentrations in the muscle as well as S6K1 phosphorylation increased after eccentric exercise and could be inhibited from exposure to l-butanol. Finally, they reexamined previous work done on an in vivo model where growth was induced in the tibialis anterior following eccentric exercise but not in the soleus following concentric exercise (Baar, K. & Esser, K. 1999). After further analysis they discovered that mTORC1 was activated in the tibialis anterior which correlated with PA concentrations within that muscle. Inversely, mTORC1 remain unchanged in the soleus. This evidence clearly shows how mTORC1 can be activated from increases in PA completely independent of the PI3K-PKB pathway (O'Neil, T. et al, 2009; Rasmussen, B. 2009). A review by Troy A. Hornberger published in 2011 reaffirmed all of these points (Hornberger, TA. 2011).

CHAPTER THREE: METHODS

Subjects

Fourteen resistance-trained men (mean ± SD; age 22.7 ± 3.3 yrs; height: 1.78 ± 0.10m; weight: 89.3 ± 16.3 kg) volunteered to participate in this randomized, double-blind, placebo-controlled, repeated measures study. The subjects had at least 1 year of recreational resistance training experience in the bench press and squat exercises and had to be training both exercises consistently within the last month prior to beginning the study. Subjects were required to stop all other supplementation during the study. Participants also had to be creatine free for at least one month prior to the start of the study to account for the creatine washout period. All subjects were required to read and complete a medical history report to ensure their safety and eligibility for the study. All participants were informed of the purpose and any possible risks associated with this investigation. They were also required to read and sign an informed consent prior to participation. The proposal and all procedures were approved by the New England Institutional Review Board.

Study Protocol

This study was conducted as a double-blind, randomized design. The investigation lasted for a total of 8 weeks. Subjects reported to the Human Performance Laboratory (HPL) for pre and post testing. They were tested for 1RM max strength in the bench press and squat exercise. Subject's dominant leg vastus lateralis thickness and pennation angle were measured using ultrasound imaging technology.

Their body fat percentage as well as lean tissue mass was measured using dual-energy X-ray absorptiometry. Subjects were required to fill out a dietary recall sheet containing everything they consumed for a three day period (2 normal weekdays & 1 normal weekend day). Their diet was analyzed to ensure proper caloric intake was met. This gave us an indication whether the subjects were taking in enough calories to promote a gain in muscle mass.

Supplement

Subjects were given bottles containing 320-mg capsules containing either PA or PL. The supplement is marketed as Mediator® 50P , ChemiNutra, MN, and contained 50-60% phosphatidic acid, 5-15% phosphatidylcholine, 5-15% phosphatidylethanolamine, 1-5% of lysophosphatidylcholine, and 1-5% of N-acyl phosphatidylethanolamine (NAPE). The placebo contained rice-flour in a similar configuration. Subjects took 5 capsules a day at the same time for 8 weeks and reported to the HPL for refills every three weeks. Subjects were also given 4 bottles a week of a collagen protein blend mixed with Powerade®. This proprietary blend was taken 30 minutes post exercise every week.

Workout

An 8 week 4 day split routine hypertrophy style workout was implemented to obtain optimal results in increasing muscle mass and fat loss. The subjects used 70% of their 1-RM max as their weight for the bench press and squat exercises. The weight for all the assistive exercises was determined by the subject as long as it stayed within the proper repetition ranges (10-12). Subjects were required to use 90 seconds of rest between each set for all exercises.

Max Testing

Subjects were tested for maximal strength [one repetition-maximum (1-RM)] in the bench press and squat exercises at the beginning and end of the study. Subjects were given multiple opportunities to reach their true maximal strength in both exercises. A successful bench press attempt had to be performed by touching the chest with the bar and lifting the weight back to a rack position without a spotter's assistance to complete the repetition. Subjects did have the option of a lift-off upon their request. A successful squat attempt was assessed on proper technique and depth. Adequate depth was determined by the knee and hip joint being parallel to the floor.

Dexa Scan

Total body fat free and lean tissue mass was measured by dual-energy X-ray absorptiometry (DEXA) using a General Electric Lunar Prodigy Series X Scanner in the Wellness Research Center at the University of Central Florida. Dexa scans were conducted on each subject prior to the study and upon completion to assess body composition changes that occurred over the course of the program. Results from the dexa were expressed as total and regional body fat percentage, fat mass (g), and lean tissue mass (g). A trained X-ray technician performed all scans throughout the study.

