Effect of High Fat vs High Sugar Diet on mTor Signaling Pathway in Skeletal Muscle of Male and Female Mice

Zereque Powell

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Effect of High Fat vs High Sugar Diet on mTor Signaling Pathway in Skeletal Muscle of Male and Female Mice

By: Zereque Powell

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

Major: Health Sciences

The University of Memphis
May 2023
Abstract

Background: Obesity and MetS has been shown to negatively impact protein synthesis rates. 

Objective: Determine the effects of sex and high fat/high sugar diets on mTOR signaling pathways. 

Methods: 15 male and 15 female wild type C57BL/6 mice were randomized into 3 groups: High Fat (45% fat), High Sugar (60% carb) and Control. While continuing diets, body weight and body composition were monitored. Following sacrifice, muscles were examined for changes in the mTOR signaling pathway. 

Results: Mice consuming the HF diet had the highest levels of weight gain. Females expressed higher levels of expression of P-AMPK. Levels of REDD1 were significantly higher in females than males with no effect of diet. 

Conclusion: There was a sex difference in body weight and muscle mass. Sex had an effect on 4EBP1 but not S6 levels.
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<tr>
<td>4EBP1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>DEPTOR</td>
<td>Domain-containing mTOR-interacting protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eEF2</td>
<td>Eukaryotic elongation factor-2</td>
</tr>
<tr>
<td>Fbw8</td>
<td>F-box/WD repeat-containing protein 8</td>
</tr>
<tr>
<td>HF</td>
<td>High Fat</td>
</tr>
<tr>
<td>HS</td>
<td>High Sugar</td>
</tr>
<tr>
<td>Grb10</td>
<td>Growth factor receptor-bound protein</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose Tolerance Test</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin Receptor Substrate-1</td>
</tr>
<tr>
<td>IRS2</td>
<td>Insulin Receptor Substrate-2</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>mLST8</td>
<td>Mammalian lethal with SEC13 protein 8</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mSIN1</td>
<td>mammalian SAPK interaction protein 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MTROC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>Pras40</td>
<td>Proline rich Akt substrate</td>
</tr>
<tr>
<td>REDD1</td>
<td>Regulated in development and DNA damage responses 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpS6</td>
<td>Ribosomal protein S6</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium lauryl Sulfate</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis Anterior</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline with Tween</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous Sclerosis Complex 2</td>
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**Introduction**

2020 estimates show that 42.5% of adult Americans are obese (BMI $\geq$ 30 kg/m$^2$) and 73.5% of Americans are considered overweight or obese (BMI between 25.0-30 kg/m$^2$)\(^1\). Obesity is associated with multiple adverse health implications and the risk of chronic health diseases. An associated disorder that correlates with this increase in obesity in the United States is metabolic syndrome. Metabolic syndrome is a cluster of conditions that occur together, increasing risk of heart disease, stroke and type 2 diabetes\(^2\). The previously mentioned conditions include increased blood pressure, high blood sugar, excess body fat around the waist and abnormal cholesterol or triglyceride levels\(^3\). The prevalence of metabolic syndrome is expected to rise alongside the global obesity epidemic assuming current trends stay the way they are today. Research has shown that it is more likely that males will develop metabolic syndrome during the lifespan than females\(^4\). Compared to males, females have a higher body fat percentage and carry their fat subcutaneously while males have increased visceral fat in the area surrounding the organs in the body cavity. This increase in visceral fat has been linked to a multitude of detrimental health issues including heart disease and diabetes\(^5\). In addition, females expend more total energy from fat oxidation than males. Males on the other hand, expend more of their total energy from carbohydrate oxidation\(^6\). The basal metabolic rate is metabolically different between the sexes, resulting in differences in how the body reacts to different diets\(^6\). Currently, it is still unclear how diet may affect male and female metabolism and the development of metabolic syndrome in different ways.

In addition to metabolic disorders, the growing obesity epidemic impacts the overall muscle mass within these same populations. With obesity, there is muscle atrophy that occurs decreasing the overall size of the muscle and its overall strength\(^7\). This decrease in muscle mass in obesity can be tracked through the lifespan as well. From adolescence through adulthood, it
has been shown that obesity does have negative impact on overall skeletal muscle. Due to the fact that skeletal muscle functions as a motor for movement as well as whole body metabolism, there are apparent issues that lie within obesity induced muscle atrophy. The turnover of proteins including the synthesis of proteins is partially responsible for change occurring within skeletal muscle mass. The inverse of this process of synthesizing new muscle, would be the metabolic process of muscle protein breakdown, which refers to the degradation of muscle protein. These two processes operate together to determine to what extent skeletal muscle mass is maintained. If the synthesis exceeds muscle protein breakdown, muscle hypertrophy occurs. Whereas if the breakdown exceeds the levels synthesis, a net loss of muscle mass would occur.

