Endurance Exercise Stimulates Protein Synthesis Independent of Insulin and has a Permissive Effect on Leucine's Ability to Stimulate 4E-BP1 and S6K1 in Uncontrolled T1DM Animals

Antonio Sandro Serino

Thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements
for the degree of

Master of Science

Graduate Program in Kinesiology and Health Science
York University
Toronto, Ontario

September 2010

Your file Votre référence
ISBN: 978-0-494-80629-6
Our file Notre référence
ISBN: 978-0-494-80629-6

ABSTRACT

Diabetes can cause profound changes in skeletal muscle mass in young growing animals. This impaired muscle mass can occur in part as a result of reduced rates of protein synthesis that occur with diabetes. In healthy animals, endurance exercise (EE) can represent a stimulus for inducing skeletal muscle protein synthesis (PS) and hypertrophy. In diabetic rats it is unknown if a similar type of EE can stimulate increases in rates of PS and help normalize skeletal muscle growth. Using a 90% partial pancreatectomy model, this study investigates how three weeks of volitional wheel running affects PS, mTORC1 signalling and skeletal muscle growth in young adolescent T1DM muscle. Four week-old male Sprague-Dawley rats were randomly assigned to have either a Sham (S; n=18) or pancreatectomy surgery. Following a one week recovery period, the rats that received a pancreatectomy surgery, now diabetic, were randomly assigned to one of two groups: a sedentary Diabetic group (D; n=14) or Diabetic Exercise group (DE; n=12). The animals in the DE group were given free access to a running wheel. Compared to the S group, both the D and DE groups were hyperglycemic and displayed attenuated body mass gain by day 7 and 14, respectively (both $P<0.05$). Despite no differences in body size, gastrocnemius mass was significantly greater in the DE animals (1.19 ± 0.06 g) as compared to the D animals (0.95 ± .04 g) ($P<0.05$). Histochemistry analysis revealed that fibre loss occurred predominantly in diabetic animals and that this impairment was not present in DE animals. It is unknown if EE can improve the ability of other interventions, such as the ingestion of the amino acid leucine, to stimulate PS in diabetic muscle. To measure EE's effect on leucine ingestion, acute changes in PS rates and mTORC1 signalling following leucine administration were

tested in sedentary and diabetic animals. Prior to euthanization (day 28), all animals were fasted for 18 hours and one half of the animals in each of the S, D and DE groups received a 0.48g/kg gavage of leucine (+) while the other half received an equivalent volume of water (-). In the fasted state, DE had higher rates of PS in mixed gastrocnemius muscle than D (DE 4.42 % ± 0.84 vs. D 2.12 % ±0.33, $P<0.05$) and similar rates of PS to that observed in S (5.81 % ± 0.55, not significantly different from DE). The higher PS rates in DE compared to D occurred despite no changes in basal (-) 4E-BP1 or S6K1 phosphorylation. However, following leucine gavage (+), DE rats had increases in both 4E-BP1 (~1.5- fold) and S6K1 (~4 fold) phosphorylation levels, while D rats had attenuated responses in these mTORC1 signalling markers to leucine gavage. Protein synthesis rates in the DE (+) animals (6.20 % ± 0.63) were found to be significantly higher than the D (+) animals (2.92 ± 0.39) ($P<0.05$) and trended to be higher than the DE (-) animals (0.06). PS rates observed in the DE (+) animals were not significantly different from those observed in the S (+) and S (-) animals. This study shows that low volume endurance exercise normalizes PS rates and muscle growth in skeletal muscle of young rats with T1DM. These findings illustrate the critical role that regular exercise can have on skeletal muscle growth during periods of hypoinsulinemia in T1DM.

ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor, Dr. Michael Riddell, who supported me throughout my thesis with his patience and knowledge while allowing me to develop my own individuality as a researcher. One simply could not wish for a better or friendlier supervisor. I must also thank my co-supervisor, Dr. Olasunkanmi Adegoke, who provided me with constant guidance and support throughout my Master's program. Your tireless technical assistance helped me to succeed and without your help this project would not have been possible.

To my lab mates, Jonathan Campbell, Anna D'Souza, Carly Gordon, Jacqueline Beaudry, Ashley Peckett, Lisa Chu, Andrei Szigiato, Yaniv Shpilberg, thank you for your support in and outside the lab, and most of all, for our friendship and memories. I feel very fortunate to have been able to work along such great people.

Last but not least, to my family who has supported me along the way, thank you for your continuous love and encouragement over the years.

TABLE OF CONTENTS

LIST OF FIGURES

Manuscript Figures:

Supplemental Figures:

LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E binding protein
AA	Amino acids
AKT	Serine/threonine protein kinase Akt
AMPK	Protein kinase, AMP-activated, beta 1 non-catalytic subunit
ANOVA	Analysis of Variance
EDL	Extensor Digitorum Longus
EE	Endurance Exercise
eiF4A	RNA helicase (ATP dependent)/eIF4A
EIF4B	Eukaryotic translation initiation factor 4B
eIF-4E	Eukaryotic Translation Initiation Factor 4E
eIF4G	Eukaryotic translatión initiation factor 4G
D	Diabetic animal (sedentary)
D (-)	Diabetic that received a placebo gavage
D (+)	Diabetic that received a leucine gavage
DE	Diabetic Exercise Animal
DE (-)	Diabetic Exercise Animal that received a placebo gavage
DE (+)	Diabetic Exercise animal that received a leucine gavage
GβL	G-protein Beta-subunit-like protein
GH	Growth Hormone
HVPS34	Phosphoinositide-3-kinase, class 3
IGF-1	Insulin-like Growth Factor
MAP4K	Mitogen-activated protein kinase kinase kinase kinase 3
mRNA	Messenger RNA
mTORC1	Mammalian Target of Rapamycin
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
PI3K	Phosphatidylinositol 3 kinase
PS	Protein Synthesis
RAG	Recombination activating gene
RAPTOR	Regulatory Associated Protein of mTOR
REDD	DNA-damage-inducible transcript 4
RHEB	Ras homolog enriched in brain
S6K1	Ribosomal protein S6 kinase
S	Sham
S (-)	Sham animal that received a placebo gavage
S (+)	Sham animal that received a leucine gavage
T1DM	Type 1 Diabetes Mellitus
TSC	Tuberous sclerosis

INTRODUCTION 1.0

Type 1 diabetes mellitus (T1DM) is commonly associated with numerous pathological complications, such as macrovascular disease, neuropathy, nephropathy, and retinopathy (1). Diabetic skeletal muscle disease or 'myopathy', a much less studied complication of poorly controlled diabetes, is also a common clinical condition characterized by lower muscle mass, weakness and overall reduced physical capacity (2-6). It is well known that untreated T1DM is associated with abnormalities in protein metabolism and this protein loss may be a major cause of symptoms observed in diabetic myopathy (7, 8). A better understanding of protein metabolism in the skeletal muscle of T1DM patients and the factors that drive protein metabolism impairments in the skeletal muscle of T1DM patients is critical for the treatment of diabetic myopathy.

