Effect of Fatty Acids and Insulin on Syncytin-1 and 4E-BP1 in Skeletal Muscle

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved November 2017 by the
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ARIZONA STATE UNIVERSITY
December 2017

ABSTRACT

Obesity impairs skeletal muscle maintenance and regeneration, a condition that can progressively lead to muscle loss, but the mechanisms behind it are unknown. Muscle is primarily composed of multinucleated cells called myotubes which are derived by the fusion of mononucleated myocytes. A key mediator in this process is the cellular fusion protein syncytin-1. This led to the hypothesis that syncytin-1 could be decreased in the muscle of obese/insulin resistant individuals. In contrast, it was found that obese/insulin resistant subjects had higher syncytin-1 expression in the muscle compared to that of the lean subjects. Across the subjects, syncytin-1 correlated significantly with body mass index, percent body fat, blood glucose and HbA1c levels, insulin sensitivity and muscle protein fractional synthesis rate. The concentrations of specific plasma fatty acids, such as the saturated fatty acid (palmitate) and monounsaturated fatty acid (oleate) are known to be altered in obese/insulin resistant humans, and also to influence the protein synthesis in muscle. Therefore, it was evaluated that the effects of palmitate and oleate on syncytin-1 expression, as well as 4E-BP1 phosphorylation, a key mechanism regulating muscle protein synthesis in insulin stimulated C2C12 myotubes. The results showed that treatment with 20 nM insulin, 300 µM oleate, 300 µM oleate +20 nM insulin and 300 µM palmitate + 300 µM oleate elevated 4E-BP1 phosphorylation. At the same time, 20 nM insulin, 300 µM palmitate, 300 µM oleate + 20 nM insulin and 300 µM palmitate + 300 µM oleate elevated syncytin-1 expression. Insulin stimulated muscle syncytin-1 expression and 4E-BP1 phosphorylation, and this effect was comparable to that observed in the presence of oleate alone. However, the presence of palmitate + oleate diminished the stimulatory effect

of insulin on muscle syncytin-1 expression and 4E-BP1 phosphorylation. These findings indicate oleate but not palmitate increased total 4E-BP1 phosphorylation regardless of insulin and the presence of palmitate in insulin mediated C2C12 cells. The presence of palmitate inhibited the upregulation of total 4EB-P1 phosphorylation. Palmitate but not oleate increased syncytin-1 expression in insulin mediated C2C12 myotubes. It is possible that chronic hyperinsulinemia in obesity and/or elevated levels of fatty acids such as palmitate in plasma could have contributed to syncytin-1 overexpression and decreased muscle protein fractional synthesis rate in obese/insulin resistant human muscle.

DEDICATION

I dedicate this work to the Gods, who have given me inner peace when I needed them at stressful times. I would especially dedicate this work to Anand R S who has been through my tough and hard times. I also want to dedicate this work to my mom who has always been my emotional support and has never given up on myself even when I have doubted myself at times. I thank my family and friends who have supported and motivated me always. I thank Harini Murali for all the discussions and help. I would like to thank Rajesh Niti for all his help. I would like to thank Vivek for his financial support and being a great friend. I also want to thank Raam, Adi, Ashwin and Harshad along with Abhi and Anu who have always been there for me when I needed them. Thanks to School of Life Sciences administrative staff for all their help and kindness. Finally, I would like to thank Dr. Katsanos who has been very understanding of me and being a great adviser.

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INTRODUCTION:

Detrimental effects of obesity on skeletal muscle

Obesity associated with insulin resistance and chronic inflammation is a major long-term risk factor for metabolic syndrome conditions such as Type-2 Diabetes (T2D) and cardiovascular disease (CVD). Millions of people in the overweight to moderately obese population (based on body mass index – BMI) have normal blood glucose concentrations but remain undiagnosed as pre-diabetic (Zhang et al., 2009). This population ultimately ends up showing the complications of T2D such as atherosclerosis, neuropathy, nephropathy, retinopathy, and foot ulcers, requiring immediate and expensive medical care. Thus, understanding the metabolic features of obesity and translating these findings to therapeutic approaches is of key importance for decreasing the burden of metabolic syndrome.

The prominent features of obesity associated metabolic syndrome is the lack of glucose and lipid homeostasis with subsequent elevation in their levels in blood, leading to further pathological complications in multiple tissues such as cardiomyocytes, endothelial cells (Clark et al., 2003). The main site for maintaining glucose homeostasis in the body is muscle, which is known to be altered in obesity leading to decreased glucose uptake. Obesity associated specific alterations in skeletal muscle metabolism are documented as impaired insulin stimulated protein synthesis (Tardif et al., 2014). Decrease in muscle protein synthesis correlates with increase body fat accumulation with age despite

maintenance of stable body weight (Abdelhafiz & Sinclair, 2016). A progression of this condition leads to decline in muscle mass (known as sarcopenia) in elderly, and is termed as “sarcopenic obesity” (Roubenoff, 2000). Since sarcopenia impacts muscle movement function and associated energy expenditure, it leads to a sedentary lifestyle leading to further body fat accumulation thus forming a vicious cycle of obesity and sarcopenia which reinforce each other (Roubenoff, 2000).

The exact reason behind the decrease in muscle protein synthesis in obesity are unknown. However what is known that when resistance training is performed during a period of energy restriction in obese older individuals, muscle protein synthesis was conserved (Smith & Villareal, 2012). Since resistance training is associated with increased myogenesis (Frese et al., 2015), it will be an ideal strategy to overcome “sarcopenic obesity”. However, what are the mechanisms that impair myogenesis in obesity remains unknown. In this perspective understanding the correlation and regulation of myogenesis mediators in obesity could provide novel lifestyle or therapeutic strategies towards addressing sarcopenic obesity and insulin sensitivity.

Muscle overview and myogenesis

Muscle is one of the soft tissues found in animals. Cytoskeletal filaments such as actin and myosin make up a predominant portion of muscle cells which regulate contraction by changing the length as well as the shapes of the cell by sliding past one another. Muscle exhibit characteristics such as excitability (responding to neural stimuli), contractibility

(able to shorten in length), extensibility (able to increase in length) and elasticity (able to return to its original shape and size after contraction/extension). It also performs key functions in the body such as facilitating motion, maintenance of posture, thermogenesis and glycogen storage (Jensen, Rustad, Kolnes, & Lai, 2011). To carry out these wide-ranging tasks, muscles have developed in to two broad categories: voluntary and involuntary muscles. Voluntary muscles include skeletal muscles which are attached to bones and help in the movement of the skeletal structure based on specific voluntary neural inputs. Involuntary muscles such as smooth muscles in the walls of blood vessels, intestine and cardiac muscles carry out their function independently of voluntary inputs.

Muscle cells are developed from their precursor cells, called myocytes, which differentiate into long, tubular cells called myotubes by a process known as myogenesis. Alignment of multiple myotubes gives rise to a muscle fiber that comprises the muscle tissue. During the process of myogenesis, the myocytes fuse together to form myotubes in voluntary skeletal muscles, but not in involuntary muscles. Undifferentiated myocytes remain within the muscle fiber as myosatellite cells and undergo myogenesis in the presence of specific stimuli such as exercise or muscle injury (Wang & Rudnicki, 2011).