**Introduction to Protein Synthesis**

Protein synthesis is a process that leads to the creation of new protein molecules. The two major components of this process are known as transcription and translation. Transcription refers to the synthesis of deoxyribonucleic acid (DNA) to ribonucleic acid (RNA). During this phase, the molecule is transcribed from the DNA and a copy of the information needed to create a protein is sent via messenger molecule (mRNA). Translation refers to the synthesis of RNA to a protein. Ribosomes inside of the cytoplasm begin to synthesize protein following the process of transcription. mRNA is decoded within a ribosome and continues to produce the encoded polypeptide. This polypeptide then folds into an active protein. Protein translation has three specific steps that lead to the creation of proteins. The first being initiation in which the ribosome is assembled around the target mRNA. Following initiation, elongation occurs which is the phase of the protein synthesis that provides growth of polypeptide chains. Lastly, termination occurs in which a stop codon is reached, and a ribosome releases a polypeptide. The ribosomal complex remains following this and begins to translate the next mRNA. However, this entire process of
mRNA translation is regulated by mTOR. Translation itself is mostly controlled at the level of initiation, which is also regulated by the mTOR (mammalian target of rapamycin) signaling pathway.

**mTOR Pathway**

The mTOR is a crucial protein kinase within the body, regulating cell proliferation, autophagy and apoptosis through multiple signaling pathways. mTOR has the ability to sense the cellular environmental and alter cellular processes such as cell growth, autophagy and apoptosis accordingly. It can therefore control anabolic and catabolic signaling of skeletal muscle mass.

The result of this control leads to the modulation of muscle hypertrophy and atrophy.

mTOR is made up of two different mTOR complexes, mTORC1 and mTORC2. These complexes have mTOR as a common catalytic subunit, but both have their own specific components that make them different from one another. mTORC1 is made up of mTOR, 40 kDa proline rich Akt substrate (PRAS40), the DEP domain containing mTOR-interacting protein (DEPTOR) and the mammalian lethal with SEC13 protein 8 (mLST8). The second complex, mTORC2, shares mLST8 and DEPTOR with mTORC1 but also has its own specific components of rictor, the rapamycin-insensitive companion and mSIN1, the mammalian stress activated map kinase interacting protein.

**mTORC1**

mTORC1 specifically controls protein synthesis by activating S6 kinase 1 (S6K1) and inhibiting 4E-binding protein 1 (4EBP1). mTORC1 is a regulator in control of skeletal muscle mass following contraction and mechanical load induced hypertrophy. mTOR itself directly phosphorylates PRAS40 and DEPTOR, which leads to a reduction of interaction with mTORC1 and further activates mTORC1 signaling. Laplante et al. suggests that Raptor could impact
mTORC1 from its regulation assembly of the complex and recruiting substrates for mTOR itself\textsuperscript{15}. mTORC1 positively regulates several anabolic processes including protein synthesis. mTORC1 promotes protein synthesis by phosphorylating the initiation factor 4E (eIF4E)-binding protein and the S6K1\textsuperscript{16}. The phosphorylation of 4E-BP1 is what prevents the binding to the before mentioned binding protein eIF4E which allows it to promote cap dependent translation\textsuperscript{16}. The activation of S6K1 activity by mTORC1 forces an increase in mRNA biogenesis and the translation of ribosomal proteins through the activation of several proteins. S6 plays an important role as a part of mTOR-dependent growth program; S6 is a major component of the 40S ribosomal subunit and is able to regulate growth by affecting protein synthesis indirectly\textsuperscript{16}. The activating of mTORC1 promotes ribosome biogenesis by stimulating the transcription of ribosomal RNA\textsuperscript{16}. Akt, or protein kinase B, is also involved in this mTOR pathway. One of the targets of Akt is mTOR; Akt is able to activate mTORC1 through phosphorylation\textsuperscript{19}. This process of Akt activating mTOR begins as the lipid kinase PI3K is activated via a response from mitogenic signals\textsuperscript{20}. This leads to the phosphorylation of phosphoinositides which go on to bind to Akt\textsuperscript{20}. Following this step, Akt is activated by the phosphorylation of T208 and S473 which ultimately results in the activation of mTORC1 indirectly\textsuperscript{20}. Subsequently, mTOR controls the signaling of insulin by regulating several upstream and downstream components including growth factor receptor-bound protein (Grb10), insulin receptor substrate (IRS-1) and F-box/WD repeat-containing protein 8 (Fbw8)\textsuperscript{21}. The importance of this signaling corresponds with the fact that insulin activates protein signaling by activating eukaryotic initiation factors and eukaryotic elongation factors\textsuperscript{22}. Over time, insulin is then able to increase the cellular content of ribosome in order to augment the capacity for protein synthesis in the muscle. This rapid activation is
completed mostly through phosphoinositide 3-kinase and then insulin can elicit the phosphorylation of 4EBP1 to form initiation factors$^{23}$.

**S6K1 and 4EBP1**

The mTORC1-S6K1 pathway regulates several different cellular functions including apoptosis, glucose metabolism and protein synthesis$^{24}$. Specifically, S6K1 is a kinase that is the target substrate for S6 ribosomal protein$^{25}$. Phosphorylation of S6K1 induces protein synthesis in the ribosomes in the cells$^{26}$. S6 has been proven to be incredibly vital towards the ability to gain muscle mass. Marabita et al. examined this closely while inhibiting mTOR signaling to S6K1 using S6K1 KO mice$^{27}$. This inhibition was shown to be capable of reducing Akt induced muscle growth and force it to be insensitive to rapamycin. In a study conducted by Ruvinsky et al, mice deficient in S6 were shown to have suffered from muscle weakness$^{28}$. Ribosome protein S6 phosphorylation is a crucial determinant of muscle strength as it has a major role in regulation of myofiber growth and overall energy content. This stands to be even with specifically S6K1 though it regulates myoblast growth in a different fashion. To further strengthen this claim, the deletion of S6 has been shown to heavily suppress muscle growth adaption$^{29}$. In response to an upstream stimulation of mTORC1, 4EBP1 is phosphorylated and begins to stimulate protein synthesis$^{40}$. Both proteins 4EBP1 and S6K1 stimulate translation$^{28}$ which is a key element in protein synthesis. This occurs after mTORC1 activates these protein$^{18}$. Both downstream signaling molecules are both crucial to the entire process of protein synthesis but operate differently. While S6K1 controls cell size it does not have any effects on cell proliferation$^{28}$. Inversely, 4EBP controls cell proliferation but not the overall cell size$^{29}$. 4EBP1 phosphorylation recruits 40S ribosomal subunits. The phosphorylation of this protein results in the activation of
cap-dependent translation. Regulation and activation of 4EBP1 within skeletal muscle has an important role in muscle fiber transformation.