The impairments in protein metabolism associated with T1DM can be particularly detrimental in children, affecting normal growth and development. During childhood the ability of skeletal muscle to grow, adapt and regenerate is at a peak (9). Protein metabolism is a balance between protein synthesis and protein degradation. Increases in the overall size and strength of young growing muscle are therefore the result of positive changes in protein metabolism, which are predominantly driven by increases in rates of protein synthesis (10). In young T1DM patients, impaired rates of protein synthesis and increased rates of protein degradation may cause a lifetime of reduced physical and metabolic capacities. As a result, maintaining normal levels of protein metabolism rates should be a major goal in the treatment of T1DM in children as this can help preserve optimal growth and minimize the short and long-term effects of the disease (11, 12). To date, there have been few studies examining protein metabolism in the skeletal muscle of

paediatric T1DM patients and considerable research is required to better understand the factors that can enhance protein metabolism in these patients. Because increased protein metabolism is driven by increases in protein synthesis, it is particularly important for paediatric T1DM patients to better understand the factors that can increase protein synthesis.

LITERATURE REVIEW 2.0

2.1 Effects of Type 1 Diabetes Mellitus on Skeletal Muscle

2.1.1 Human Studies

Lower muscle mass and an overall reduced physical capacity are key trademarks of diabetic "myopathy" in children (3-6). The experimental challenges of studying this population however, have resulted in a limited amount of research concerning this aspect of the disease and its effects. Although some studies hypothesize that skeletal muscle mass loss in diabetic patients is caused by the neuropathy associated with the disease, several studies have highlighted that skeletal muscle is acutely sensitive to T1DM even prior to neuropathic complications. An early paper by Reske-Nielsen et al showed that in a group of recently diagnosed patients, fibre atrophy, disruption of Z-lines, and morphological abnormalities in mitochondria, all occurred prior to signs of neuropathy (13). Further supporting the belief that TIDM causes skeletal muscle mass loss is the study by Jakobsen et al which showed reduced muscle fibre size in newly diagnosed young males prior to insulin treatment (14) and studies which have shown that whole body protein degradation rates are significantly enhanced in TIDM adolescents as compared to non-diabetic adolescents (15). It is a well known fact that protein

metabolism is a correlate of muscle mass (16). This suggests that changes in protein metabolism may be a factor in the reduced muscle mass observed in young T1DM patients (8).

Unfortunately, no direct information is available on the effects of T1DM on regional skeletal muscle protein metabolism in paediatric populations. Normally the experimental techniques that are used to measure skeletal muscle protein metabolism require multiple tissue biopsies. These techniques are not appropriate in children, limiting investigators to the use of minimally invasive techniques that can only measure whole body protein turnover (7). Our limited understanding of protein metabolism in young T1DM patients is derived from these minimally invasive studies. Although using suboptimal methods, these studies have shown that improvements in protein metabolism can be stimulated by factors such as insulin and amino acids. These studies have also shown that improvements in protein metabolism have been achieved through reduced rates of protein degradation, while whole body protein synthesis has remained unaffected for the most part (17-19). The improved protein metabolism observed in these human studies independent of increases in protein synthesis is inconsistent with animal studies of T1DM which show that protein synthesis in the muscle tissue of young growing animals can be stimulated by insulin, exercise and amino acids (20, 21). The whole body measurements of protein metabolism used in human studies reflects changes in all bodily tissue. The different responses in the various tissues may mask the stimulatory effect of protein synthesis in skeletal muscle (7, 22-24). This methodological problem associated with the whole body tracer method of measuring protein metabolism in humans is likely the major reason for the discrepancies between human and animal studies of protein metabolism in

T1DM populations (7). Animal models, therefore, have played an essential part in understanding the specific mechanisms and effects of protein synthesis in young growing T1DM skeletal muscle.

2.1.2 Animal Models

Similar to human studies, animal studies have also shown that uncontrolled T1DM results in atrophy of diabetic skeletal muscle. In animals, this atrophy of skeletal muscle is typically fibre type-specific. Slow-twitch or type I fibres exhibit a minimal loss, while fast-twitch fibres, especially the fatigable glycolytic (type IIB) fibres, exhibit the most severe atrophy (12, 25-27). When changes in muscle mass were studied in relation to physical capacities, mixed result were achieved. Studies have shown that in T1DM animals, absolute contractile force is usually decreased. When this contractile force is expressed relative to muscle mass, however, studies have shown it to decrease, increase, or remain similar to non-diabetic control values (28). The basis for these discrepancies is not clearly understood, although it may involve the variability in duration of untreated diabetes, the broad range of muscle stimulation protocols employed or, in the case of pharmacologically induced diabetic rodents, the dose of drug administered (28, 29). Nonetheless, a relatively common finding is that muscle groups composed predominantly of slow-twitch muscle fibres are more resistant to loss of function than muscle groups composed of fast-twitch fibres. For instance, soleus muscle or isolated slow-twitch fibres of streptozotocin-diabetic rodents tend to exhibit no loss of force production, while the extensor digitorum longus (EDL) or isolated fast-twitch fibres are often reported to have impaired force production (26, 30-33)

Consistent with the fibre specific muscle mass loss and impaired physical capacities observed in T1DM rodents, fibre specific differences are also observed in the way protein synthesis rates are affected by T1DM. In muscle groups composed of glycolytic fibres there are severe reductions in protein synthesis, while in soleus, an oxidative muscle composed primarily of type I fibres the reduction in protein synthesis compared to healthy controls is almost negligible (34). The parallel change in muscle mass and protein synthesis in different fibre types in diabetic animals suggests that impaired protein synthesis can directly affect muscle size and function in T1DM animals (35-37). In the T1DM model, insulin deprivation is likely the major factor causing reduced rates of protein synthesis and ultimately impaired growth and function. However, several other hormones including glucocorticoids, insulin-like growth factor 1 (IGF-1) and growth hormone (GH) may also contribute to impaired protein synthesis in T1DM animals. The remainder of this review will focus on the effects of insulin, exercise and amino acids on protein synthesis and their role in diabetic models.