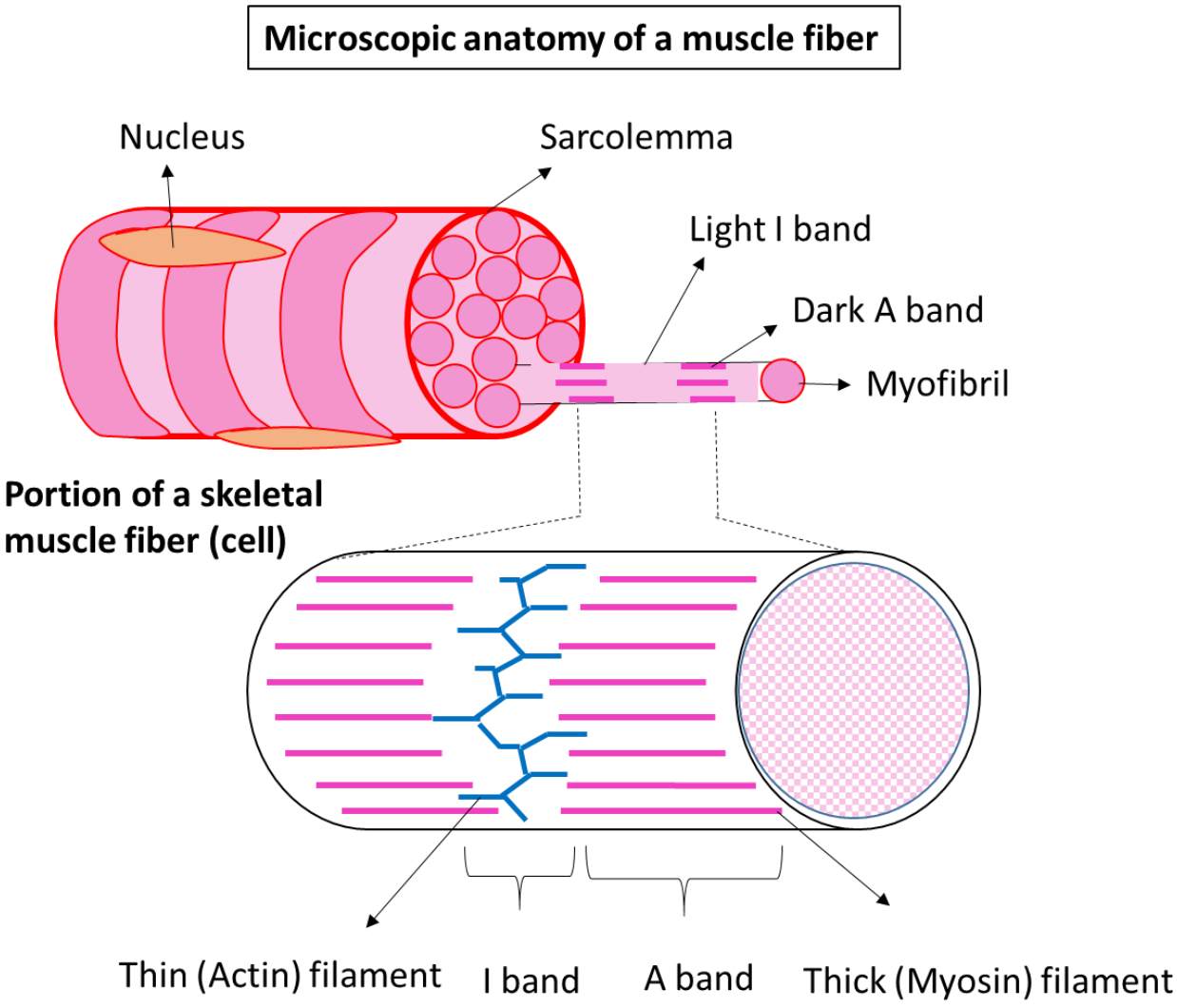
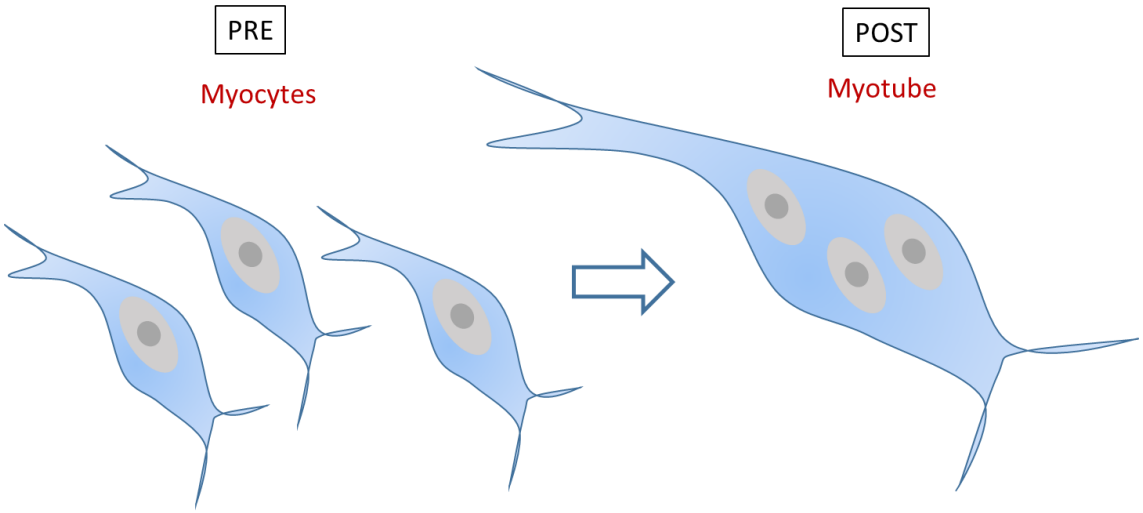


Figure 1. A schematic depiction of the anatomy of a muscle fiber. Muscle cells are multinucleated and are made up of thick myosin and thin actin filaments that are arranged in a specific pattern.

Syncytin and sarcopenic obesity

Skeletal muscles constitutes a predominant site of glucose utilization next to brain (DeFronzo & Tripathy, 2009). Considering their capacity for high uptake of glucose, skeletal muscle inevitably plays a key role in glucose homeostasis of the body, and depending on their active participation in glucose uptake which is mediated by insulin signaling in skeletal muscles. In addition, the process of myogenesis is also mediated by insulin signaling (Al-Khalili, Krämer, Wretenberg, & Krook, 2004). Cell fusion of myoblasts is a critical step during skeletal muscle differentiation (myogenesis), maintenance and repair (Yablonka-Reuveni, 2011). The cell fusion event during skeletal muscle myogenesis requires the presence of fusogenic agent that brings the cell membranes of the two fusing cells in contact. A protein known as syncytin-1 performs this function and is found in three isoforms syncytin-1, 2 and 3 (Bjerregard, Ziomkiewicz, Schulz, & Larsson, 2014). This protein is derived from the human endogenous retroviral (HERV)-derived gene (Mi et al., 2000). It was originally a virus encoded protein that helped in the fusion of the viral membrane with host cell membrane which was incorporated into human genome to end up serving the fusion events between the host cell with the loss of viral genetic components (Sapir et al., 2013). Syncytin-1 and 3 alone play a role in cell fusion within which syncytin-1 has a dominant role. This was evident when treatment of myoblasts with anti-syncytin-1 resulted in loss of cell fusion (Frese et al., 2015). In addition, the major sites of syncytin-1 expression are placental syncytiotrophoblasts, multinucleate cells from fetal trophoblasts (Mi et al., 2000), differentiating osteoclasts (Søe et al., 2011), and cancer cells (Maliniemi et al., 2013). Regarding syncytiotrophoblasts, the

fusion of cells lining the placenta prevents a direct exposure of the foetus inside the placenta from the immune system of the mother (Mi et al., 2000). Though a large body of literature is available on the levels and regulation of myogenic mediators such as myosin (Egan & Zierath, 2013), myogenin (Fu et al., 2016), desmin, alpha-actinin-2 (Houweling et al., 2017) in obesity, similar data on syncytin-1 and its regulation in skeletal muscle of obese/insulin resistant people is lacking. Moreover, syncytin-1 may be of relevance to understanding muscle protein synthesis via two possible modes 1) its receptor Neutral amino acid transporter B(0) (ASCT2) which is a key essential amino acid transporter in the cells (Sugimoto, Sugimoto, Bernstein, Jinno, & Schust, 2013) and 2) Increased syncytin-1 expression has been shown to mediate endoplasmic reticulum (ER) stress response under multiple sclerosis (Antony et al., 2007). Since amino acid starvation is a direct signal for ER stress response via elongation initiation factor – 2 alpha (eIF-2α) to decrease protein synthesis (Krishnamoorthy & Pavitt, 2001), exploring syncytin-1 expression and correlating with protein synthesis in lean and obese/insulin resistant subjects could provide insights into the downstream effects of syncytin-1. Considering its prime importance in muscle cell fusion under exercise activities such as cycling (Frese et al., 2015) and loss of myotubes by treatment of fatty acids in vitro (Yang et al., 2013), understanding the exact correlation of syncytin-1 with obesity and its regulation by insulin and fatty acids could provide novel insights into the development and treatment of sarcopenic obesity.



Treatment	POST compared to PRE
In vivo (Exercise)	• Multinuclei/mm fiber (cell fusion) ↑ • Syncytin-1↑
In vitro (Myocyte differentiation)	• Cell proliferation ↓ • Multinucleated cells (cell fusion) ↑ • Syncytin-1 ↑

Figure 2. Synctin-1's expression is essential for the fusion of myotubes from myoblasts. Exercise induced increase in syncytin-1's expression in muscle which increased myotube formation in healthy humans. Differentiation of the myoblasts invitro also showed that syncytin-1 increased in expression during myotube formation. Adapted with permission from previous report (Frese et al., 2015).

Insulin regulation of muscle protein synthesis

Insulin stimulates protein synthesis in multiple cell types including skeletal muscle. Insulin mediated-stimulation involves two types of effects: rapid and long term. The rapid effect is mediated by eIFs (eukaryotic initiation factors) and eEFs (eukaryotic elongation

factors) which are activated by the insulin. The long term effects involve an increase in ribosomal contents by the insulin (Proud, 2006). Protein synthesis activation by insulin is primarily through phosphoinositide 3-kinase - Akt axis. Downstream of this axis, regulation by protein synthesis happens in two phases. In one case, PKB phosphorylates and inhibits glycogen synthase kinase 3 which in turn is a mediator of eIF2B dependent inhibition on global protein synthesis. In the second case, PKB activates mTOR (mammalian target of rapamycin) by phosphorylating and relieving the inhibitory effect of TSC1/2 (tuberous sclerosis complex 1/2) on mTOR complex 1 (mTORC1). Translation initiation and elongation is controlled by mTORC1 via 4E-BP1 phosphorylation on threonine 37/46 position to activate protein synthesis (Proud, 2006).

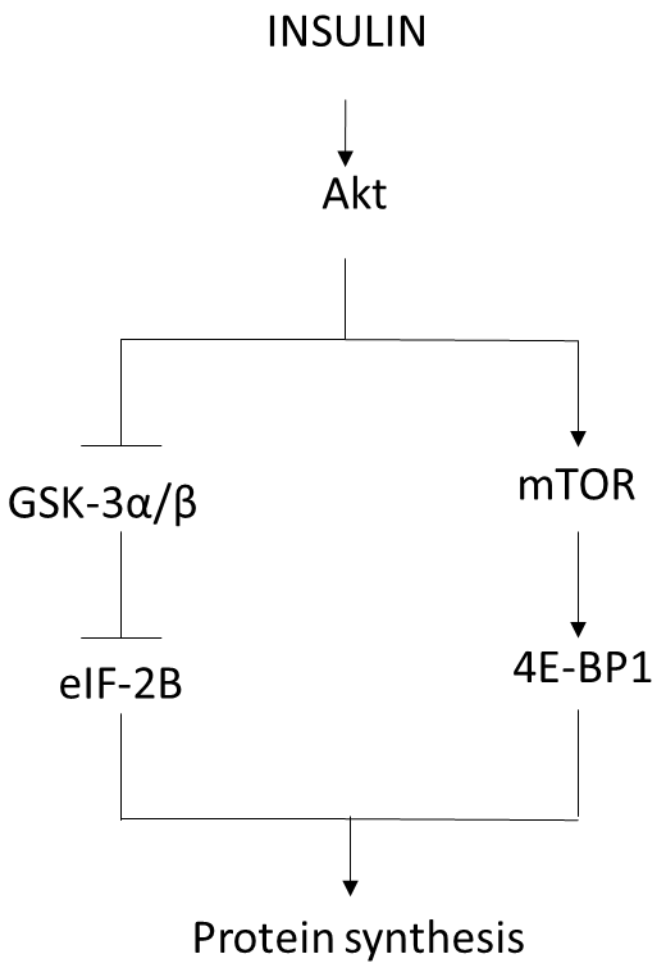


Figure 3. Overview of insulin regulation of protein synthesis.