**REDD1 and Protein Synthesis**

Upstream of the mTOR pathway lies REDD1 (Regulated in development and DNA damage responses 1). REDD1 was recently discovered as a gene that is induced by damaged DNA and hypoxia\(^3^0\) and has been reported that energy stress, glucocorticoid treatment and reactive oxygen species induce the transcription of the gene. It is found in humans, mice and *drosophila* and is expressed mainly in adult tissues\(^3^1\). A major finding that was responsible for further understanding the function of REDD1 was the discovery that it is a crucial regulator of mTOR pathway signaling during hypoxic stress by Brugarolas et al\(^3^2\). Within the body, REDD1 is rapidly degraded by the ubiquitin-proteasome system that is then mediated by the CUL4A-DDB1 ubiquitin ligase through glycogen synthase kinase 3 phosphorylation and β-transducin repeat containing protein activity\(^3^3\). Therefore, the CUL4A–DDB1–ROC1–β-TRCP ubiquitin ligase acts to regulate mTOR by modulating the stability of the REDD1 gene\(^3^3\). More research still needs to be conducted in regard to the signaling pathways and post-translational modifications that regulate REDD1, but more is being discovered in regard to mTOR inhibition by REDD1. The induction of REDD1 expression leads to the blocking of phosphorylation of S6K1 or 4EBP1\(^3^3\). The protein REDD1 serves many functions within the body including cell growth, proliferation and autophagy\(^3^4\). This multifaceted protein is also an important inhibitor of protein synthesis. Typically, the expression of REDD1 in basal conditions is relatively low but can be exponentially increased via endurance exercise and unfolding protein response\(^3^5\). REDD1 is intertwined with many diseases associated with impairment of skeletal muscle such as retinopathy, Parkinson’s, and diabetes\(^3^6\). REDD1 as an inhibitor, functions by inhibiting protein
synthesis via the activation of TSC2 and increasing the proportion of RHEB (ras homolog enriched in brain) in the GDP bound form. This is triggered by several external factors including nutrient deprivation, hypoxia, and DNA damage. With the inhibition of protein synthesis, the cells can redirect energy towards other processes that can deal with the stress that it is facing rather than the energetically expensive system, protein synthesis. Britto et al. found that were surprisingly able to show that REDD1 limits muscle loss during energetic stress by reducing glycogen depletion and additionally, the activation of AMPK. The group was also able to justify that REDD1 was necessary for the decrease of O₂ and ATP consumption in skeletal muscle via reduction of the extent of mitochondrial-associated endoplasmic reticulum membranes.

**AMPK and Protein Synthesis**

The enzyme AMPK is active in several processes in the body and plays a major role in cellular energy homeostasis. This kinase becomes active as a response to stressors that use cellular ATP storage such as hypoxia, low glucose, and heat shock. One of AMPK’s main targets is the mTORC1 pathway. In situations with low nutrient storage, the depletion of ATP causes AMPK to phosphorylate TSC1/2 to stimulate GAP activity towards Rheb which in turn cause a suppression of mTORC1. In addition to this, it has been shown that AMPK also inhibits mTORC1 by the phosphorylation of raptor. The combination of these two processes gives AMPK the ability to directly regulate mTORC1. The main driver of the interaction between AMPK centers around energy levels. The process of protein signal is a very energetically demanding process that takes approximately 2300 ATP to synthesize a protein. Therefore, during periods of low ATP levels, AMPK works to turn off the process of protein synthesis. Amino acids are another perquisite for the initiation of protein synthesis and amino acids also
have a relationship with AMPK. Pancreatic β-cells treated with high levels of leucine and glutamine showed a significantly elevated levels of mTORC1 activity while the levels of phosphorylated AMPK were diminished\textsuperscript{41}.