2.2 Skeletal Muscle Protein Synthesis (MPS)

2.2.1 mTORC1

The turnover of proteins in skeletal muscle involves the ongoing processes of protein synthesis and protein degradation. Lean body mass can be gained or lost due to changes in either of these processes, or simultaneous changes in both. A positive net protein balance occurs when protein synthesis exceeds protein degradation and a negative net protein balance occurs when protein degradation exceeds protein synthesis (38). Cellular protein stores in skeletal muscle are maintained in equilibrium; in other words in a state

where rates of protein synthesis and protein degradation are kept balanced. However, there are many physiological and pathophysiological influences that can alter this synthesis-degradation equilibrium (39). For instance, healthy skeletal muscle has well-known sensitivities to several environmental cues such as hormones, nutrients and mechanical loading. Environmental cues such as these normally converge at the cellular level to signal a common target, namely the mammalian target of rapamycin complex 1 (mTORC1) (21).

mTORC1 is a serine/threonine kinase whose signalling pathway has been shown to have a wide range of functions. It receives and integrates many different upstream signals that function as either activators or inhibitors of its activity. For this review, we are specifically interested in how mTORC1 plays a prominent role in the regulation of cell growth as a result of signalling from insulin, amino acids and exercise (40). Activation of the mTORC1 from these environmental cues results in both acute and long-term up-regulation of protein synthesis through mediating changes in the initiation phase of messenger RNA translation (41). Two of the best characterized substrates of mTORC1 are the 70 kDa ribosomal protein (rp) S6 kinase (S6K1) and eukaryotic initiation factor (eIF)4E-binding protein 1 (4E-BP1) (40). It is these two substrates that ultimately cause changes in mRNA translation initiation.

When un-phosphorylated, 4E-PB1 normally binds to eIF4E to form a 4E-BP1-eIF4E complex. When this occurs, eIF4E cannot bind with eIF4G, and consequently mRNA cannot bind to the ribosome to initiate mRNA translation (42). The phosphorylation of 4E-BP1 by mTORC1 releases eIF4E from the 4E-BP1-eIF4E complex and allows eIF4E to bind with eIF4G forming an eIF4E-eIF4G complex, which allows mRNA to bind with

the ribosome and initiate mRNA translation (43). There is a positive linear relationship between the amount of eIF4E-eIF4G complex and rates of protein synthesis (44). In one-dimensional SDS/PAGE, three bands corresponding to 4E-BP1 can be resolved, and these bands have been designated α, β and γ in order of decreasing electrophoretic mobility. The increase in phosphorylation of 4E-BP1 caused by insulin and amino acids is associated with a shift from the α to the β and γ bands. The 4EBP1 bound to eIF4E migrates mainly as the α band with some as the β band, whereas the phosphorylated forms of 4E-BP1, which do not bind to eIF4E, migrate as the β and γ bands (45, 46). In addition, mTORC1 also phosphorylates and activates the protein kinase S6K1. Although, in rodents and humans alike, there is a positive correlation between the level of S6K1 phosphorylation and increases in muscle mass, its exact mechanism remains to be elucidated. S6K1 has as many as nine possible phosphorylation targets. Of these possible targets, the most understood is S6K1's ability to activate eIF4B and PDCD4. PDCD4 in its un-phosphorylated state binds to eIF4A and eIF4G inhibiting the mRNA binding step (20, 47).

2.2.2 Insulin and Leucine

The fact that protein synthesis rates can be increased by the consumption of a mixed macronutrient meal has been well established (48, 49). Increases in insulin concentrations and amino acids in plasma following a meal are likely the two critical components that help stimulate this increase in protein synthesis in skeletal muscle. Insulin stimulates mTORC1 through phosphoinositide 3-kinase (PI3K) and AKT (or protein kinase B). Following phosphorylation from insulin, AKT acts to phosphorylate the TSC1 (tuberous sclerosis complex 1)–TSC2 complex. In the basal state, the TSC1–

TSC2 complex normally acts as a brake on mTORC1 signalling. Insulin mediated phosphorylation of TSC1–TSC2 protein complex relieves its inhibitory action on mTORC1 and allows for activation of translation initiation through 4E-BP1 and S6K1 (50). Similarly, amino acids work to not only provide substrate for protein synthesis, but also stimulate a number of cell-signalling pathways important in the regulation of mRNA translation through mTORC1 (40). Insulin and amino acid induced stimulation of mTORC1 are critical to skeletal muscle growth and health (10).

The branched chain amino acid leucine seems to be the most potent amino acid with regards to stimulating protein synthesis (51). In fact, leucine alone can fully substitute a meal in stimulating mTORC1 signalling, thereby accelerating mRNA translation initiation and stimulating protein synthesis (52). In Anthony et al.'s study they showed that following the administration of an oral leucine gavage the activity of the mTORC1 signalling pathway in muscle is increased, in a similar way that it would after a meal feeding (53, 54) Similar to meal feedings, leucine is also known to increase a subjects insulin levels (55). This raises questions as to whether the effects of leucine on protein synthesis are independent of insulin level increases. A series of studies has tried to explain whether leucine and other amino acids can stimulate PS through insulin-independent mechanisms.

In a study by Anthony et al. rats that had been deprived of food for eighteen hours were administered, via an oral gavage, one of four different solutions: saline, carbohydrate, leucine, or a combination of carbohydrate and leucine. The administration of leucine to the food-deprived rats was shown to stimulate protein synthesis, whereas the administration of carbohydrate alone had no effect. More importantly, leucine plus

carbohydrate had the same effect as leucine alone. This finding suggests that increasing insulin levels when administering leucine does not have the effect of further increasing protein synthesis levels beyond the levels observed when leucine alone is administered. The administration of leucine did, however, produce a slight, transient rise in plasma insulin concentrations in this study (54, 56). To further examine insulin's effect on leucine's ability to stimulate protein synthesis, this same group of investigators examined the effect of leucine on protein synthesis when the small increase in plasma insulin concentration, which was observed in the previous study, was blocked. In order to block the increase in insulin, somatostatin, an inhibitor of pancreatic hormone release, was used prior to the administration of leucine to maintain insulin concentrations at the fasting basal level. When insulin concentrations were maintained at the fasting basal level the effect of leucine on protein synthesis was attenuated (55). In addition, the somatostatin treatment also appeared to inhibit increases in 4E-BP1 and S6K1 phosphorylation that are normally observed with the ingestion of leucine. This study concluded that the transient increase in plasma insulin concentrations caused by the administration of leucine to food deprived rats may have a permissive effect on leucine's ability to stimulate protein synthesis.

It is important to note that the amount of leucine administered in the above mentioned studies was highly concentrated (approximately 1.35g of leucine per kg of bodyweight (g/kg)). This amount is insoluble in water and when administered as a suspension is equivalent to the typical amount of leucine that rats fed ad libitum would intake over a 24 hour period. Crozier et al. conducted a study to examine the effects of lower amounts of leucine. In their study, food-deprived rats were administered leucine in

amounts ranging from 0.068 to 1.35 g/kg. Their results showed leucine induced protein synthesis stimulation was possible with as little as 0.135 g/kg of leucine, which is only 10% of the amount used in Anthony et al.'s studies. In fact, 0.135 g/kg of leucine was able to stimulate protein synthesis to the same level as the higher leucine concentrations, although the higher leucine concentrations did appear to increase 4E-BP1 and S6K1 phosphorylation levels. Their results also showed that plasma insulin concentration increases were only observed with the higher concentrated amounts of leucine (57). The conclusion suggested by this study is that leucine can increase protein synthesis independent of increases in plasma insulin concentrations. Overall, these results suggest that leucine can stimulate protein synthesis largely through insulin-independent mechanisms, although basal insulin levels are important to obtain leucine's optimal effect on protein synthesis.