Role of fatty acids in muscle protein synthesis

Fatty acids have been shown to influence insulin sensitivity and protein synthesis in a manner depending on their saturation and chain length (Kalhan, 2009). The fatty-acid composition of membranes is a cellular factor that has been shown to influence the action of insulin in cells (Weijers, 2012). Studies of insulin sensitivity indicate that increasing the content of polyunsaturated fatty acids within cell membranes in cultured cells increases

membrane fluidity (Brand et al., 2010), the number of insulin receptors (Le Marchand-Brustel et al., 2003), and the action of insulin (Manco, Calvani, & Mingrone, 2004). When the saturated fatty acids concentration in the membrane increase there is a converse effect (Grunfeld et al., 1981). Mechanistically, long-chain polyunsaturated fatty acids within membrane phospholipids have been shown to influence insulin receptor signaling and glucose transporter translocation, through effects on the physical properties of their surrounding lipid environment (Weijers, 2012).

The fatty acid composition in the body fat stored in adipocytes in the form of triglycerides in lipid drops consists of (C16) palmitate synthesized via fatty acid synthase complex in the cytosol and its extension to (C18) stearate mediated by the elongase isoform - ELOVL6 in the endoplasmic reticulum followed by its desaturation to (C18:1) oleate (Shi et al., 2017). Among palmitate, stearate and oleate, oleate constitutes the major fraction of triglyceride composition in lipid drops of adipocytes followed by palmitate (Listenberger et al., 2003). Though an effect of chain elongation and desaturation in membrane fluidity and resulting changes in cellular signaling is known, knowledge of the functional significance of these different fatty acids in circulation on syncytin-1 mediated myogenesis is lacking. This is critically applicable in cases where there is increased circulation of free fatty acids released from adipocyte lipolysis in obesity (Duncan, Ahmadian, Jaworski, Sarkadi-Nagy, & Sul, 2007). A previous study from our lab showed that circulating non-esterified fatty acids (NEFA) extracted from the serum of obese resulted in decreased translation efficiency of muscle β1-F1-ATPase compared to NEFA from lean subject

suggesting the key role played by circulating fatty acid on protein synthesis (Tran et al., 2016). Thus, understanding the functional significance of palmitate and oleate on syncytin-1 levels and linking with insulin mediated protein synthesis mediators in muscle cells could provide novel insights into their regulation in sarcopenic obesity.

Objectives of the work

Human study:

Skeletal muscle cells are specifically evolved cells to enact physical movement in animals and they do so by utilizing glucose as a primary fuel source. To enact their function, the muscles need to increase their protein content of cytoskeletal proteins such as myosin, myogenin and a fusion of myocytes to myotubes mediated by syncytin-1. Thus, a combined effect of muscle protein synthesis and muscle fusion by syncytin-1 is required for the homeostatic function of muscle. Since defective muscle function is known to be associated with sarcopenic obesity, it can be hypothesized that either muscle protein synthesis or syncytin-1 alone or both are down regulated. Current knowledge on this aspect of skeletal muscle in obesity is lacking. Hence the first objective of the study was designed as follows:

Objective 1: To determine the expression of fusogenic protein syncytin-1 and the rate of protein synthesis in skeletal muscle in lean and obese/insulin resistant human subjects.

Hypothesis 1: It was hypothesized that expression of fusogenic protein synctyin-1 will be decreased in obese/insulin resistant human subjects. There will be a positive correlation between syncytin-1 expression and the rate of protein synthesis in obese/insulin resistant human subjects.

Cell culture study:

Previous evidence has shown the influence of fatty acids such as palmitate and oleate on myogenesis and insulin signaling cascade mediated muscle protein synthesis in a C2C12 myotube model of skeletal muscle in *vitro* (Kwon & Querfurth, 2015). However, evidence on the effects of fatty acids on syncytin-1 expression or the regulation of protein synthesis under physiological concentrations of insulin and/or fatty acids is lacking. Considering that alterations in myogenesis mediated by syncytin-1 may have a crucial role in defective insulin mediated muscle protein synthesis in obesity, we hypothesized that fatty acids such as palmitate and oleate will have a regulatory effect of syncytin-1 expression.

Objective 2: To determine the effect of insulin and fatty acids on insulin-stimulated syncytin-1 and 4E-BP1 expression in C2C12 myotubes.

Hypothesis 2: It was hypothesized that insulin will increase syncytin-1 expression and 4E-BP1 phosphorylation, and that concurrent treatment with fatty acids will inhibit this effect.

CHAPTER 2

RESULTS AIM 1: CORRELATION BETWEEN THE EXPRESSION OF SYNCYTIN-1 AND MUSCLE PROTEIN SYNTHESIS IN OBESITY IN HUMAN SUBJECTS

Considering the critical function of syncytin-1 in myogenesis and the loss of muscle function in obesity, we evaluated the levels of syncytin-1 in lean and obese/insulin resistant human subjects using western blot analysis and correlated with the overall muscle protein synthesis rate. The human subjects were classified as lean and obese based on their Body Mass Index (BMI) and body fat percentage. Glucose and glycated hemoglobin (Hb-A1c) measurements were done in the blood and insulin levels were measured in the serum samples of the subjects and, the MATSUDA index (DeFronzo & Matsuda, 2010) was calculated. In addition, fractional synthesis rate (FSR) measurements were done to determine the protein synthesis *in vivo* using labelled leucine tracer (Tran, Masters, Roust, & Katsanos, 2015). The characteristics of the human subjects are listed in Table 1, and blood/-serum biochemical parameters and muscle protein synthesis are listed in Table 2.

SUBJECT CHARACTERISTICS:

Parameters	**Lean**	**Obese**
Total (n)	6	8
Male/Female	2/4	5/3
Age (years)	30 ± 8	34 ± 9

Table 1. Characteristics of lean and obese subjects included for the study.

BLOOD AND SERUM PARAMETERS:

Parameters	**Lean**	**Obese**	**p-value**
BMI (Kg/m^2)	22.5 ± 2.9	33.6 ± 2.3	<0.001
BF (%)	24.7 ± 7.3	33.3 ± 5.5	<0.05
Total Mass (Kg)	66.9 ± 15.4	98.6 ± 12.2	<0.001
Lean Mass (Kg)	50.5 ± 5.1	67.0 ± 3.0	<0.05
Triglyceride (mg/dL)	70.8 ± 11.0	191.6 ± 42.1	<0.05
Total NEFA (mmol/L)	0.39 ± 0.1	0.41 ± 0.1	>0.05
Total Palmitate (μmol/L)	129.6 ± 17.3	120.0 ± 14.1	>0.05
Total Oleate (μmol/L)	184.9 ± 29.8	161.2 ± 29.4	>0.05
Glucose (mg/dL)	85.6 ± 3.0	101.8 ± 14.9	<0.05
HbA1c (%)	5.3 ± 0.2	5.7 ± 0.4	<0.05
Insulin (uIU/mL)	3.9 ± 1.2	10.8 ± 5.2	<0.01
Matsuda (ISI)	8.8 ± 2.5	3.5 ± 1.6	<0.001

Table 2. Blood and serum biochemical parameters of the lean and obese human subjects.

CORRLATIONS TABLE

Parameters	**Pearson correlation coefficient (r)**	**p-Value**
BMI (Kg/m^2)	0.65	<0.01
BF (%)	0.57	<0.05
Total Mass (Kg)	0.47	>0.05
Lean Mass (Kg)	0.23	>0.05
Triglyceride (mg/dL)	0.36	>0.05
Total NEFA (mmol/L)	0.09	>0.05
Total Palmitate (μmol/L)	0.10	>0.05
Total Oleate (μmol/L)	0.04	>0.05
Glucose (mg/dL)	0.55	<0.05
HbA1c (%)	0.61	<0.05
Insulin (uIU /mL)	0.37	>0.05
Matsuda (ISI)	- 0.54	<0.05

Table 3. Correlation between syncytin-1 expression and blood and plasma biochemical parameters.

Western blot analysis showing significant increase in syncytin-1 levels in the obese/insulin resistant subjects (Figure 4).