**Male Sex Hormone and Muscle Protein Synthesis**

The fact that there are obvious physical and anatomical differences in musculature of males and females leads to the conclusion that there must be differences in protein synthesis between the sexes. On average, a male will have a higher percentage of muscle mass than a female. This growth in muscle stems from muscle hypertrophy which increases the total volume of muscle cells\textsuperscript{42}. Possible differences in the protein synthesis rates between the sexes could explain some of the differences in muscle mass and likely involve sex hormones. Testosterone increases both the rate of muscle protein synthesis and the resulting net muscle protein balance which results in the increase in muscle mass\textsuperscript{43}. These levels of testosterone remain similar until puberty, which is the point where the amounts significantly rise in males. To explain the importance of testosterone in relation to muscle protein synthesis, Griggs et al. conducted a study giving 9 males a pharmacological dose of testosterone\textsuperscript{43}. The boosted levels of testosterone increased muscle protein synthesis in all subjects with a mean increase of 27\%\textsuperscript{43}. The data suggested that testosterone does in fact increase muscle mass through the elevation of levels of muscle protein synthesis. Testosterone has a major impact on the mTOR pathway and its downstream targets. Testosterone increases the phosphorylation of mTOR, S6K1 and 4EBP1 and activates the mTOR pathway\textsuperscript{44}. The removal of testosterone has been shown to alter the rates of protein synthesis. Serra et al. strengthened this claim in a study looking at the difference between testosterone deprived and testosterone treated male mice\textsuperscript{45}. Over the course of 6 weeks, the decrease in testosterone reduced mTOR activation therefore lowering the rate of phosphorylation
in the downstream targets S6K1 and 4EBP1. In contrast, the groups with supplementation went on to show sustained mTOR activity compared to control and the reduced testosterone mice. Further emphasizing this claim, Jiao et al. was also able to clearly show reduced mTOR activity in castrated rats. Leucine was unable to increase muscle protein synthesis in this reduced testosterone group and again diminished levels of 4EBP1 and S6 were shown. Evidence exists regarding reduction in testosterone levels decreasing mTOR signaling. Additionally, research has been conducted to justify that increased testosterone does increase protein synthesis levels. Chen et al. examined this via a study providing testosterone to hypertensive rats. In the testosterone boosted groups, mTOR, S6K1 and 4EBP1 were expressed at higher levels than the control groups.

In terms of upstream targets, REDD1 levels are also modified with increased or decreased testosterone levels. White et al. monitored this by monitoring the signaling differences in mice with testosterone supplementation and reduction. In the groups with reduced testosterone, REDD1 levels were increased. Inversely, in the groups with added testosterone, the levels of REDD1 within the mRNA were significantly lower than the reduced group. Wu et al. was able to find similar results while looking Male Wistar rats. The effects of testosterone attenuated increases in REDD1 that was in skeletal muscle and the testosterone reduced groups yielded the reverse effects. The other upstream target being monitored, AMPK, has been shown to have increased phosphorylation with the addition of testosterone. Troncoso et al. used cultured cardiomyocytes and stimulated them with a dosage of testosterone, monitoring the effects over 24 hours. Also, the group was able to find the inhibition of AMPK blocked glycolysis induced by testosterone.

Female Sex Hormones and Muscle Protein Synthesis
While studies have been shown to suggest that testosterone boosts muscle protein synthesis levels, it has been shown that estrogen may have similar effects on muscle protein synthesis\textsuperscript{51}. Currently, research is available that gives evidence that in males, impaired skeletal muscle development is a direct side effect of overall loss of androgens\textsuperscript{52}. In a study conducted by MacLean et. Al., in mice with androgen receptors knocked out, muscle mass was severely decreased in males however, remained normal in females\textsuperscript{53}. The information gained from this gave the ability to conclude that androgens play a role in the ability to gain muscle mass for males in mice. Even without the same levels of testosterone, is females have been shown to have no sex difference in protein synthesis rate\textsuperscript{54}. Fujita et. Al. tested this by conducting a study with males and females given the same diet and exercise regimen\textsuperscript{55}. The results of this study were able to conclude that the levels of protein turnover following a bout of exercise was the same for the males and females. The link can also be seen as rates of muscle protein synthesis drop drastically following menopause when levels of estrogen drop\textsuperscript{56}. In addition, it was shown that when young mice lose estrogen their levels of skeletal muscle are reduced\textsuperscript{57}. mTOR has been proven to be a critical part of estrogenic signaling. Estrogen itself activates mTOR signaling\textsuperscript{58} and conversely mTORC1 is a important activator in estrogen receptor transcriptional activity. mTOR promotes growth factor mediated estrogen receptor activation by phosphorylation of S167\textsuperscript{59}. This phosphorylation is mediated by the mTOR effector S6K1 which is vital in estrogen receptor dimerization. This expression of estrogen promotes the expression of S6K1\textsuperscript{60}.

While obvious differences exist in muscle mass between males and females, research has yet to find evidence of major differences in the rates of protein turnover through the lifespan in relation to sex hormones. A possible area of focus for this could be how muscle protein synthesis
responds to high stress situation, such as an array of metabolic disease stemming from high fat or high sugar diet.

**Diet and Impact on Protein Synthesis**

Whole body protein mass is regulated in relation between protein synthesis and protein degradation. In order for muscle mass to be gained, there must be a net positive between these two factors. Food intake has been shown to affect whole body and tissue protein. Both high fat and high sugar diets can lead to diabetes which has been shown to negatively affect protein metabolism. Obesity often is a consequence of these diets and typically coincides with insulin resistance which regulates protein metabolism. Kimbal et. Al. conducted a study in dogs that were able to show that insulin was able to inhibit the effects of muscle protein breakdown and boost the rates of muscle protein synthesis. In the reverse of this, it appears that deficiencies in insulin will reduce muscle protein synthesis and result in muscle loss. Insulin deficiency causes a loss of muscle mass that can only recovered with insulin therapy.