2.2.3 Amino Acid Regulation of mTORC1

Compared to insulin regulation of mTORC1, very little is known of how amino acids are sensed and how this information is detected by mTORC1. A number of different molecules have been implicated to play a role in this amino acid sensing. Three molecules have been specifically identified as regulators of mTORC1 signalling, namely, human vacuolar protein sorting 34 (hVps34, a class III PI3K) (58), a family of four related small GTPases called Rag proteins (59, 60) and a mitogen-activated protein 4 kinase kinase kinase kinase 3, MAP4K3 (61). Although, it is not understood through which mechanism amino acids are sensed and detected by mTORC1, it is likely that multiple regulators are required.

Several studies have looked specifically at the role hVps34 plays in the regulation of mTORC1 by amino acids. Unlike growth hormones that act through the class I PI3K pathway, amino acids mediate their effects through a separate class III PI3K, hVps34. This appears to be the case as inhibition of hVps34 blocks amino acid stimulation of S6K1 and over expression of hVps34 activates S6K1 in the absence of insulin stimulation. It was determined that hVps34 is upstream of mTORC1 through studies that found that hVps34 was not inhibited by the mTORC1 inhibitor rapamycin. Furthermore knockdown of hVps34 does not inhibit AKT or block insulin stimulated phosphorylation of TSC2 (62). Recent studies in mammalian cells have shown that in response to amino acids, hVps34 activates mTORC1 through increased cytoplasmic calcium. Gulati et al showed that amino acids induce a rise in intracellular calcium which causes direct binding of calcium/calmodulin to hVps34 and leads to mTORC1 activation (58). Interestingly, it should be noted that hVps34 is also known to play a critical role in autophagy (63). Therefore, this presents some controversy with regards to the role of hVps34 as a positive regulator of mTORC1. It may be possible that there are two distinct class III PI3K protein complexes, one associated with autophagy and one associated with positive regulation of mTORC1. However, this remains to be determined.

A second mechanism for the stimulation of mTORC1 by amino acids requires a family of four related small GTPases, Rag proteins. Rag proteins interact with mTORC1 in the presence of amino acids. However, Rag does not directly stimulate mTORC1 activity; instead it promotes translocation of mTORC1 to an endo membrane compartment, an event that promotes the interaction of Rheb and mTOR. Rag's role in amino acid sensing and activation was proven when it was shown that expression of

constantly active mutant forms of Rag eliminates the need for amino acids to activate mTORC1 and expression of a dominant negative Rag inhibits mTORC1 activity in the presence of amino acids (60). Despite this evidence, it is not known if Rag proteins themselves act as amino acid sensors, or if there is some other mediator involved (59, 60). Again, the idea that amino acids can stimulate a rise in calcium has been proposed to play a role in the response of Rag proteins. If calcium is a key mediator in this pathway, this would provide an interesting link between activation of both Rag protein and hVps34.

Finally, mitogen-activated protein kinase kinase kinase kinase 3, MAP4K3 has been identified as another upstream regulator of mTORC1 signalling. Similar to the other amino acid sensors, MAP4K3 activity is positively regulated by amino acid supply, but is not regulated by insulin or rapamycin (61, 64). Therefore, this means that MAP4K3 works as an intermediate step between increased amino acid supply and mTORC1 activity. Specifically, it has been shown that phosphorylation on Ser170 by amino acids regulates MAP4K3 activity and this phosphorylation is critical for the activation of mTORC1. Conversely, Ser170 appears to become de-phosphorylated when amino acids are not present resulting in loss of MAP4K3 activity and signalling to mTORC1 (64). Further work is required to elucidate how substrates and other proteins associate with MAP4K3 in an amino acid sensitive manner to up regulate mTORC1.

Although a definitive target for amino acid activation of mTORC1 has not been established, it does appear that multiple mechanisms might be involved. Amino acids and mTORC1 may be linked through a series of different pathways that allow the cell to

adapt to various environments. More work is needed to help assemble the various amino acid sensors into an understandable amino-acid-sensing model.

2.2.4 Exercise

Exercise produces many changes in protein metabolism and turnover in skeletal muscle (65-67). Acute changes in protein metabolism caused by exercise are driven by energy needs and amino acid availability, and as a result these changes are largely catabolic. During exercise and immediately after a bout of exercise, rates of protein degradation in muscle are elevated and a net negative balance between the rates of protein synthesis and protein breakdown results (67). The magnitude of these catabolic processes during and immediately after exercise is determined by the type of exercise, such as endurance or resistance and nutritional state. Both endurance and resistance exercise increase rates of protein degradation in a similar fashion following a bout of exercise. However, there are differences in the way protein synthesis is affected directly after each bout of exercise. As exercise reduces rates of muscle protein synthesis (68, 69). Exhaustive endurance exercise inhibits mTORC1 pathways. The mechanisms for this may be through increased eIF4E binding to 4E-BP1 and therefore reduced binding of eiF4E with the eIF4G initiation complex (68-71) and increased activity of the AMPK (72). When AMPK is stimulated, this leads to the phosphorylation of TSC2, formation of the TSC1/TSC2 complex, and inhibition of mTORC1. The reduction in protein synthesis following endurance exercise remains until energy is restored or nutritional stimulation is provided. Reductions in rates of PS occur in proportion to the duration and intensity of the activity. Once energy levels are restored following recovery from endurance exercise, rates of protein synthesis are increased and rates of protein degradation are decreased.