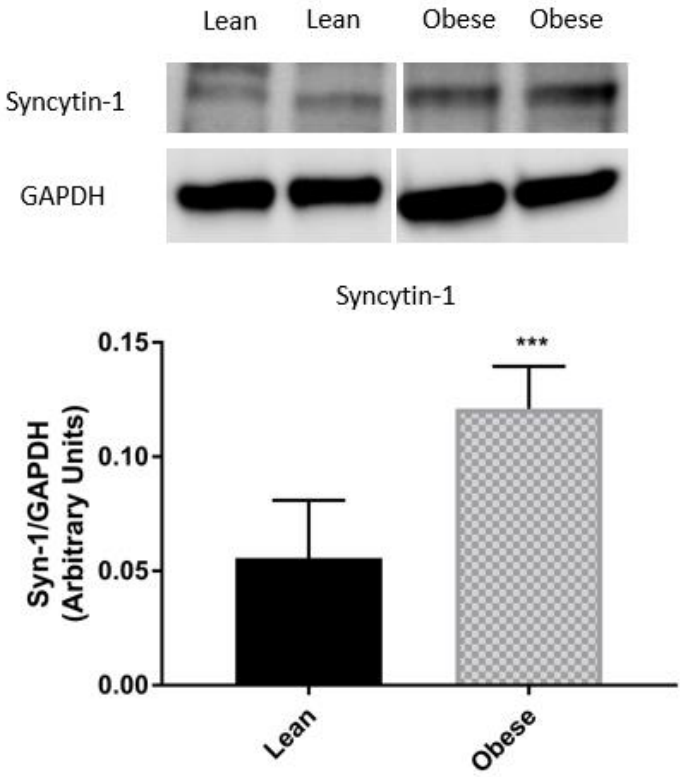


Figure 4. Western blot analysis of syncytin-1 in skeletal muscle of lean and obese/insulin resistant subjects. A) Western blot image of syncytin-1 and housekeeping gene GAPDH, which was used as loading control. B) Average densitometry values of syncytin-1/GAPDH determined in skeletal muscle of the lean and obese/insulin resistant subjects represented in a bar graph.

There was inverse correlation between syncytin-1 levels and muscle protein synthesis of skeletal muscle in the human subjects (Figure 5). In addition, a significant correlation was observed for syncytin-1 expression with BMI, percent body fat, blood glucose concentration, HbA1c, and MATSUDA insulin sensitivity index (Table 2).

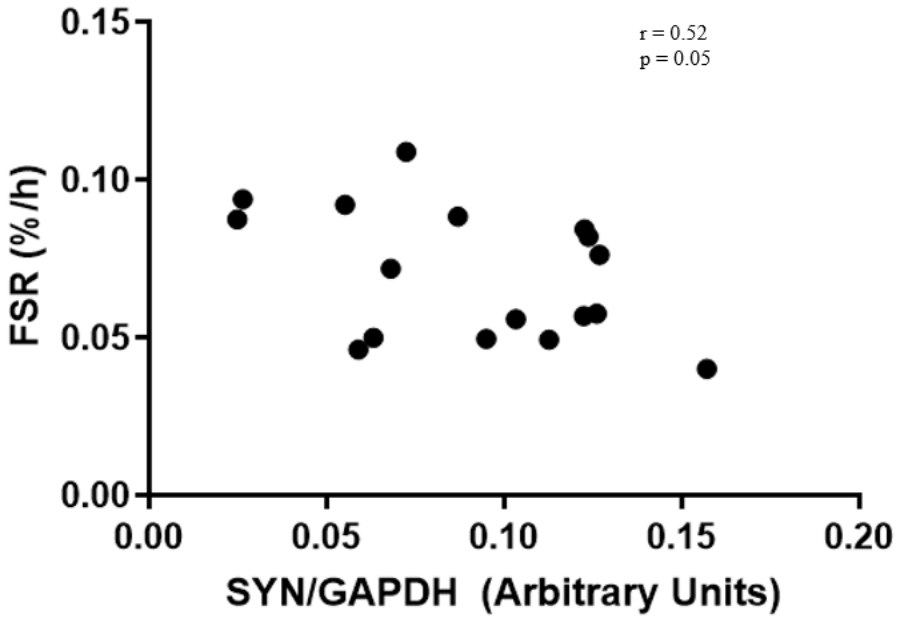


Figure 5. The graph shows significant correlation between syncytin-1's expression and fractional synthesis rate (FSR) of muscle proteins across lean and obese/insulin resistant subjects.

The findings from the human studies showed an increase in syncytin-1 level with decrease in muscle protein synthesis in obese/insulin resistant humans. Since insulin and fatty acids have been known to influence muscle protein synthesis, we sought to understand their effects on syncytin-1 using an *in vitro* model (following chapter).

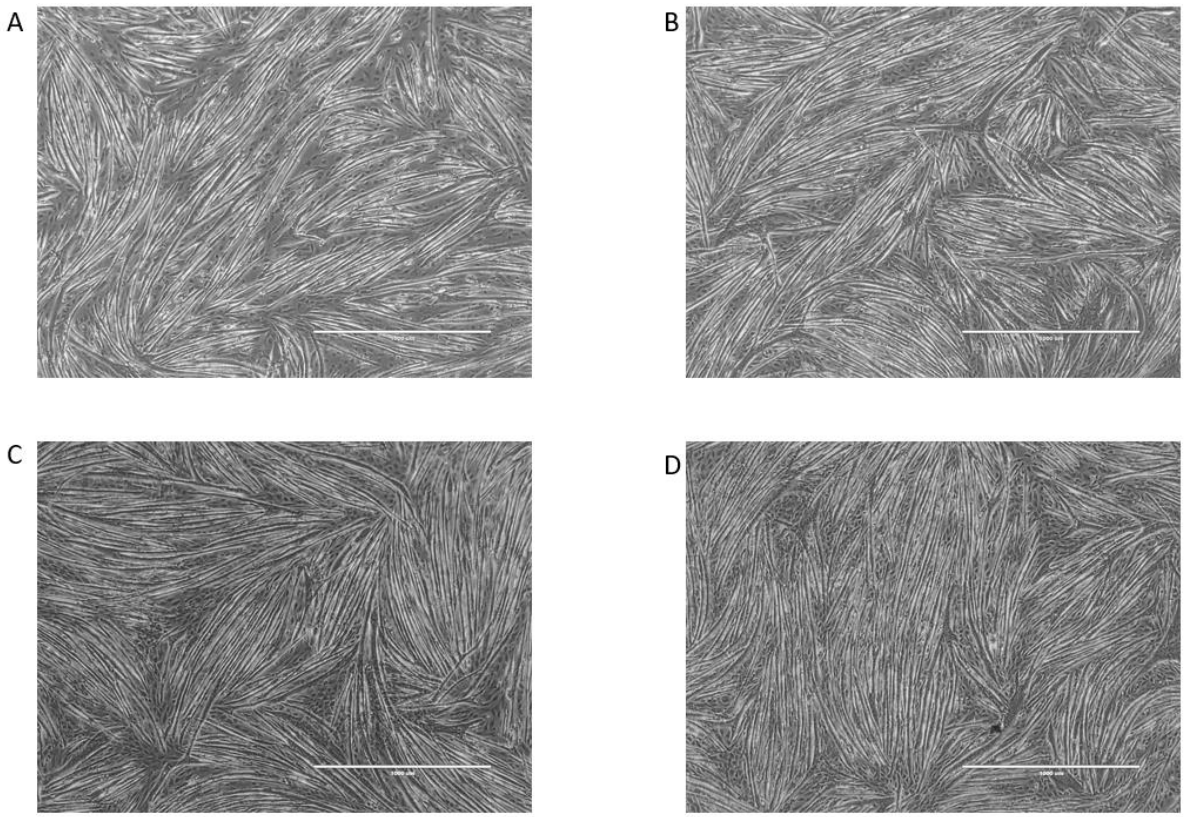
CHAPTER 3

RESULTS AIM 2: EFFECT OF FATTY ACIDS IN THE PRESENCE AND ABSENCE OF INSULIN ON THE REGULATION OF SYNCYTIN-1 EXPRESSION AND 4E-BP1 PHOSPHORYLATION AND EXPRESSION

To understand the link between increased syncytin-1 expression and obesity in muscles, we undertook an *in vitro* study using C2C12 mouse muscle cell line. The cells were cultured as myoblasts and differentiated into myotubes in the presence of 2% horse serum (Figure 6). The differentiation of myoblasts to myotubes has been shown to express syncytin-1 protein which mediate the fusion of cells (Frese et al., 2015). Since the progression of obesity has its greatest impact on differentiated muscles which in turn leads to sarcopenia (Dorrens & Rennie, 2003), we investigated the treatment of differentiated C2C12 cells with key fatty acids palmitate and oleate in the presence and absence of insulin (20 nM). We evaluated the levels of protein synthesis marker 4E-BP1 phosphorylation and myotube fusion marker syncytin-1 expression *in vitro*. The immune-blot densitometry levels were normalized to myosin heavy chain (MYH) expression as loading control as GAPDH showed a variation between treatments (Figure 7). In the absence of insulin, 300 µM oleate treatment and a combination of 300 µM oleate + 300 µM palmitate treatment produced an increase in total 4E-BP1 phosphorylation compared to control (Figure 9). In the presence of 20 nM insulin by itself produced a significant increase in total 4E-BP1 phosphorylation which was maintained in the presence of 300 µM oleate (Figure 9). The increase in total protein 4E-BP1 levels was observed only in the case of 20 nM insulin, 300 µM palmitate + 20 nM insulin and 300 µM oleate + 300 µM palmitate treatments (Figure

10). However, there was no significant differences in the ratio of phosphorylated 4E-BP1 to total 4E-BP1 (Figure 11).

Analysis of syncytin-1 expression in differentiated C2C12 myotubes in the absence of insulin showed a stimulatory effect of 300 µM palmitate and 300 µM oleate + 300 µM palmitate on syncytin-1 expression (Figure 8). 20 nM insulin treatment alone also produced a significant up regulation of syncytin-1 expression which was maintained in the presence of 300 µM oleate (Figure 8).