Diets high in fat and sugar can have many negative impacts on the body. These types of diets typically lead to obesity, which can lead to a plethora of diseases including stroke, coronary heart disease and type 2 diabetes. Diets with high fat and sugar content can also lead to alterations in protein metabolism which can have its own side effects. Studies have shown that obesity in correspondence to high fat diet has been associated with abnormalities in leucine turnover leading to disruptions in protein synthesis. Bae et.al. was able to monitor several protein signaling pathways in a study conducted with sixty male Sprague Dawley rats fed with a control and high fat diet (40% fat) with exercise intervention. After the conclusion of 8 weeks, the muscle protein levels of IRS-1, IRS-2 and mTOR within the high fat group were significantly lower than the control. Additionally, the mTORC1 protein levels were significantly lower in the
high fat diet mice, and had significantly lower levels of S6K1. This high fat diet had an overall negative effect on how insulin was used in the skeletal muscle. In another study involving high fat diet and its impact on protein synthesis, Tsintzas et al. explored this in older adult men. In a study conducted with 8 overweight men, a high fat diet was implemented and followed over the course of 2 weeks. At the conclusion of this time period, there were differences with mTOR levels and specific amino acids however, there were no detrimental effects on the skeletal muscle protein in response to the diet. With the increase of obesity in result of unhealthy high fat diet, Nair et. Al. was able to track the increase of whole body proteolysis that ultimately led to an impaired response in the antiproteolytic response to insulin. Insulin’s action as an anabolic hormone that suppresses protein breakdown and stimulating protein synthesis of protein synthesis. Obese rats on high fat diets have been shown to be more prone to ectopic muscle lipid accumulation that can lead to a decrease in muscle protein anabolism. Additionally, the fat gained through a high fat diet can affect the quality of the skeletal muscle which leads to reduced muscle strength due to a disruption of typical protein synthesis function.

High sugar diets have also shown to have similar effects on protein synthesis as high fat diets. Anderson et al. was able to further prove this looking at mice given high sugar diets over the course of nine weeks. Following the conclusion of the study, the high sugar diet impaired the activation of skeletal muscle protein synthesis in response to nutrient ingestion and additionally reduced basal rates of synthesis of hepatic proteins when compared to a control diet. This evidence proved that alterations in protein metabolism in high sugar diets do exist. The idea that high sugar diets negatively affect protein metabolism is also consistent in Drosophila. Kemppanien et al. was able to exhibit a deficiency in protein synthesis in flies given a high sugar diet intervention. In this diet, the flies developed abnormalities in their amino acids which
triggered growth arrest and inhibition of protein synthesis through the mTOR pathway independent of the effects of ATP depletion\textsuperscript{44}. Further research is given from Mosoni et al\textsuperscript{74} while looking at chronic intake of sugar in Wistar rats. The sugar fed rats had significantly reduced stimulation of protein synthesis compared to the starch fed rats that were used for comparison. Protein synthesis was measured via flooding dose method and was able to track decreases in ribosomal activity, decreased postprandial responses of muscle protein synthesis and fractional synthesis rates. This study was also able to show that sugars caused inflammation that also altered muscle protein synthesis levels in the rats.

**Gaps in Knowledge**

Currently, it is known that there are modifications to the mTOR signaling pathway in respect to general western diets. Additionally, research has shown reduction in quality of skeletal muscle within populations living with obesity and diabetes. However, gaps of information are present regarding the sexual dimorphism of on how these conditions may impact protein synthesis. Significant research has been completed with a focus on high fat diet and protein, but the additional factor of high sugar remains relatively untouched. Considering the growing amounts of high sugar diets across the world, a primary interest in investigating protein synthesis with this diet is crucial. Continuing with the existing gaps in knowledge, there is an absence of substantial evidence displaying if protein synthesis differs between the sexes with differing dietary intakes. To address these areas, the mTOR signaling pathway will be monitored by measuring upstream regulators, AMPK and REDD1, and downstream regulators, 4EBP1 and S6K1, in male and female C57BL/6 mice after 12 weeks of either a high fat or high sugar consumption. Collecting data on this will be beneficial for the growing obesity epidemic and being able to create a better skeletal muscle outcome in these populations.
Hypothesis

We hypothesize that the mice fed with the high fat diet will have decreased mTOR signaling than the mice with the high sugar diets. When comparing the diets among the sexes, we hypothesize that males will have greater reductions of mTOR signaling than the females.

Methods

30 male and female mice were purchased at 7 weeks of age from Envigo (Cumberland, Virginia). Mice had the opportunity to acclimate to university of Memphis facilities for one week. Mice were placed in cages with five mice per cage. At 8 weeks of age, mice were switched from standard rodent chow to one of three purified diets; the High Fat (HF) : 45% Fat, 20% Protein, 35% Carbohydrate, High Sugar Diet (HS): 60% Carbohydrate (60%) sugar, 20% protein and a Control Diet (CON): 20% fat, 20% protein and 60% carbohydrate (Research Diets Inc, New Brunswick, NJ). Mice were weighed every three days to monitor health and track weight gain over time. Body composition was monitored with MRI testing to determine fat mass and lean mass biweekly (EchoMRI-100, Houston, TX). At the conclusion of the dietary intervention at 12 weeks, mice were euthanized by CO₂. Cervical dislocation was used as a secondary means of euthanasia. 30 minutes prior to euthanasia, mice were injected with 0.04 µm/g of total body weight of puromycin. Liver, spleen, kidney, intestine, adipose tissue, skeletal muscle, and the heart were collected immediately after euthanasia and frozen in liquid nitrogen or stored in formalin.