Ultrasound

Vastus lateralis (VL) muscle architecture (thickness & pennation angle) was assessed in the dominant leg of each subject at rest using B-mode ultrasound imaging with a 12 MHz linear transducer (General Electric LOGIQ P5) in the HPL at the University of Central Florida.

Subjects were positioned on their side with knee slightly flexed. The length of the upper limb was measured from the central lateral side of the patella to the iliac crest. Measures of pennation angle and muscle thickness were taken along this line in the first subject at a location approximately half way up the upper limb. Measurements were taken at 5cm section along this marked line. These areas of measurement were then standardized in accordance with limb length of the following subjects. Each subject was measured prior to their 1RM max squat test at the beginning and end of the study. Pennation angle was determined as the crossing between the deep aponeurosis and the fascicles. The muscle thickness was determined as the distance between deep aponeuroses and superficial tissue of the VL muscle.

Statistical Analysis

Magnitude based inferential analyses were used as an alternative to normal parametric statistics to account for the small sample size (PA = 7; PL = 7). Several studies have supported magnitude based inferences as a complementary statistical tool to null hypothesis testing for reducing interpretation errors (Batterham, AM. & Hopkins, WG. 2005; Hopkins, WG, Batterham, AM, Marshall, SW, and Hanin, J. 2009).

The precision of the magnitude based inference was set at 90% confidence limits, using a p-value derived from an unpaired t-test and the threshold values remained constant at ± 0.2 for the small sample size. Data is represented as mean $\pm$ SD.