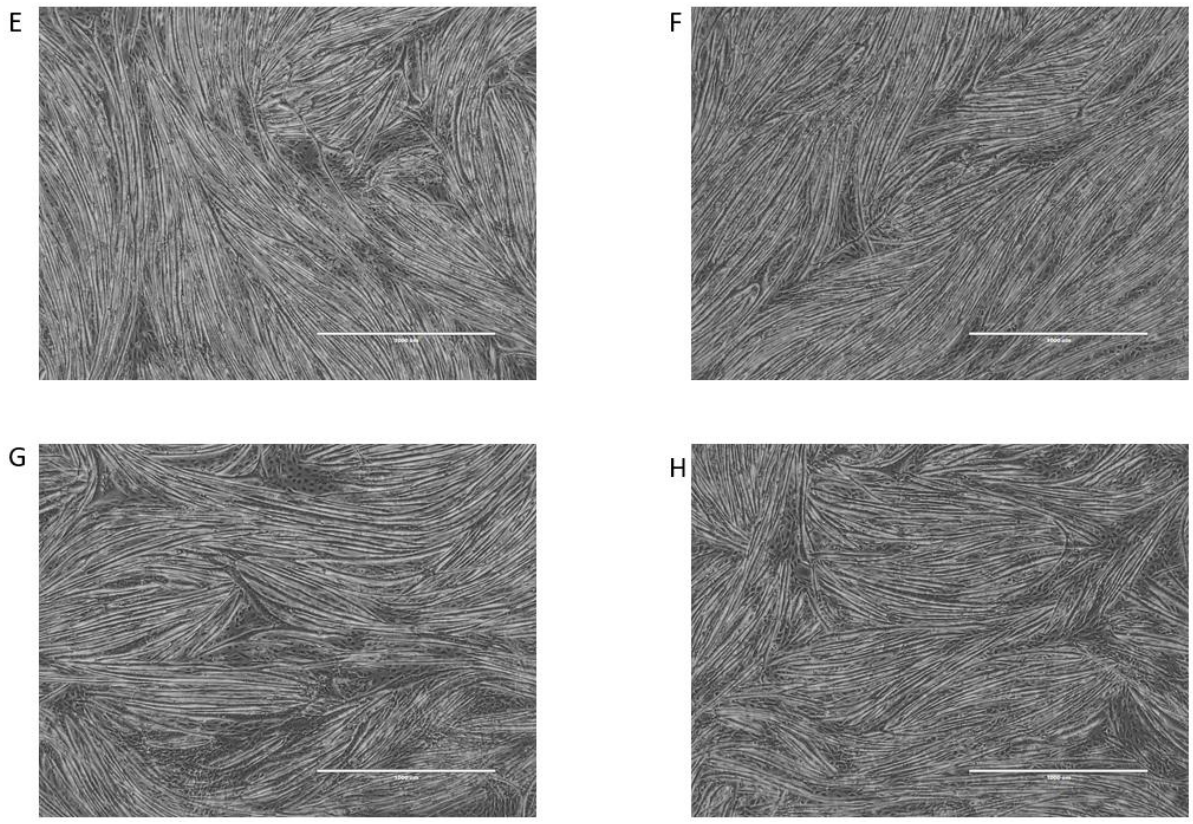


Figure 6. C2C12 cells images taken at 40X magnification, after treatment of cells with A) No insulin B) 20 nM Insulin C) 300 µM Palmitate D) 300 µM Palmitate + 20 nM Insulin E) 300 µM Oleate F) 300 µM Oleate + 20 nM Insulin G) 300 µM Oleate + 300 µM Palmitate H) 300 µM Oleate + 300 µM Palmitate + 20 nM Insulin for 24 hours. The bar in the figure indicates 1 mm.

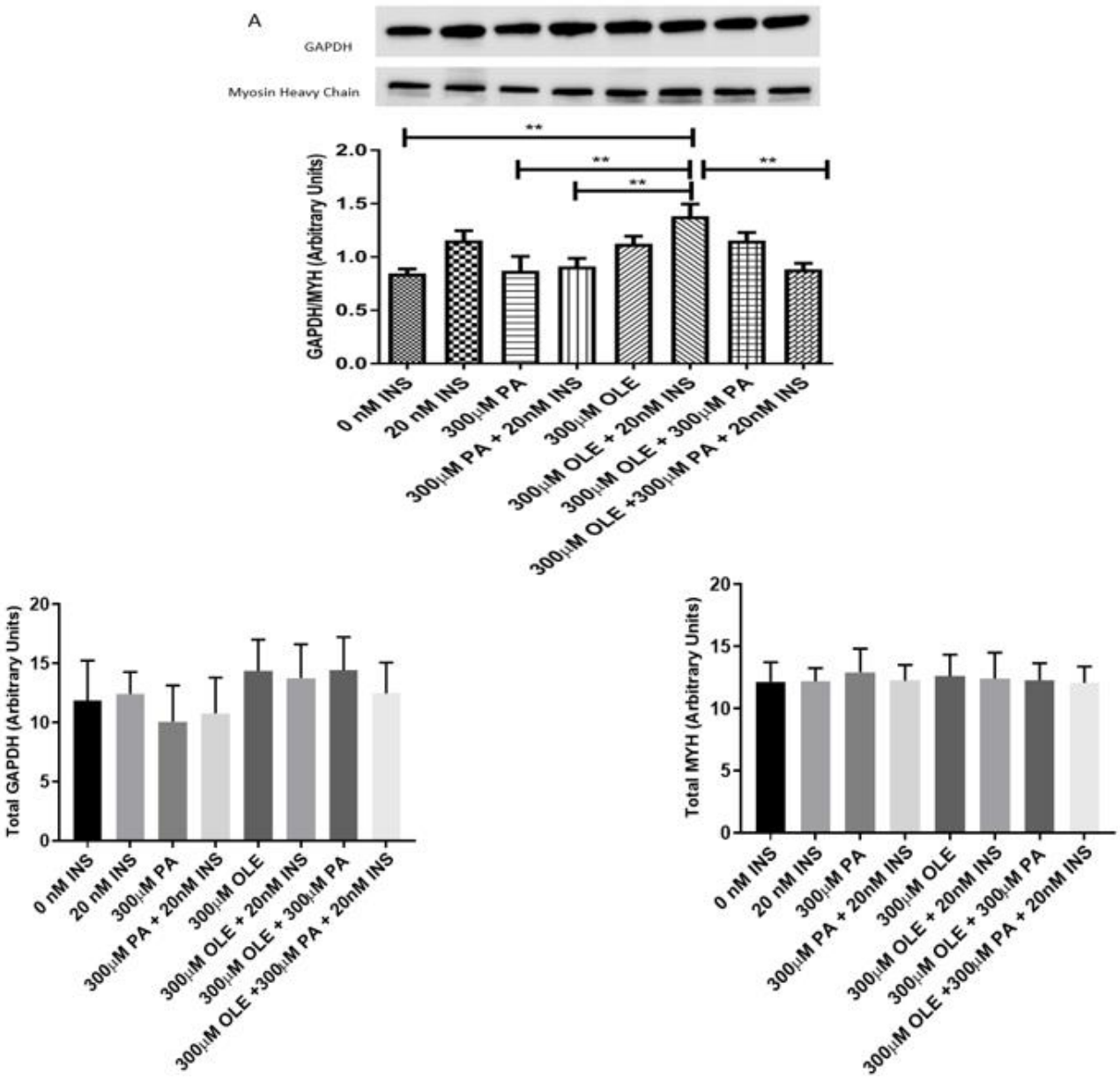


Figure 7. Western blot bands and densitometry analysis of GAPDH/Myosin Heavy chain showing the variation of GAPDH expression with the treatment of fatty acids and insulin on C2C12 cells.

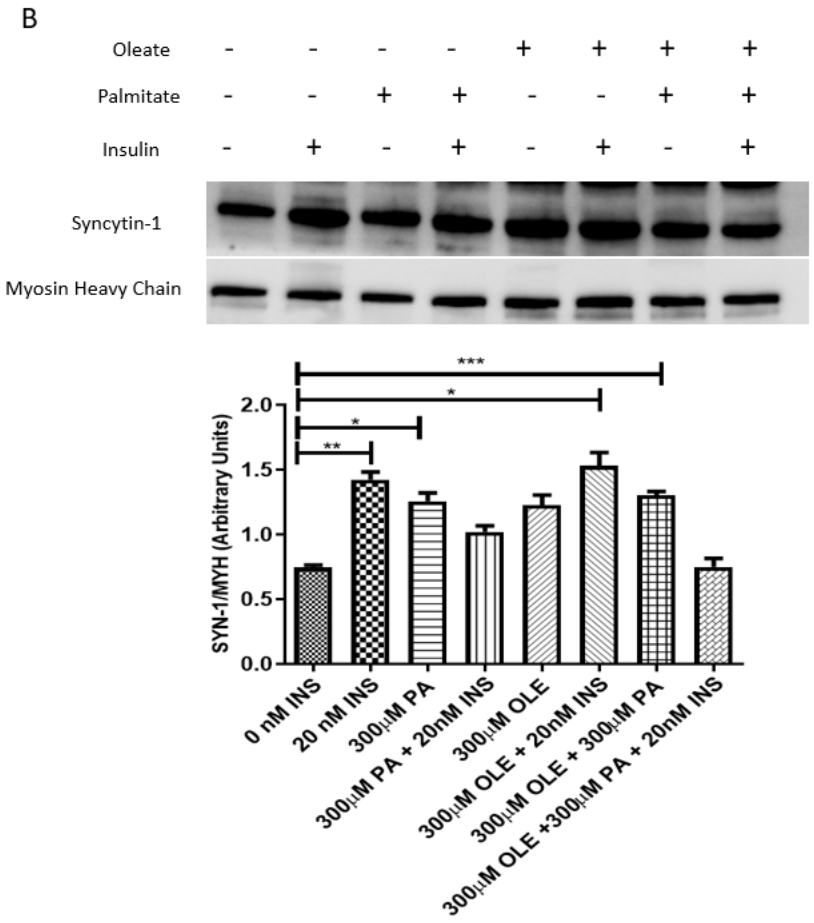


Figure 8. Western blot bands images and densitometry analysis of syncytin-1/MYH. Syncytin-1 expression in differentiated C2C12 myotubes showed a stimulatory effect of insulin, palmitate and in the combined treatment of oleate + insulin and palmitate + oleate.