Western Blot: Western blot analysis was performed on muscle tissue to evaluate the differences in protein expression levels. Portions of skeletal muscle cut into fourths and were homogenized in a 10x Mueller buffer and protein concentrations were measured using the Bradford method. Homogenates (40 µm) were loaded on 10% SDS-polyacrylamide gels, separated, and then
transferred overnight to polyvinylidene difluoride membranes. Ponceau staining was used to confirm gel transfer and equal loading. Membranes was blocked Tris-buffered saline with 0.1% tween 20 (TBST) and 5% milk for 1 h at room temperature. Primary antibodies for phosphorylated (P)-4EBP1 (Cell Signaling, Danvers, MA) and P-S6 for (Cell Signaling, Danvers, MA) Total 4EBP1 (Cell Signaling, Danvers, MA) and total S6 (Cell Signaling, Danvers, MA), actin (Cell Signaling, Danvers, MA) and puromycin (Millipore, Temecula, CA) were used and incubated for 24h in 5% TBST milk at 4ºC. Secondary HRP-conjugated antibodies (Cell Signaling, Danvers, MA) were used and incubated in 5% TBST milk at room temperature for 1 hour. Enhanced chemiluminescence reagent (BioRad, Hercules, CA) was used to visualize protein on iBright1500 (ThermoFisher Scientific, Waltham, MA). Blots were analyzed by measuring the optical density of each band using ImageJ software (NIH, Bethesda, MD). All phosphorylated proteins blots were normalized to non-phosphorylated control.

**RNA Isolation and qPCR:** To isolate RNA from the gastrocnemius muscle, tissue was homogenized in 3-5 mL of Trizol (TRIzol™ Reagent, Life Technologies, Carlsbad CA). Total RNA was extracted from Trizol solution with the addition of chloroform. Extracted RNA were washed with 75% ethanol and dissolved in water, then quantified using a Nanodrop (ThermoFisher Scientific, Waltham, MA). For qPCR measurement of mRNA transcripts, 1 µg of RNA was reversed transcribed into cDNA. The Applied Biosystem High Capacity RNAs to cDNAs kit (ThermoFisher Scientific, USA) was used to synthesize cDNAs from total RNA. The cDNA was mixed with forward and reverse primers for the intended gene target and SYBR Green qPCR master mix (PowerUp™ SYBR™ Green Master Mix, ThermoFisher Scientific, USA). The reaction was run in the RT-qPCR thermocycler (Applied Biosystems QuantStudio 6 Flex Real-Time PCR System, Thermo Fisher Scientific) by using the RT- program.
(QuantStudio™ Real-Time PCR Software) The 2-ΔΔCT method was used determine changes in gene expression between treatment groups.

**Statistics**

A two-way ANOVA was used to determine the effects sex and diet. Repeated measures ANOVA was used to analyze changes in data over time. Tukey post hoc analysis was used to examine interactions. GraphPad Prism 8 was used for all statistical analysis. Significance will be set at \( p \leq 0.05 \).

**Results**

**Body Weight Results**

Body weight (BW) was assessed at the time of euthanasia. There was an effect of time on BW (Figure 1, \( p<0.0001 \)). Males at 6wk consuming the controls diet weighed more than females at 6wks (\( p=0.005 \)). After 6 weeks on the respective diets, males consuming a HF diet weighed significantly more than all other groups at 6wks, whereas there was not significant difference in females consuming the HF diet (\( p=0.99 \)). Males and females consuming the high sugar diet were not different than their respective controls at 6wks (\( p>0.99 \)). After 12wks males consuming the HF diet weighed significantly more than all other groups (Figure 1; \( p<0.001 \)), whereas females consuming HF diet for 12wks did not display increased body weight compared to controls (\( p=0.74 \)). Females weighed significantly less than males consuming control diet at 12wks (\( p=0.0004 \)). Males and females consuming the high sugar diet were not different than their respective controls at 12wks (males \( p=0.90 \), females \( p=0.97 \)).

**Lean Mass Results**

Every two weeks the lean body mass of the mice was measured by EchoMRI. At the conclusion of 12 weeks, there was a main effect on sex (\( p<.0001 \)), with the males gaining more
lean muscle mass than the females (Figure 2). Additionally, there was an effect of diet (p<.0001). For males, no significance differences were detected between the HF and HS (p=0.9993), HS and CON (p=.9993) and lastly HF and CON (p>.9999). Similarly, for females there were no significant differences between HF and HS (p=.9928), HF and CON (p=.9430) and HS and CON (p=.9934). Multiple comparisons were able to show a significant difference in lean mass (p=.0069) between the Male HF and Male HS group. For females, there was no significance (p=.1780) in lean muscle mass in the HF and HS groups.