CHAPTER FOUR: RESULTS

We used a paired sample t test to assess changes in eight variables from pre to post testing. Arms and legs soft tissue mass (ALSTM) changed significantly in both groups (PL = 0.94 ± 0.86; PA = 1.13 ± 0.67). Changes in pre to post squat strength were significant among both groups (PL = 12.6 ± 6.12; PA = 17.2 ± 6.7). Vastus lateralis (VLpre/post) muscle thickness (cm) increased in both groups from pre to post testing (PL = 0.365 ± 0.329; PA = 0.3 ± 0.251). Muscle quality (MQLEGS) also increased over the course of the study in both groups (PL = 0.384 ± 0.382; PA = 0.56 ± 0.326). There was a significant difference in both groups in skeletal muscle index (SMI) from pre to post (PL = 0.289 ± 0.266; PA = 0.362 ± 0.326). There was no significant difference in bench press (BENCHpre/post) exercise among the PL group (Figure 2) but there was a significant difference in the PA group pre to post (PL = 3.56 ± 7.034; PA = 6.155 ± 6.101). No significant changes were seen in the PL group in lean soft tissue (LST) from pre to post testing (Figure 3) however, there was a significant increase in LST from pre to post testing among the PA group. No significant change occurred in leg lean mass (LEGLM) among the PL group (Figure 4) but, there was a significant increase in LEGLM with in the PA group. Magnitude based inferential analyses to further examine the effects of PA on lean mass (LM), squat strength (SQ), and bench press (BP) over time. Differences between the PL and PA group in BP strength were unclear. Interestingly, PA was shown to be likely beneficial over PL in increasing lean mass and squat strength.
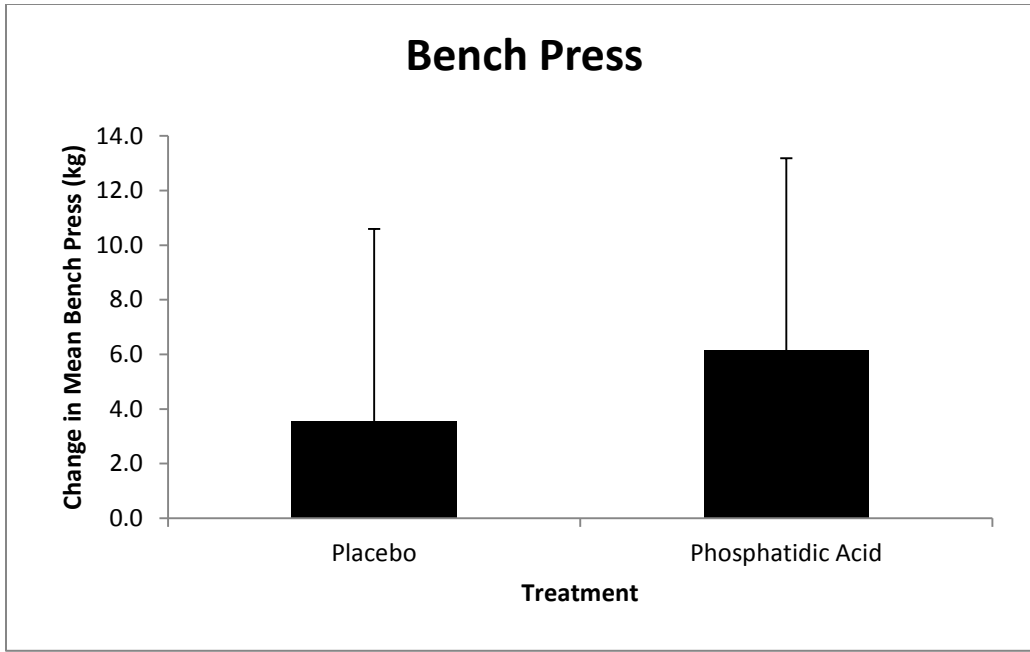
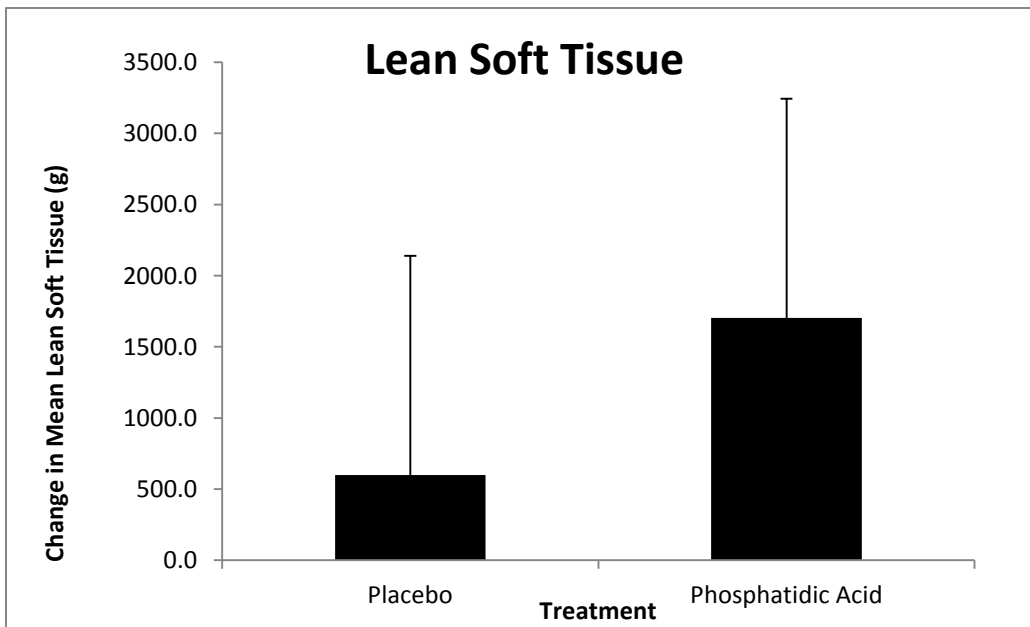Figure 2: Mean changes in bench press strength by treatment group.