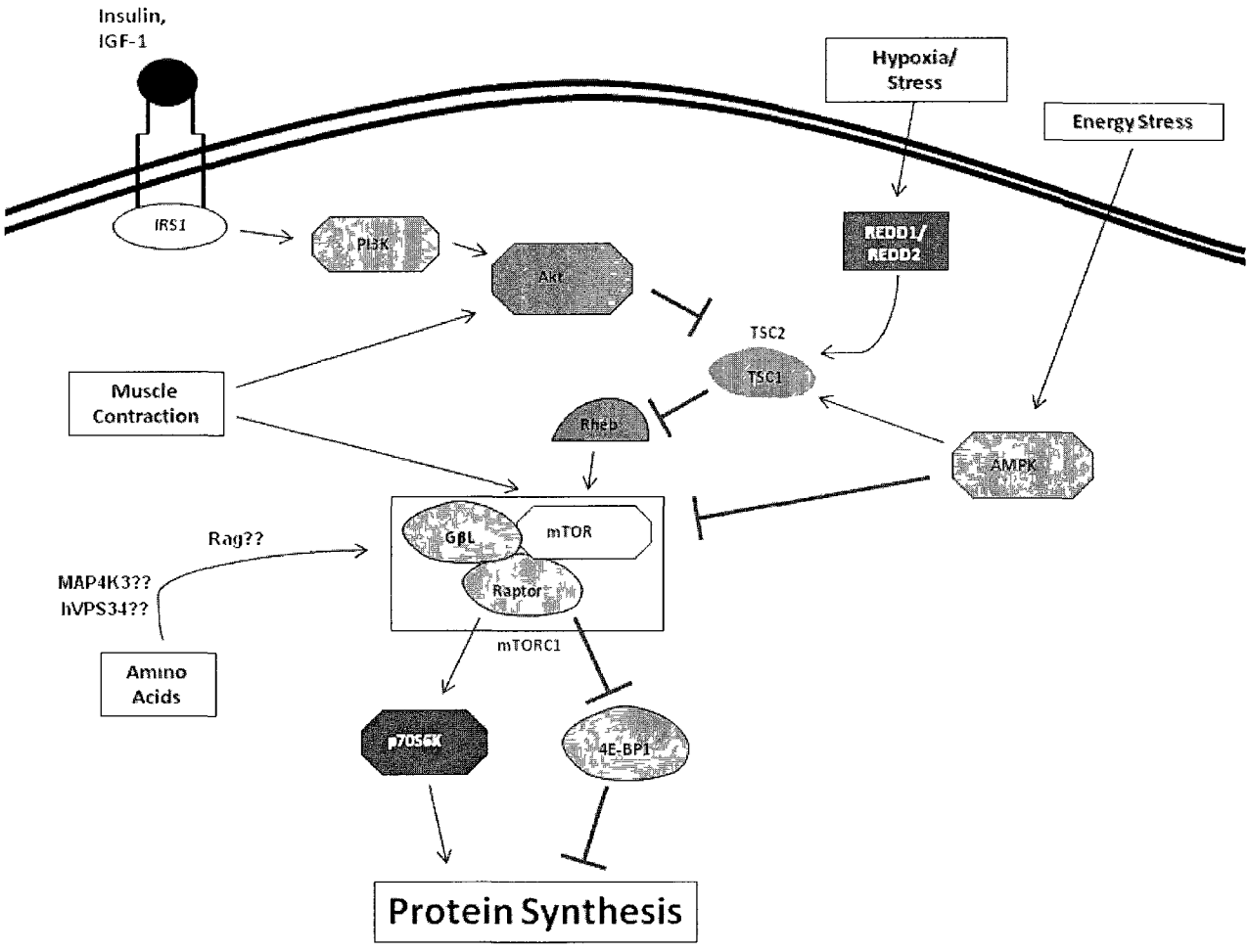
Resistance exercise, on the other hand, has the effect of increasing muscle protein synthesis soon after the bout of exercise and for a period of up to 48 hours. This increase in protein synthesis appears to be mediated through PI3-kinase and AKT signalling. A well known study by Bodine et al. was the first to provide convincing data implicating the mTORC1 signalling pathways in controlling translation initiation and promoting skeletal muscle hypertrophy in response to contraction (73). The study shows that chronic overloading of the plantaris muscle through synergistic ablation of the soleus and gastrocnemius, leads to increased phosphorylation of AKT and mTORC1. The central role of mTORC1 in resistance exercise-induced hypertrophy is shown when the drug rapamycin is used in vivo. Rapamycin, which effectively inhibits mTORC1, completely blocks the muscle hypertrophy associated with synergistic ablation (73) thereby highlighting mTORC1's role. Similar to this pioneering study, numerous other studies have shown increased phosphorylation of both 4E-BP1 and S6K1 in response to muscle loading in both acute and chronic bouts of resistance exercise in rodents and in humans alike (20, 66, 74-77).

2.2.3.1 Exercise and Leucine

Studies have shown that following endurance exercise plasma leucine levels may change. For example, in young athletes, when exercised to exhaustion, plasma leucine concentrations decrease dramatically and protein synthesis levels are acutely lowered (67, 78). For this reason, leucine supplementation may be beneficial in helping to increase protein synthesis rates post exercise. Gautsch et al. conducted a study which had rats run on a treadmill for 2 hour periods. This study showed that at the end of a 2 hour exhaustive run rats experienced a 25% decrease in muscle protein synthesis rates, when

compared to fasted controls that did not exercise. The exercised animals were then provided with a series of recovery drinks to evaluate the impact of carbohydrate and protein on muscle protein synthesis. An electrolyte drink containing glucose and sucrose increased blood glucose and insulin concentrations as well as muscle glycogen content but produced no recovery of muscle protein synthesis (79). However, a complete meal containing protein (or leucine alone) produced complete recovery of muscle protein synthesis within the first hour after the exhaustive bout of exercise. Supplemental leucine increases intracellular leucine concentrations, which directly stimulates mTORC1 allowing for post-exercise recovery of muscle protein synthesis (69). Another study showed that leucine, following a bout of endurance exercise, can stimulate muscle protein synthesis independent of increased plasma insulin (68).

When compared to non-exercised controls, rats that undergo resistance exercise do not experience a decrease in skeletal muscle protein synthesis rates (67). These rats also do not experience decreased levels of leucine after resistance exercise. A study by Mackenzie et al confirmed this by showing that following high-resistance contractions, mVps34 activity is stimulated by an influx of essential amino acids such as leucine, which may prolong mTORC1 signalling and contribute to muscle hypertrophy (80). Supplemental leucine allows for the muscle to achieve maximum protein synthesis and anabolic recovery (81).



Simplified scheme depicting a model through which both positive and negative factors can contribute to mTORC1 signaling and protein synthesis in skeletal muscle. mTORC1 functions as a central element in a signaling pathway involved in the control of cell growth and proliferation and is regulated by a wide variety of cellular signals, including growth factors, amino acids, muscle contraction, and stress conditions. Activation of mTOR results in phosphorylation of several downstream targets. For the protein mTOR to activate its signaling cascade, it must form the ternary complex mTORC1 containing mTOR, RAPTOR (Regulatory Associated Protein of mTOR) and G-BetaL (G-protein Beta-subunit-like protein.) Adapted from Miyazaki and Esser 2009 (21, 82).

Type 1 Diabetes and Skeletal Muscle Protein Synthesis

2.3.1 Insulin and Leucine

Shortly after insulin was discovered, it was observed that insulin treatment improved the muscle wasting of patients suffering from uncontrolled insulin-dependent diabetes (7, 8). Despite this well known anabolic effect of insulin on muscle protein in diabetic patients, controversy still existed as to what its mechanism of action was. There is still no clear consensus as to whether the primary effects of insulin involve a stimulation of muscle protein synthesis or just an inhibition of muscle protein breakdown in young diabetic patients (17, 19, 83). In diabetic animals, there is evidence that insulin can stimulate protein synthesis in vivo. Pain et al showed in two separate experiments that rates of protein synthesis were decreased in the muscle of diabetic rats, and that insulin treatment helped increase these rates (36, 84). Furthermore, it appeared in these studies that low doses of insulin were as effective as higher doses in achieving an anabolic response (36). Increasing insulin levels did not significantly increase rates of protein synthesis. This finding suggests that insulin typically has a permissive as opposed to stimulatory effect on skeletal muscle protein synthesis in diabetic rats. Therefore, insulin may be necessary to help nutrients such as amino acids stimulate protein synthesis.