Muscle Weight Results

After 12 weeks on the respective diets muscle mass was measured. There was an effect of diet on tibialis anterior (TA) muscle mass (Figure 3, p=0.002), with the high fat diet increasing TA weight. There was an effect of sex on TA muscle mass with the males having larger muscle weight than the females (Figure 3A, p<0.001). Multiple comparisons showed TA muscle mass was significantly heavier in the HF compared to the HS group (p=.0076). There was an effect of diet on soleus muscle mass (p<0.001), with the high fat diet increasing soleus weight. There was an effect of sex on soleus muscle mass with the males having larger muscle weight than the females (Figure 3B, p<0.001). Multiple comparisons showed soleus muscle mass was significantly heavier in the HF group compared to the HS group (p<.0001). There was an effect on diet on gastrocnemius muscle mass (p=0.0103), with the high fat diet increasing their muscle mass the greatest amount. There was an effect of sex on gastrocnemius muscle mass (Figure 3C, p<0.001) with the males having larger muscle weight than the females. Multiple comparisons showed gastrocnemius muscle was significantly heavier in the HF group compared to the HS group (p=.0075). In the extensor digitorum longus (EDL) there was an effect of sex on muscle mass (p=0.001); however, there was no effect of diet (Figure 3D, p=0.1321). These data support
increased muscle mass in males regardless of diet and that a high fat diet tends to increase muscle mass in oxidative and mixed fiber type muscle in both sexes but did not increase muscle mass in highly glycolytic muscle.

**Protein Signaling**

Upstream and downstream regulators of protein synthesis were measured in gastrocnemius of mice. For the Phosphorylated AMPK, there was no effect on sex (p=0.9844) or diet (p=0.2936) (Figure 5A). The ratio of Phosphorylated S6 to Total S6 was also not affected by sex (p=0.7807) or diet (p=0.6939) (Figure 5B). Similar results were also seen for the ratio of Phosphorylated 4EBP1; no effect of sex (p=0.8753) or diet (p=0.2269) (Figure 5C) was detected. The incorporation of puromycin into newly synthesizing polypeptides was measured using the SUnSET technique. There was no effect on sex (p=.07424) or diet (p=0.5306) on protein synthesis measured by puromycin incorporation (Figure 5D).

**REDD1**

REDD1 mRNA expression was measured in the gastrocnemius muscle. There was an effect of sex (p=0.0331), but not on diet (p=0.2982) on Redd1 gene expression (Figure 6).
Discussion

The rate of obesity and diabetes continues to rise across the globe. This increase in popularity of the Western diet has resulted in an obesity epidemic that has resulted in the increase of MetS. Due to its association with insulin resistance, and insulin’s important role on protein metabolism, it is expected that obesity would have severe consequences on protein synthesis. One of the direct consequences of obesity is muscle atrophy, which decreases muscle mass, strength, and quality. It has been well studied that there are negative effects on skeletal muscle with increase in body fat. This stems from the rates of protein degradation being higher than that of protein synthesis. Obesity causes an impaired system of muscle protein remodeling and eventually leads to anabolic resistance compared to non-obese individuals.

Beginning with weight gain, several studies have shown high fat mice to have quickly gained mass and diabetes compared to control diets. Melhorn et al found similar results in which males on HF diets were able to reach statistical significance in weight gain by Day 9 of diet implementation compared to the control. Similarly, we were able to find similar results (Figure 1) showing that male mice on the HF diet increased their body weight at the fastest rate compared to the control. This gain in weight was also significant compared to the weight gained to the mice in the high sugar diet. These studies showed consistent results, our data supported this while also measuring the weight gain in both sexes. Frequently only males are studied in these high fat diet studies, but we were able to compare both males and females to observe the still not completely understood differences within MetS. Diet did not alter males and females lean mass; however males had significantly more lean mass than females.

The entirety of protein synthesis is made up of two steps, transcription and translation. The initial step of transcription operates by producing making RNA. This mRNA can leave the
cell’s nucleus and binds with the ribosome to help form protein. Within skeletal muscle, alterations in protein synthesis have been observed. Several studies have been able to prove that compared to lean individuals, the rate of protein synthesis is significantly lower in those with obesity. Guillet et al was one of the primary groups able to show the alterations via isotope tracers of amino acids. Within mixed muscle protein, the overnight fast showed a reduction in the rate of protein synthesis in humans with obesity compared to leaner ones. Following this initial finding, several other groups were soon able to confirm that within obesity, there is a slowing of the rate of protein synthesis. Conversely, Hulston et al was able to find evidence stating otherwise in a study that included resistance exercise. The study found no significance within protein synthesis levels in obese and lean human subjects, showing that exercise could potentially be a factor in evening protein synthesis levels within obese populations. Similar but with a different approach in exercise type, Serrano et al added the external factor of aerobic exercise and was able to find that mixed muscle protein synthesis was not different among lean and obese untrained subjects. While there are differing opinions on protein synthesis when exercise is added, it remains consistent that without exercise intervention, there is a disruption in the flow of protein synthesis rates.

To stimulate protein synthesis, mTORC1 must phosphorylate S6K1 and 4EBP1 to allow for translation to function properly. Phosphorylated 4EBP1 and S6K1 are known reliable markers of activated mTOR signaling. Within our groups we were not able to find difference in phosphorylated S6. Within our groups we were not able to find difference in phosphorylated S6 but did note a reduction in these markers in females that was not seen in males. This difference could be associated with the elevated levels of AMPK found within the female mice compared to that of the males. Bolster et al was the first to discover changes in translation initiation and
overall skeletal muscle protein synthesis as a reaction to AMPK activation\textsuperscript{82}. After AMPK activation by AICAR treatment, immediate suppression of protein synthesis was seen in mixed muscle. With AMPK being an energy sensor, and protein synthesis being the energetically taxing process that it is, there appears to a negative correlation in AMPK and protein synthesis levels. While only male mice were observed, the mechanism in principle remains the same. An elevated level of AMPK, a protein synthesis inhibitor, resulted in a reduction of protein synthesis downstream targets.