Figure 3: Mean changes in total body lean soft tissue by treatment group.
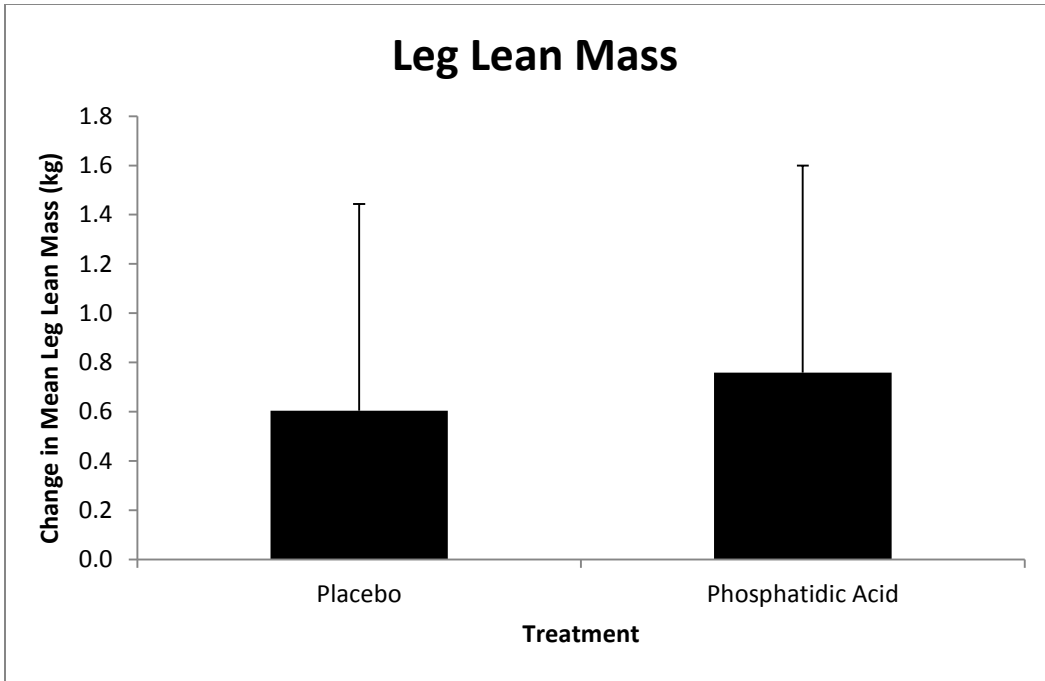
Figure 4: Mean changes in leg lean mass by treatment group.

CHAPTER FIVE: DISCUSSION

The findings of this study suggest that Phosphatidic Acid supplemented every day for 8 weeks 15 minutes prior to a hypertrophy workout may be capable of enhancing strength and lean soft tissue in resistance trained men. This is the first study to examine the effects of exogenously supplied PA combined with a resistance training protocol in humans. While PA did appear to affect overall total body lean soft tissue, the biggest changes were seen in the legs. PA did not significantly affect lean soft tissue in the arms. Interestingly, the PA group had a significant increase in bench press strength compared to the PL group but not in the squat exercise when compared to the PL group (Table 1).

Table 1: Pre and post mean changes between treatment groups.

Number	DELTA Measurements	Placebo			PA		
		Mean	Std. Dev.	Sig.	Mean	Std. Dev.	Sig.
1	ALSTMpre – ALSTMpost (g)	0.944	0.867	0.028	1.130	0.670	0.004
2	SQUATpre – SQUATpost (lbs)	12.600	6.120	0.002	17.20	6.700	0.000
3	VLpre – Vlpost (cm)	0.365	0.329	0.026	0.300	0.251	0.018
4	MQLEGSpre - MQLEGSpost	0.384	0.382	0.037	0.560	0.326	0.004
5	SMIpre - SMIpost	0.289	0.266	0.028	0.362	0.209	0.004
6	**BENCHpre – BENCHpost (lbs)**	3.560	7.034	**NO**	6.155	6.101	0.037
7	**LSTpre –LSTpost (g)**	598.710	1539.790	**NO**	1702.850	1324.35	0.014
8	**LEGLMpre – LEGLMpost (g)**	0.604	0.840	**NO**	0.759	0.306	0.010

The exact reason for this is unknown. It's possible that the subjects in this study all had substantially more training experience in the bench press exercise than the squat exercise. This would explain the effect PA had on bench press strength. If the participants were not as experienced in the squat exercise they would all have improvement in strength which could be

increased effectively with a good training protocol without the need of a supplement such as PA. The training protocol was designed and used based on previous research. High training volume was incorporated because of its correlation with increases in lean tissue (Hendrick, A. 1995; McDonagh, M. & Davies, C. 1984). This is normally accomplished by using a medium to high amount of repetitions (8 to 12), for three to six sets per body part (Ostrowski, K.J., Wolson, G.J., Weatherby, R., Murphy, P.W., Lyttle, A.D. 1997; Tesch, P.A. 1992; Fleck, S.J. & Kraemer, W.J. 1987; Hendrick, A. 1995; Herrick, A.R., & Stone, M.H. 1970). The specific mechanism of action remains unclear. It is possible that PA consumption could be more effective when paired with the use of a resistance training program geared more toward strength (4-6 reps) rather than hypertrophy.