Conversely in studies that have shown increased protein synthesis with insulin, amino acid availability has played an important part in insulin's ability to induce increases in skeletal muscle protein synthesis in diabetic models. In the few human studies that have been able to show increased whole body protein synthesis following hyperinsulinemia, elevated levels of amino acids were nearly always present (23, 24, 85).

This poses the question as to what the specific roles of amino acids, such as leucine are in stimulating protein synthesis in diabetics. To date only one study has specifically looked at the interaction between leucine and insulin in diabetic animals. This work was conducted by Anthony et al in alloxan treated rodents (86). Following the alloxan treatment, unlike in healthy rodents, plasma insulin concentration levels in diabetic rats did not change after an oral leucine administration. Similar to work that predated this study; this study showed that protein synthesis in the gastrocnemius muscle of these diabetic rats was severely reduced. Interestingly, Anthony et al.'s study showed that protein synthesis can be stimulated by an oral administration of leucine independent of insulin in diabetic animals. However, the magnitude of the increase in diabetic rats was much less than in control animals. In an attempt to restore these reduced levels of protein synthesis following an oral leucine gavage, insulin was administered in both physiological and super physiological doses. Interestingly, despite increased mTORC1 signalling, protein synthesis levels remained below those observed in sham animals that were also administered a leucine gavage.

Overall, the results presented by Anthony et al suggested that independent of insulin, leucine enhances protein synthesis through an independent signalling pathway that does not involve activation of 4E-BP1 or S6K1. However, the levels of protein synthesis observed in diabetic animals following leucine independent of insulin were significantly lower than levels observed in healthy animals also administered leucine. Only with the addition of insulin was protein synthesis and mTORC1 signalling restored to baseline levels observed in sham animals that were not administered leucine. Increases

in protein synthesis to levels observed in sham animals given leucine were not possible in diabetic rats even with hyperinsulinemia.

2.3.2 Exercise, Insulin and Diabetes

Some early studies by Goldberg et al. suggested that insulin is not necessary for the muscle hypertrophy in diabetic rats that occurs in response to overload synergistic ablation (87, 88). These results are consistent with another set of studies by Farrell et al. which used moderately diabetic rats and showed increased protein synthesis after acute resistance exercise. Farrell et al. also showed that an increase in muscle mass can occur in these moderately diabetic rats if chronic resistance exercise is performed over many weeks (89-91). These findings, however, are inconsistent with the work of Fedele et al. which showed that in severely diabetic rats, no change in muscle protein synthesis is observed in response to acute resistance exercise (92, 93). Taken together, the results of the studies summarized here suggest that a certain concentration of plasma insulin is required for both increased protein synthesis and increased muscle mass in response to resistance exercise.

There is a question as to whether there are compensatory factors that can assist in the stimulation of muscle protein synthesis during severe hypoinsulinemia. IGF-1 is a likely candidate because it can stimulate protein synthesis independent of insulin (94) and muscle IGF-1 concentrations increase with exercise. In the abovementioned studies using moderately diabetic rats, it was shown that IGF-1 increased in diabetic animals but not healthy animals. This is proof of the IGF-1 compensatory mechanism which helps restore muscle mass in exercised diabetic muscle devoid of insulin (95). To prove this point further, a study by Fedele et al. showed that passive immunization against IGF-1 before,

during, and after resistance exercise in moderately diabetic rats abolishes the exercise-induced stimulation of protein synthesis that was previously observed (96).

The effects of endurance training on protein synthesis in a model of diabetes to date have not been explained. This is true despite the fact that endurance exercise training is recommended for patients with diabetes mellitus because it improves glucose control and reduces other risk factors (97). It is interesting that the effects of endurance exercise have not been tested considering that in normal subjects, endurance exercise training increases capillarity (98) and improves vasodilatation in skeletal muscle. The increased capillarity and improved vasodilatation in skeletal muscle can in turn improve blood flow (99), which is required for the delivery of amino acids to skeletal muscles and the stimulation of protein synthesis (100). The improved delivery of amino acids to skeletal muscle in diabetic rats may help stimulate protein synthesis. Furthermore, endurance exercise has the potential to increase IGF-1 (96, 101), which as mentioned above, has the potential to increase protein synthesis and maintain muscle in diabetic muscle.

2.3.3 Elevated Glucocorticoids with Diabetes

Changes in levels of glucocorticoids associated with uncontrolled diabetes have been well documented (102) and should be considered as glucocorticoid hormones generally have negative effects on protein metabolism. In skeletal muscle, the effects of glucocorticoids are generally opposite to those of insulin as they produce a catabolic state. Several studies have shown how the administration of exogenous glucocorticoids in animals causes loss of body weight (103, 104), marked atrophy of skeletal muscle (105), and a reduction in rates of protein synthesis. Mechanistically, glucocorticoids act

to repress initiation of mRNA translation by decreasing phosphorylation of 4E-BP1 and S6K1 (106-108). This repression of 4E-BP1 and S6K1 can be explained by two novel repressors of mTORC1 signalling, namely REDD1 and REDD2 (regulated in development and DNA damage) (109). REDD1 functions upstream of TSC2 to down-regulate mTORC1 signalling in response to glucocorticoids. Therefore, this mTORC1 repressor is likely to play an important role in the control of translation initiation in skeletal muscle under a number of physiological and pathophysiological conditions such as diabetes (110).

PUROSE AND SPECIFIC OBJECTIVES 3.0

Overall Thesis Purpose

In paediatric T1DM populations, early detection and intervention to treat diabetic myopathy are of critical importance to ensure optimal muscle growth is not jeopardized. The overall purpose of this thesis was to test the efficacies of endurance exercise and leucine as non-pharmacological interventions for the treatment of diabetic myopathy.

Specific Objectives

1) To determine if volitional endurance exercise can effectively increase mTORC1 signalling, PS and muscle mass in uncontrolled T1DM rats.

2) To determine whether endurance exercise alters the muscular response to acute oral ingestion of leucine in diabetic animals.

Hypotheses

I hypothesized that the hypoinsulinemic state in uncontrolled T1DM will induce diabetic myopathy. This myopathy will be manifested through decreased body and skeletal muscle mass in glycolytic fibres. Exercise will help attenuate some of the detriments caused by this hypoinsulinemic state by increasing mTORC1 signalling and protein synthesis rates independent of insulin. Leucine supplementation will help to further enhance the effects of exercise on protein synthesis and mTORC1 signalling.

MANUSCRIPT 4.0

Volitional endurance exercise stimulates protein synthesis and has a permissive effect on leucine's ability to stimulate 4E-BP1 and S6K1 in uncontrolled T1DM animals.