The levels of REDD1 were not affected by diet but significant difference was seen between sexes which follows results of previous findings. One of the major contributors to REDD1 differences with the mice can be linked to testosterone. Males produce a significantly higher amount of testosterone, and this sex hormone has been linked to decreased amount of REDD1 found inside of skeletal muscle. While measuring examining the gastrocnemius muscle in male mice, Wu et al found data suggesting that the upregulation of REDD1 mRNA is blocked by testosterone via transcriptional regulation by androgen receptors\textsuperscript{83}. REDD1 has been confirmed to be an estrogen receptor target gene that possesses several estrogen response elements within the promoter\textsuperscript{84}. Further explaining the possible reasoning for greater REDD1 found within the muscles in females, is the fact REDD1 is more efficiently activated in females than males, according to Baida et al who measured these rates while studying the skin. REDD1 has been found to be induced at earlier timepoints and at larger concentrations overall due to its relationship with estrogen\textsuperscript{85}.

**Conclusion**

Recent studies have repeatedly stated that high fat and high sugar diets can have a significant impact on protein synthesis in humans. There is still a lack of complete understanding in how
this is different among the sexes. Our study showed that high fat diets increase body weight significantly compared to other groups, while having no significance in lean muscle with the other sexes. In REDD1 mRNA, there was a found main effect in sex but not diet which correlates with previous findings. Our findings showed an increase of P-AMPK in females compared to males, and as a result lower levels of protein synthesis marker 4EBP1 than male counterparts. With Metabolic Syndrome becoming a more prominent issue in society and its negative role in relation to muscle mass, it is important to understand the mechanisms regulating these changes to develop valid therapeutics. Information gained on diet composition to better maintain muscle mass balance would be a benefit to a large population of individuals.

**Limitations and Future Directions**

One limitation to our study would be the number of mice we had for each diet group. With more mice, the variance that individual mice had would have less of an impact on the overall statistics. The diets implemented on the mice did not completely align with the typical Western diet. A group with a diet composition of high fat and sugar group would be more similar to the Western diet that has become popularized. Protein synthesis was measured in a fasted state. Fasting is known to suppress protein synthesis so all changes may not be able to be tracked due to fasting induced suppression of protein synthesis. Future studies should include anabolic stimulus. Lastly, mTOR itself could have been measured directly instead of measuring the indirect markers of the pathway. Future studies may consider adding an aspect of exercise to observe if there are differences in activation of the mTOR pathway with an anabolic stimulus.
References


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## Appendix

### Tables and Figures

Table 1. Composition of High Fat, High Sugar and Control Diets.

<table>
<thead>
<tr>
<th></th>
<th>Carbohydrate</th>
<th>Protein</th>
<th>Fat</th>
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<tbody>
<tr>
<td>High Fat</td>
<td>35%</td>
<td>20%</td>
<td>45%</td>
</tr>
<tr>
<td>High Sugar</td>
<td>60(40 sugar%)%</td>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>Control</td>
<td>60%</td>
<td>20%</td>
<td>20%</td>
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Table 2. Gene primers for qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
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</thead>
<tbody>
<tr>
<td>REDD1</td>
<td>CAAGGCAAGAGCTGCCATAG</td>
<td>CCGGTACTTAGCGTCAGGG</td>
</tr>
<tr>
<td>Actin</td>
<td>GGCTGTATTCCTCCATCG</td>
<td>CCAGTTGGTAAACATGCCATGT</td>
</tr>
</tbody>
</table>
Figure 1. Body Weight of all mice at 6wk and 12wk timepoints. a= Significant from Male CON 6wk, b=Significant from Male HF 6wk, c=Male HS 6wk, d=Significant from Male CON 12wk, e=Significant from Male HF 12wk, f=Significant from Male HS 12wk, g=Significant from Female 6wk CON, h=Significant from Female 6wk HF, i=Significant from Female 6wk HS, j=Significant from 12wk CON, k= Significant from Female 12wk HF, l=Significant from Female 12wk HS, Z=Significant to all groups. All data are presented as mean ± SEM. Significance was set at p<0.05
Figure 2. Lean Mass over the course of 12 weeks in all mice groups collected via EchoMRI. All data are presented as mean ± SEM. Significance was set at p<0.05.
Figure 3. Mass of Tibialis Anterior (A), Soleus (B), Gastrocnemius (C), and EDL (D) in all mouse groups at time of sacrifice. All data are presented as mean ± SEM. Significance was set at p<0.05.
Figure 4. Protein Expression of mTOR signaling pathway measured in the gastrocnemius after 12 weeks on high fat or high sugar diets in male and female mice. AMPK (A), Phosphorylated 4EBP1 (B), Phosphorylated S6 (C), Total 4EBP1 (D), Total S6 (E), Puromycin (F).
Figure 5. Relative protein expression of Phosphorylated AMPK (A), Phosphorylated S6: Total S6 (B), Phosphorylated 4EBP1: Total 4EBP1 (C) and Puromycin (D) quantified from western blot images. All data are presented as mean ± SEM. Significance was set at p<0.05.
Figure 6. REDD1 mRNA measurements in gastrocnemius muscle. All data are presented as mean ± SEM. Significance was set at p<0.05