There is no known appropriate dosage of PA needed to illicit noticeable gains in strength or lean soft tissue mass. There are also concerns that exogenously supplied PA is unable to cross the cellular membrane barrier due to the changes it would undergo through digestion (Foster, D. 2007). In addition, the total sample size was very small (PL = 7; PA = 7) which affected the statistical power of the results. It is important for future studies to explore several issues. The sample size must be increased substantially to achieve solid statistical power. The effects of different PA doses on lean tissue and strength gains must be examined. Also, a protocol should be developed to expose whether or not PA can be taken up into the cell to bind to mTOR for protein synthesis activation. The timing of PA is also unclear. It is unknown as to whether there is a specific time to take PA before or after an exercise bout that would enhance the effects. In addition, the study length of 8 weeks may not be long enough. Future studies should look at PA supplementation over a longer time frame (12-15) week period. There could also be responders and non-responders to PA similar to creatine. During this study, subjects were exercising using a

facility of their choice. This limits the ability to control absolute compliance. It must be

controlled in future studies to verify complete adherence to the program.

Conclusion

In conclusion, PA supplementation was determined to be likely beneficial at improving

SQ and LM over PL by 26% and 64%, respectively (Table 2). The strong relationship between

changes in total training volume and LM in the PA group suggest that greater training volume

most likely lead to the greater changes in LM, however, no such relationship was found with PL

group. For the BP data, the PA group resulted in a 42% greater increase in strength over PL,

although the effect was considered unclear.

Table 2: Change in PA strength and body composition measures using magnitude-based inferential statistics.

Change in Measure				
	PA (mean + SD)	PL (mean + SD)	Difference (+90% CL)	Qualitative Inference
LM(Kg)	1.7±1.3	0.6±1.5	1.1; ±1.4	Likely Beneficial
SQ(Kg)	17.2±6.7	12.6±6.1	4.5; ±6.0	Likely Beneficial
BP(Kg)	6.16±6.1	3.6±7.0	2.6; ±6.2	Unclear

Table 3: Eight-week resistance training protocol.

Monday/Thursday		Tuesday/Friday	
Exercise	Sets/Reps (RM)	Exercise	Sets/Reps (RM)
Bench Press*	1,4 x 10 – 12	Squats*	1,4 x 10 – 12
Incline DB Press	3 x 10 - 12	Lunge/Front squat	3 x 10 - 12
Seated Shoulder Press*	1,4 x 10 – 12	Leg Curl	3 x 10 - 12
Upright rows	3 x 10 - 12	Knee Extension	3 x 10 - 12
Lateral raises	3 x 10 - 12	Calf Raises	3 x 10 - 12
Shrugs	3 x 10 - 12	Lat Pulldown	4 x 10 - 12
Triceps pushdown	3 x 10 - 12	Seated Row	4 x 10 - 12
Triceps extension	3 x 10 - 12	EZ Bar Curl	3 x 10 - 12
Situps	3 x 25	Dumbbell Curls	3 x 10 - 12
		Situps	3 x 25

Table 4: Supplement ingredients and amounts of each.

Amino Acid	g AA/100g of product	Amino Acid	g AA/100g of product
Alanine	7.6	Leucine	2.8
Arginine	7.8	Lysine	3.1
Aspartic acid	5.1	Methionine	0.6
Cystine	0.0	Phenylalanine	1.9
Glutamic acid	10.5	Proline	12.2
Glycine	18.2	Serine	2.8
Histidine	1.2	Threonine	1.7
Hydroxylysine	0.5	Tryptophan	0.0
Hydroxylproline	10.8	Tyrosine	0.6
Isoleucine	1.4	Valine	2.0

Practical Application

While more research is needed to elucidate mechanism of action; the current findings suggest that in experienced resistance trained men supplementing 750mg PA per day for 8 weeks may likely benefit greater changes in muscle mass and strength compared with resistance training only.

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