Antonio S. Serino[1,2], Olasunkanmi AJ Adegoke[1,2], Sana Zargar[1,2], Carly S. Gordon[1,2], Andrei-Alexandru Szigiato[1], Thomas J Hawke[2,3] and Michael C Riddell[1,2].

[1]School of Kinesiology and Health Science and [2]Muscle Health Research Centre, York University, 4700 Keele St., Toronto ON. Canada, M3J 1P3; [3]Department of Pathology & Molecular Medicine. McMaster University, Hamilton ON. Canada, L8N 3Z5.

Running Title: Endurance exercise and protein synthesis in adolescent diabetic muscle

Key Words: type 1 diabetes mellitus, endurance exercise, leucine, mTORC1, protein synthesis, partial pancreatectomy, hypertrophy

Correspondence and request for materials should be addressed to:
Michael C. Riddell, Ph.D.
School of Kinesiology and Health Science, Muscle Health Research Centre
York University, 4700 Keele St.,
Toronto ON. Canada, M3J 1P3
Phone: 416-736-2100 ext 40493
Email: mriddell@yorku.ca

Contribution by the Authors

In this thesis I (Antonio Sandro Serino) was involved in the research development with my supervisor (Dr. Michael Riddell) and co-supervisor (Dr. Olasunkanmi Adegoke). I conducted approximately 85% of all its components. These components included pancreatectomy surgery, animal characteristic measurements (i.e. blood glucose, body weight, running distances ect.), isotope injection, gavage, tissue harvest, analysis of protein synthesis, western blotting, assays and data analysis. I was responsible in helping establish the pancreatectomy-endurance exercise model and establishing the protein synthesis protocol in my laboratory. Assistance was provided by Carly Gordon (a former graduate student in Dr. Riddell's laboratory) in teaching me how to perform the pancreatectomy surgery used in this study. Sana Zargar (a graduate student in Dr. Adegoke's laboratory) provided assistance and guidance in measuring fractional protein synthesis rates and Dr. Adegoke provided guidance in overall project development and assisted in the analysis of the protein synthesis data. Andrei-Alexandru Szigiato (A summer NSERC student in Dr. Riddell's laboratory) performed all cryosectioning, histochemical staining with the assistance of Carly Gordon. I performed all of the western blot analysis independently. Dr. Riddell is the primary investigator and supervisor of this project. The first draft of this thesis was written by me and the final draft includes edits and recommendations from the thesis advisory committee (supervisor Dr. Michael Riddell and Co-supervisor Dr. Olasunkanmi Adegoke).

Introduction

From the time of its early discovery, type 1 diabetes mellitus (T1DM) has been associated with impairments of protein metabolism and muscle wasting. When not managed intensively, T1DM patients experience elevated rates of skeletal muscle degradation and an impaired ability to increase skeletal muscle protein synthesis (PS) (7). Unfortunately, glycemic control is often suboptimal in youth with the disease (111) and this occurs at a time when there would normally be large gains in muscle development (28). Animal models of T1DM have become essential tools for understanding the muscle loss associated with the disease and the molecular pathways involved. Studies using diabetic animals have clearly demonstrated that diabetes results in muscle growth impairments, and specific fibre-type loss (11, 12). They have also shown that diabetic animals have severe reductions in PS rates (34, 36, 84). Reduced rates of PS can be a major cause for the changes in skeletal muscle morphology found in these models of T1DM (11). Increasing PS through nutrition and exercise may provide an effective way to help restore and maintain muscle mass in the T1DM model (112). Unfortunately, numerous endocrine and non endocrine disturbances in T1DM appear to prohibit increases in PS through reductions in mTORC1 signalling and other mechanisms (28). Strategies that complement insulin therapy and increase PS in T1DM, such as exercise and/or nutrient feeding, therefore, need to be explored.

In healthy animals, resistance exercise can provide an effective means to increase skeletal muscle PS and hypertrophy (21, 41, 65). However, when tested in partially pancreatectomized rats, mixed results have been reported. Although, in moderately diabetic animals a single bout of resistance exercise appears to stimulate PS (90, 91),

when a similar training program was tested in severely diabetic pancreatectomized rats, a bout of resistance exercise was not effective in stimulating rates of PS (92, 93). The severe hypoinsulinemia observed in severely diabetic animals appears to prohibit resistance exercise from inducing PS. These resistance exercise studies, however, only provide partial conclusions on the potential of exercise to increase PS rates in diabetic muscle. The effect of different forms of exercise, particularly endurance exercise, on diabetic animals requires investigation. In healthy animals, three weeks of volitional wheel running can represent a potent stimulus for inducing skeletal muscle PS and hypertrophy (113). In diabetic rats, while endurance exercise has been shown to help improve glucose control, it is unknown if it can stimulate rates of PS and normalize skeletal muscle growth.

The effects of endurance exercise on muscle mass in the diabetic animal may not be limited to contraction stimulated increases in basal (unstimulated by nutrients) PS rates. Endurance exercise may also cause favourable physiological changes in muscle such as increased blood flow and increased insulin-like growth factor 1 (IGF-1) release (96, 101). These changes may help improve the effects of interventions such as leucine administration that also increase protein synthesis (114). The amino acid leucine is a well known regulator of PS in-vivo (48, 57, 115). In healthy rats, leucine is a potent regulator of the mammalian target of rapamycin (mTORC1). By activating mTORC1, leucine effectively stimulates increases in rates of PS through changes in the initiation of mRNA translation (48, 116). In a study by Anthony et al., however, it was shown that T1DM reduces the effects of leucine in-vivo (86). The effects of endurance exercise on leucine stimulated PS in T1DM is unknown. Therefore, the goal of this study was two-

fold: 1) To determine if volitional endurance exercise can effectively increase PS and muscle mass in uncontrolled T1DM rats; and 2) To determine whether endurance exercise alters the muscular response to acute oral ingestion of leucine in diabetic animals.

Methods

Ethics Statement

All experiments were approved by the York University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines.

Animal Characteristics

Young, male Sprague Dawley rats (~age 1 month, 45-55g) were purchased from Charles River Laboratories (Montreal, QC, Canada). All animals had a seven day habituation period to a 12:12-hour light-dark cycle (lights on at 0800 and lights off at 2000) in a temperature (22–23°C) and humidity (50–60%) controlled room. The animals (body mass 100-120g) were then randomly assigned to one of three groups: Sham operated (S) (n =18), pancreatectomized (or diabetic) (D) (n =14) and pancreatectomized exercised (or diabetic exercise) (DE) (n= 12).