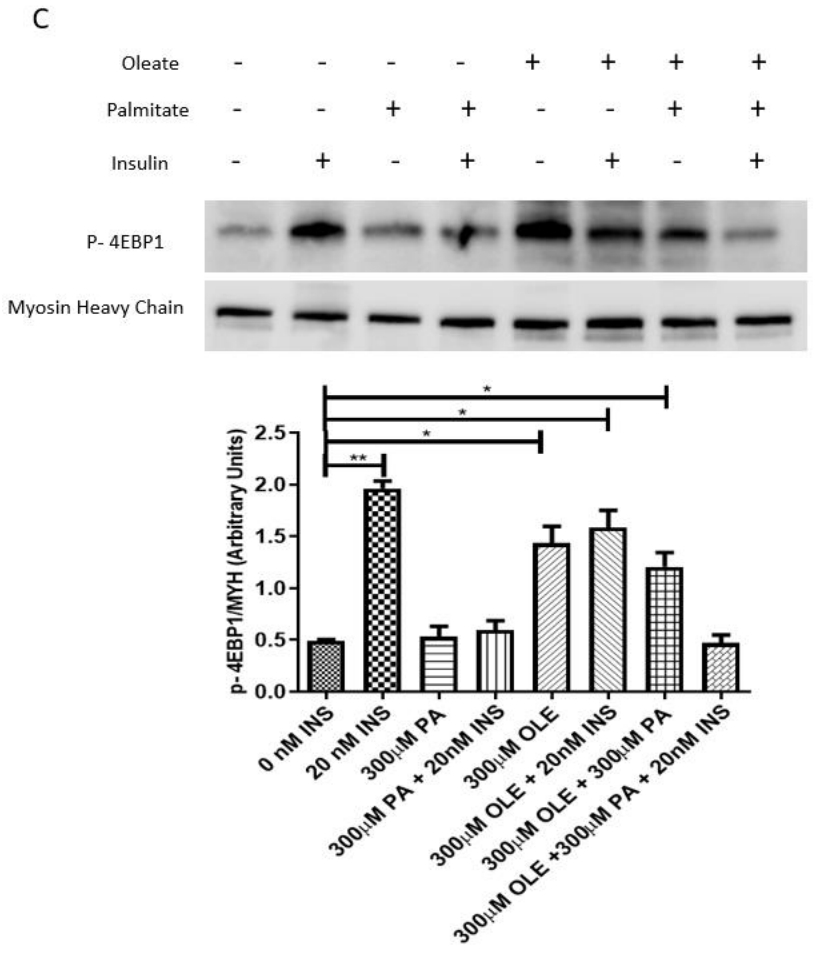


Figure 9. Western blot bands and densitometry analysis of total 4EBP1 phosphorylation/MYH showing insulin has a stimulatory effect on total 4E-BP1 phosphorylation. The presence of oleate alone or along with insulin/palmitate also produced a significant increase in total 4E-BP1 phosphorylation compared to control.

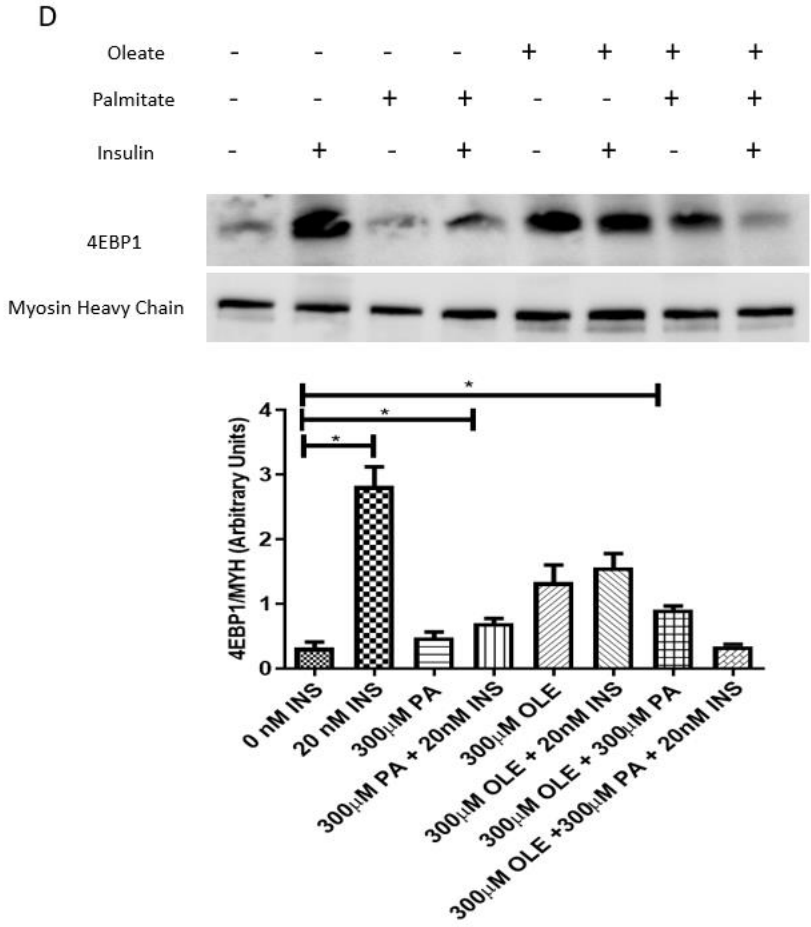


Figure 10. Western blot bands and densitometry analysis showing the levels of total protein 4E-BP1 were altered between the treatments in the same manner as total 4E-BP1 phosphorylation.

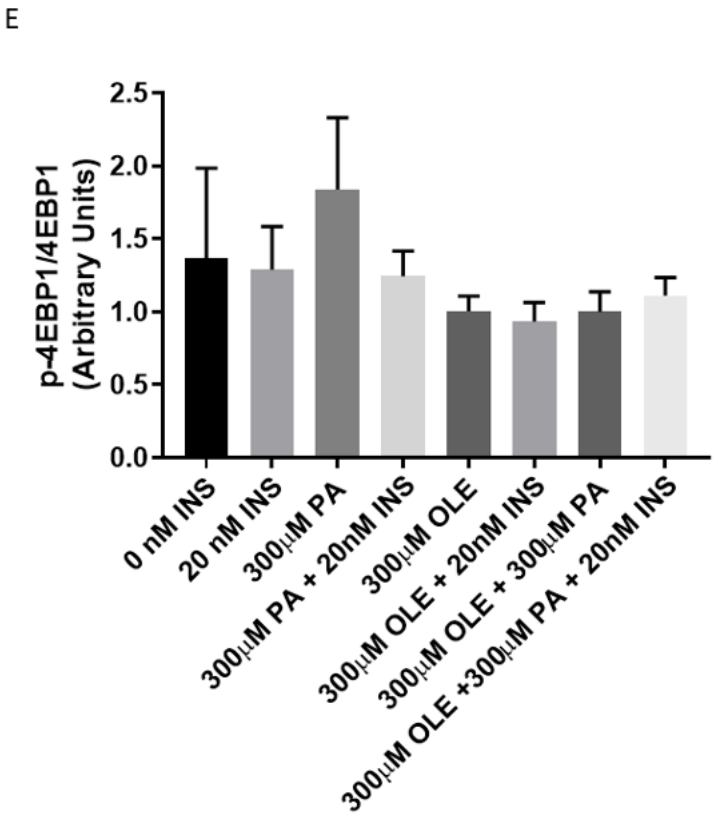


Figure 11. Western blot bands and densitometry analysis showing the levels of 4E-BP1 phosphorylation levels to total 4E-BP1. There were no significant differences between groups.

CHAPTER 4

DISCUSSION

Human study:

The study aimed to determine the effects of obesity on the regulation of the fusogenic protein syncytin-1 expression. In our studies with lean and obese/insulin resistant human subjects we found an inverse correlation between syncytin-1 expression and muscle protein synthesis in humans. Interestingly, this was contrary to our hypothesis, considering the myogenic role of syncytin-1 in the fusion of myoblasts to myotubes we hypothesized a decrease in syncytin-1 in obesity which could directly explain sarcopenia in obesity. Increased syncytin-1 expression in obesity could rather signify two possibilities: first possibility is an alteration of amino acid transport which could be mediated by syncytin-1 receptor ASCT2. ASCT2 is a key amino acid transporter (Sugimoto et al., 2013) of essential amino acids such as leucine and isoleucine whose alterations could result in GCN2-eIF-2α dependent inhibition of protein synthesis in the presence of amino acid deprivation (Krishnamoorthy & Pavitt, 2001). Second possibility could be an increase in ER stress through NO synthase expression. In the astrocytes, NO synthase expression, is mediated by overexpression of old astrocyte specifically induced substance (OASIS) in multiple sclerosis (MS) which has shown to increase syncytin-1 expression (Antony et al., 2007).

Though it is known ASCT2 is a receptor for syncytin-1, it might be a possibility that increased levels of syncytin-1, as observed in obesity, could block amino acid transport

by ASCT2. Considering the stimulatory effect of amino acids such as leucine in facilitating muscle protein synthesis (Wilson et al., 2010), the blocking of amino acid transport by ASCT2 in the presence of elevated syncytin-1 (Frese et al., 2015) could offer a direct explanation for the decreased muscle protein synthesis as observed in obese/insulin resistant humans via GCN2-eIF2α mediated signaling (Krishnamoorthy & Pavitt, 2001). However, prior studies in our lab found that eIF2α phosphorylation (not shown here) was not affected in muscle of obese/insulin resistant humans, casting doubts on this possibility.

Considering the second possibility, higher syncytin-1 expression has been linked in the brain of individuals with MS (Antony et al., 2007). It has been shown that increased syncytin-1 expression was correlated with increased expression of endoplasmic stress mediator, old astrocyte specifically induced substance (OASIS) in MS brains. The overexpression of OASIS in cultured astrocytes have been shown to increase inducible NO synthase expression and concurrently downregulate ASCT-1, a receptor for syncytin-1 and a neutral amino acid transporter. siRNA mediated suppression of syncytin-1 expression prevented the negative effects on ASCT-1 (Antony et al., 2007). Considering the potential role of amino acid availability and ER stress in regulating protein synthesis via mTOR and eIF-2α signaling, a combined effect of syncytin-1 expression on amino acid transport and ER stress is possible and may account for the negative correlation between protein synthesis and syncytin-1 expression.

Cell culture study:

The *in vitro* findings on the C2C12 muscle cell line in the absence of insulin show a positive impact of oleate, either alone or in combination with palmitate, on total 4E-BP1 phosphorylation dependent protein synthesis. Interestingly, palmitate did not have a significant effect on total 4E-BP1 phosphorylation. This contrasts the findings by Kwon and Querfurth, 2015, in which 300 µM palmitate decreased total 4E-BP1 phosphorylation and was rescued by 300 µM oleate in C2C12 myotubes. The differences in the outcome may be due to the experimental design in our study which includes an additional treatment of 100 nM insulin stimulation for 15 minutes in all the groups before lysis of cells to record the insulin stimulated response. As expected, 20 nM insulin alone was sufficient to increase total 4E-BP1 phosphorylation which was maintained only in the presence of 300 µM oleate. This positive effect of 300 µM oleate, rather than palmitate, on insulin mediated total 4E-BP1 phosphorylation is an original finding with respect to insulin-fatty acid interaction in regulating protein synthesis signaling.

Regulation of syncytin-1 by fatty acids in the absence of insulin showed that 300 µM palmitate and a combination of 300 µM palmitate + 300 µM oleate produced an increase in the expression of syncytin-1 indicating a stimulatory effect. However, in the presence of 20 nM insulin, only 300 µM oleate produced a stimulatory effect. Interestingly, the combination of 300 µM palmitate + 300 µM oleate in the presence of 20 nM insulin negated the stimulatory effect of insulin on syncytin-1 expression as well as total 4E-BP1 phosphorylation. Moreover, in the absence of insulin though 300 µM palmitate produced

an increase in syncytin-1 expression it did not produce an increase in total 4E-BP1 phosphorylation as done by insulin. On the contrary, 300 µM oleate in the absence of insulin produced similar effects on syncytin-1 expression and total 4E-BP1 phosphorylation as done by insulin. The study by Kwon and Querfurth, 2015 indicate that neither palmitate nor oleate produced significant differences in mTOR phosphorylation but only had significant effects downstream of mTOR via p70S6K and 4E-BP1 phosphorylation. The only existing evidence from the literature that supports our findings on insulin's regulation of syncytin-1 in skeletal muscle is from a study which showed that insulin-like growth factor (IGF) signaling has been shown to activate syncytin-1 expression in cancer cells via AKT-mTOR-Estrogen Receptor α (ERα) pathway (Strissel et al., 2008). These findings implicate that insulin's regulation over syncytin-1 and 4E-BP1 follow a common axis up to a certain point (possibly mTOR) and branch out beyond it. Moreover, in the presence of 20 nM insulin, signaling that branches towards 4E-BP1 can be prevented from stimulation by 300 µM palmitate either alone or in combination with 300 µM oleate but not by 300 µM oleate alone. The signaling that branches towards syncytin-1 expression requires a combination of 300 µM palmitate + 300 µM oleate to negate the stimulatory effect of 20 nM insulin. These results highlight a novel differential regulation of insulin signaling cascade towards 4E-BP1 and syncytin-1 via palmitate and oleate. This suggests an additional regulation of syncytin-1 in addition to its known regulation by glucagon-cAMP-PKA cascade (Knerr et al., 2005). In the light of *in vitro* findings, the elevated syncytin-1 in obesity *in vivo* could be correlated with MATSUDA index (Table 2), which is a measures of insulin sensitivity. Moreover, the obese/insulin resistant subjects showed

an increased trend of insulin levels (although not significant – $p<0.05$) but might have been enough to have an impact on syncytin-1 expression and its associated correlation with muscle protein synthesis. In this perspective, chronic hyperinsulinemia and/or the levels of palmitate in the blood might have contributed to the decreased muscle protein synthesis. An additional perspective of the results from human and i*n vitro* studies is that exercise has been shown to elevate the levels of syncytin-1 (Frese et al., 2015) and decrease in muscle protein synthesis with reduction in total 4E-BP1 phosphorylation (Dreyer et al., 2006). Interestingly, the elevation of syncytin-1 is a transient response during the differentiation of myoblasts into myotubes (Frese et al., 2015) analogous the inhibition of 4E-BP1 to conserve the ATP consumed towards protein synthesis (Dreyer et al., 2006). This physiological response to exercise fits with the action of palmitate which has been shown to lower the ATP supply reserved for protein synthesis (Nisr & Affourtit, 2016) and thereby might explain its inhibitory effect of total 4E-BP1 phosphorylation but stimulatory effect on syncytin-1 expression.

Further studies on this interplay between fatty acids and insulin signaling cascade in regulating syncytin-1 expression and total 4E-BP1 phosphorylation (Figure 13) are required.

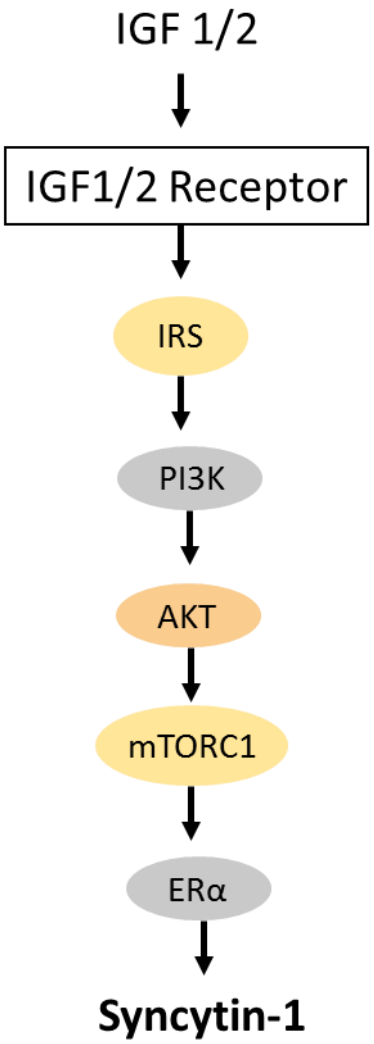


Figure 12. Pathway showing the link between insulin like growth factors and syncytin-1, and their modifications by fatty acids. Adapted from previous report (Strissel et al., 2008).

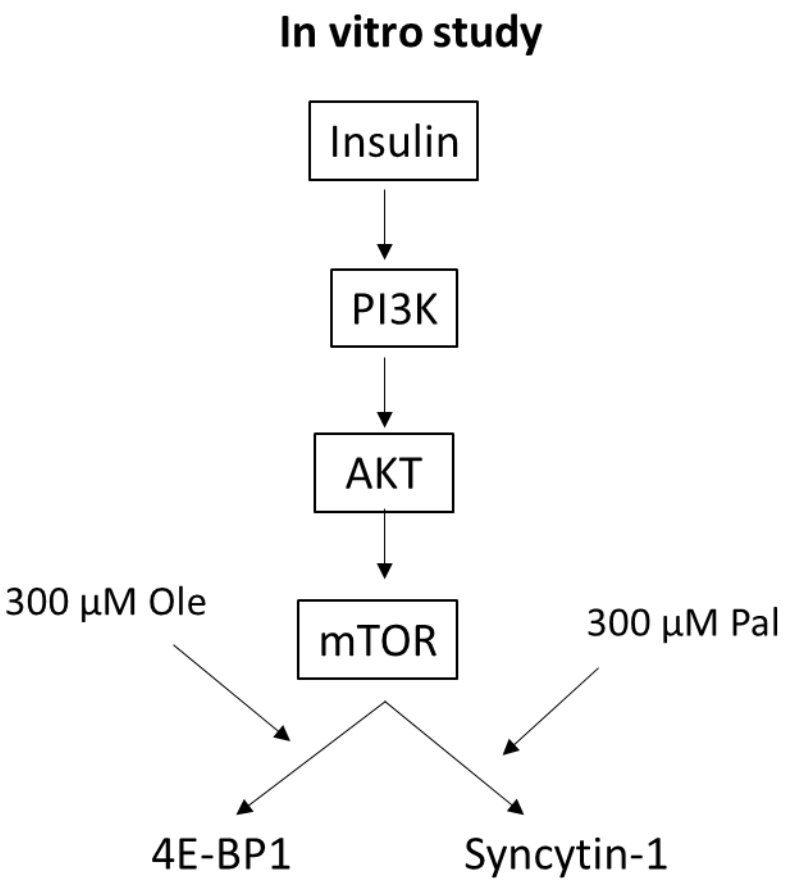


Figure 13. A potential model regulating syncytin-1 and 4E-BP1 through fatty acids and insulin.

Conclusion

Our studies show that increased syncytin-1 expression correlates with decreased muscle protein synthesis in obese/insulin resistant humans. Furthermore, palmitate/oleate differentially regulate insulin's effect on total 4E-BP1 phosphorylation and syncytin-1 expression in C2C12 myotubes. Specifically, the insulin signaling cascade that branches towards 4E-BP1 can be inhibited by 300 μM palmitate either alone or in combination with 300 μM oleate but not by 300 μM oleate alone (all in the presence of 20 nM insulin). Whereas, the signaling regulating syncytin-1 expression requires a combination of 300 μM palmitate + 300 μM oleate to be inhibited in the presence of 20 nM insulin.

CHAPTER 5

METHODS AND MATERIALS

Study participants

Lean (BMI < 25 kg/m^2) and obese (BMI >30 kg/m^2) subjects were recruited to take part in the experimental procedures described below. Initial screening over the phone was conducted to determine subjects that were healthy and met the study criteria. They were invited to Clinical Studies Infusion Unit (CSIU) in Mayo Clinic in Scottsdale, Arizona for further screening. Physical activity was used as a parameter to screen subjects and those who were physically active less than two days per week (i.e., sedentary individuals) were included in the study. Conditions of heart disease, diabetes, liver or kidney disease, elevated blood pressure (chronic, systolic, > 150 mmHg; diastolic, >100 mmHg), peripheral vascular disease, smoking, weight-loss regimen participation, acute illness, use of nutritional supplements and prescription or over the counter medications were used as exclusion criteria. The CSIU was used for screening, which included a medical history, electrocardiogram, urine tests, physical exam and standard blood chemistry tests. Oral glucose tolerance test (OGTT) was used to determine Insulin sensitivity and by calculating the Matsuda insulin-sensitivity index (DeFronzo & Matsuda, 2010).

Experimental design

The participants were informed to avoid any form of exercise, alcohol consumption and maintain a regular diet for three days prior to study visits. All the

studies were started at ~7:30 am at CSIU, after a 10-h overnight fast, and after verbally confirming compliance with the exercise and diet instruction. A catheter was placed into an antecubital arm vein for infusion of a stable isotope of a leucine tracer, and another in a retrograde fashion in a dorsal hand vein for blood sampling. The heated-hand technique was used for blood sampling. Infusion of L- [2,3,3,4,5,5,5,6,6,6-$^{2}H_{10}$]leucine, 0.15 μmol·kg FFM^{-1}·min^{-1} (priming dose, 6.4 μmol·kg FFM^{-1}), was initiated and was continued till the end of the study. Synthesis rate of overall muscle protein was determined using "d10-to-d9-leucine approach" (Tran et al., 2015) from muscle biopsy samples collected at 120 and 300 mins after the start of d-10 leucine infusion. The muscle biopsy samples were also used to study relevant markers of protein metabolism (i.e., eukaryotic translation initiation factor 4E-binding protein 1) and fusion (i.e., syncytin-1). The tissue samples were cleaned of any visible fat and connective tissue and were immediately stored in liquid nitrogen. Blood samples were collected at specified time points for the determination of d9-leucine enrichment, and as previously described (Tran et al., 2015).

Cell culture and differentiation

C2C12 mouse myoblasts were grown in growth media containing Dulbecco's Modified Eagle's Medium (DMEM) with 20 % Fetal Bovine Serum (FBS) and 1 % antibiotic- antimycotic. After 95% confluency, cells were differentiated in differentiation media containing DMEM with 2% Horse Serum (HS) and 1 % antibiotic- antimycotic for 5 days till the cells were spindle shaped (Kwon & Querfurth, 2015).

Fatty acid preparation and treatment

200 mM stock solution of palmitate and oleate were prepared using sodium oleate and palmitate which were dissolved in 50% ethanol for 30 minutes at 70 °C. 10 % solution of fatty acid free- BSA was prepared in Phosphate Buffer Saline (PBS) for conjugation purposes. 5mM stock solution of BSA conjugated fatty acid solutions (Palmitate and Oleate) were prepared by adding 200 mM stock solution of oleate and palmitate in 10 % fatty acid free BSA. Conjugation of the fatty acid and BSA was done at 37 °C for 1 hour before cell treatment.

C2C12 cells were incubated in Serum free DMEM media for 2 hours before the treatment of fatty acids. The conjugated fatty acid – BSA serum was filtered and added to serum free DMEM media. Cells were either treated with 300 μm palmitate or 300 μm oleate with or without 20 nM insulin for 24 hours. Before harvesting, cells were stimulated with 100 nM of insulin for 15 minutes.

Harvesting cells

Cells were washed with ice-cold PBS twice after removing the media. 100 μl of RIPA (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) lysis buffer with protease and phosphatase inhibitors were added to each well of a 6-well plate. After 5 minutes, the cells were scrapped off the wells using cell scrappers and collected in centrifuge tubes. Cells were centrifuged at 14,000 rpm for 15 minutes in

4ºC to collect the supernatant which was used for various assays, such as protein estimation and immunoblot analyses.

Protein estimation

Cell lysate protein concentrations were measured using Coomassie® protein assay reagent kit purchased from Pierce Biotechnologies. Briefly, BSA standards were used in the concentrations of 0 mg, 0.2 mg, 0.4 mg, 0.6 mg, 0.8 mg, 1 mg for standard curve measurements. Cell lysates were diluted in the ratio of 1:40 (Lysate: Mili Q water). 10 µl of the standard or the unknown lysates were loaded into a 96 well plate. 190 µl of the Coomassie Bradford reagent was added and the plate was incubated at room temperature for 10 minutes before measuring the absorbance at 595 nm in a spectrophotometer.

Human muscle samples

Approximately 50 mg of muscle sample was homogenized in RIPA (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) (1mL/100mg tissue) cell lysis buffer with protease and phosphatase inhibitors. The homogenate was centrifuged at 14,000 rpm for 15 minutes at 4ºC to collect and store the supernatant at - 80 °C.

Protein immunoblot assays

A marker related to protein synthesis signaling, 4E-BP1, 4E-BP1 phosphorylation and fusion protein marker syncytin-1 were separated using SDS-PAGE and quantified by immunoblotting. Specifically, the supernatant was mixed with 2x lamellae buffer and boiled at 95 °C for 5 minutes. Protein concentration of ~ 40 ug of muscle homogenate and 15 ug of cell lysate from C2C12 myotubes were separated by gel electrophoresis on Any kD polyacrylamide gels (Mini-PROTEAN, Bio-Rad Laboratories, Inc.). Biotinylated ladder was used as an internal control along with the blue ladder (Precision Plus, Bio-Rad Laboratories, Inc.). The gels were transferred onto nitrocellulose membrane in case of human muscle homogenates and polyvinylidene difluoride membranes in case of C2C12 cell lysates for 1 hour at 90 V. After the transfer, membranes were blocked for 1 hour at room temperature with TBST + 5% non-fat dry milk (Syncytin-1) or TBST + 5% BSA (4ebp1, p-4ebp1). Then the membranes were incubated with primary antibodies at 4^{o}C overnight. The primary antibodies used were: anti-4E-BP1 (Cat. # 9452), anti-phospho-4E-BP1 (Thr$^{37/46}$) (Cat. # 9459) (all from Cell Signaling Technology) and anti-syncytin-1 (Cat. # orb100573 from biorbyt). A dilution of 1:1000 was used for all the primary antibodies in TBST + 5% BSA except for syncytin-1 which was used at a dilution of 1:250 in TBST. Anti-GAPDH antibody (Cat. # 600-401-A33, Rockland Immunochemicals) was used to quantify GAPDH, which was employed as a loading control for human muscle lysate samples. In case of C2C12 lysates, skeletal muscle anti-myosin (Cat. # 32732, Santa Cruz Biotechnologies) was used to quantify myosin (i.e., MYH 1/2/4/6).

After the primary antibody incubation, the membranes were washed with TBST and incubated with secondary antibody for 1 hour at room temperature. Secondary antibodies used were anti-rabbit (Cat. # 205718; Abcam) (for 4E-BP1, 4E-BP-1 Phosphorylation and GAPDH) and anti-mouse (Cat. # sc-516102; Santa Cruz Biotechnology) (for myosin heavy chain) IgG HRP-linked antibodies. A dilution of 1:2000 (anti-mouse) or 1:5000 (anti-rabbit) in TBST was used as secondary antibodies. Excess secondary antibodies were washed with TBST. Clarity™ Western ECL Blotting Substrate (Bio-Rad, Hershey, PA) was used to visualize positive bands.

Non-Esterified Fatty Acid Extraction

Total Non-Esterified Fatty acids (NEFA) were extracted using LC-NH_2 Columns (Sigma-Aldrich, Hershey, MA). Lipids that were bound to hexane-conditioned LC- NH_2 columns were separated with chloroform. Chloroform was used to wash the columns while chloroform/isopropanol (2:1) was used to clear the neutral lipids. 2% acetic acid/diethyl ether was used to elute NEFA, and a continuous stream of nitrogen gas was used to evaporate solvents.

Calculations

The precursor-product approach was used in measuring the fractional synthesis rate (FSR; $\%\cdot hour^{-1}$) of overall muscle protein in the human studies, and using the calculations described here (Tran et al., 2015).

Statistics

One-way repeated measures ANOVA was used to compare mean of interest. Dunnett's comparison test was used as a post-hoc test for comparing individual group responses with a control. Unpaired t-test was used to compare lean and obese/insulin resistant groups. Correlation between muscle protein synthesis and human synctin-1 densitometry in the human studies was performed using the Pearson Product-Moment Correlation. Data are presented as means ± SEM. The level of significance was set at $P \leq .05$ and all statistical tests were two-sided. Commercially available software (GraphPad Prism version 7.03, GraphPad Software, La Jolla, CA) was used in performing statistical analyses.

Human studies approval

All human studies were carried out after obtaining approval by the Institutional Review Board at Mayo Clinic. Each study participant was informed of the potential risks and benefits associated with participation in the study and written consent was obtained prior to inclusion in the study.

Copyright materials

Permission was obtained for all copyrighted material (i.e., figures used from the work of previously published articles).

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