Pancreatectomy Surgery

A pancreatectomy model of diabetes was selected on the basis of previous studies showing that the effects of streptozotocin are not beta-cell specific (29) and therefore may cause distinctive contractile, metabolic, and phenotypic properties that are not present in non-pharmacological models of diabetes. On the seventh day following their arrival (Day 0), the animals underwent surgery to either remove 90% of their pancreas

(Diabetic and Diabetic Exercise) or to have a sham procedure (Sham) (117). For this, animals were anesthetized with 2% inhaled isofluorane and an incision was made extending from the xyphoid process of the sternum to hip level. Using cotton tip applicators, the splenic, gastric and duodenal regions of the pancreas were removed leaving all major vessels intact so as not to compromise the surrounding organs. The area within 2 mm of the common bile duct, extending from the duct to the first part of the duodenum, was left intact and classified as the residual pancreas (~10% of the initial pancreatic mass). Sham animals underwent the same surgical procedure but with no pancreas removal, only titillation of the pancreas with a cotton tip applicator. Following surgery, the animals recovered in standard rodent cages and received oral antibiotic treatment (amoxicillin) for seven days to help prevent infection.

Design

The sham and diabetic groups were housed 2 animals per cage and remained in their home cages for the entire duration of the study. On Day 7 post surgery, DE rats were separated and individually given 24 hour access to a standard running wheel (Harvard Apparatus). Blood glucose levels were measured (0900) via tail nick three times a week to follow glycemic levels but to minimize animal handling and stress. Body mass was also measured three times per week. Wheel revolutions were counted daily and multiplied by the wheel circumference (106 cm) to obtain daily running distances. On Day 26, fed plasma samples were collected in heparinized tubes (#16.443 SARSTEDT) via tail nick at 08:00h for a basal corticosterone measurement. All animals were given food (Lab Diet 5012) and water ad libitum.

On Day 27, all animals were food deprived for approximately 18 hours. Half of the animals in the Sham and D and DE groups (6-9 per group) were randomly selected and administered a leucine solution by oral gavage. To account for differences in running distances, Diabetic Exercise animals were pair matched according to their average running distance over the three weeks in which they had access to the running wheel. One animal from each matched pair in the Diabetic Exercise group was administered a leucine gavage, while the other was given a water gavage (i.e. nutrient placebo). For the leucine solution, 20 g of leucine (L-Leucine, cat # L8912, Sigma-Aldrich Canada), the maximum amount that could be successfully dissolved at 36° C, was dissolved in 1L of double distilled water. Standardized volumes of the solution (24 mL/kg) were administered. This amount of leucine gavage (0.48g/kg) was calculated to be approximately 40% of what is consumed in a 24 hour period by sham animals. For the water gavage, animals were administered an equivalent volume of double distilled water to control for the stress response that is produced by gavage administration (118). Twenty minutes after the oral gavage protein synthesis was measured using the flooding dose method developed by Garlick et al. (119). L-[2,3,4,5,6-^{3}H] phenylalanine (TRK648, GE Healthcare Canada) was combined with unlabelled phenylalanine (150mM in PBS, #5202 EMD Chemicals Canada) to give a 150mM 50μCi/ml solution of L-[2,3,4,5,6-^{3}H] phenylalanine to be injected into the animals. The animals were restrained in a cloth and given an intraperitoneal injection of the radioactive phenylalanine solution (1.0ml/100g of body weight). Ten minutes after the injection, the animals were sacrificed by decapitation. Blood was collected; gastrocnemius and soleus muscles were quickly removed and frozen in liquid nitrogen. Elapsed time from the point of injection to the

time of muscle freezing was recorded for each animal and taken as the duration of isotope incorporation. All samples were frozen at -80°C and subsequently pulverized at the temperature of liquid nitrogen.

Determination Muscle Protein Synthesis

The muscle powder of approximately 100 mg of gastrocnemius was homogenized in 1 mL of cold 2% $HClO_4$ and centrifuged at 2000 g for fifteen minutes. The supernatant was collected and to it ~ 0.5 mL of saturated potassium citrate was added. This sample was centrifuged at 2000 g for fifteen minutes and was used to determine the specific radioactivity of free phenylalanine in the precursor pool. Protein-bound phenylalanine was obtained by washing the pellet with 4 mL of $HClO_4$ four times and then hydrolyzing the protein in 5mL of 6M HCl for 24 hours at 110°C. HCl was removed via evaporation using a vacuum system and the amino acids were re-suspended in 1 mL of 0.5M sodium citrate. pH was adjusted to approximately 7 with the addition of NaOH.

An enzymatic conversion of [H^3] phenylalanine to β-phenethylamine was performed to allow for the separation of phenylalanine from other amino acids that may have also been labelled radioactively such as [H^3] tyrosine. For this, approximately 1.25mL of the supernatant and 0.8 mL of hydrolysate were incubated, with 0.20 mL or 0.285 mL respectively, of a 2 units/mL L-tyrosine decarboxylase solution (cat # T7927, Sigma-Aldrich Canada) for 20 hours at 50°C. β-phenethylamine was extracted by adding 0.5 mL of 3M NaOH and 5 mL of chloroform: n-heptane (1:3). Samples were then shaken and centrifuged at 500 g for 5 minutes. The organic layer from these samples was removed and 2.5 mL of chloroform and 1 mL of 0.1 M H_2SO_4 was added. Again samples

were shaken and centrifuged at 500g for 5 minutes. The upper aqueous phase was removed and used for the determination of phenylalanine specific activity of the pellet and supernatant.

An aliquot of both the supernatant and pellet was counted in a scintillation counter to determine radioactivity. Subsequently, another aliquot of both the supernatant and the pellet were assayed for phenethylamine using a modified version of a method developed by Suzuki and Yagi (120). For this, 0.1 mL of 2 mM L-leucyl-L-alanine, 0.5 mL of 1M potassium phosphate and 0.2 mL of 50 mM ninhydrin were added to specific volumes of the supernatant and the pellet. Samples were incubated for 30 minutes at 60°C, and then cooled in ice for fifteen minutes. Fluorescence was measured using a 96 well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek, USA).

Specific activity was calculated by dividing the radioactivity of each sample by its concentration of phenethylamine ((DPM/mL)/ (nmol/mL)). Fractional rates of protein synthesis (ks) were determined using the formula ks= (SB * 100)/ (SA * t), where t is the time interval between the time of injection and the freezing of sample in liquid nitrogen expressed in days, SB is the specific activity of β-phenethylamine in the protein bound amino acids and S_A is the specific activity of β-phenethylamine in the precursor pool.

AKT and markers of mammalian target of rapamycin (mTORC1) signalling

For analysis of the 4E binding protein 1 (4E-BP1), ribosomal protein S6 kinase (S6K1) and AKT, two separate portion of gastrocnemius and soleus muscle powder (100 mg) were homogenized on ice in 10 parts buffer (in mM: 20 HEPES, 2 EGTA, 50 NaF, 100 KCl, 0.2 EDTA, 50 glycerolphosphate, pH 7.4) supplemented with 1 mM DTT, 1 mM benzamidine, 0.5 mM sodium vanadate, 10 μl/mL of each of protease inhibitor

cocktail and phosphatase inhibitor 2 cocktail (P8340, P5726; Sigma-Aldrich Canada, Oakville, ON, Canada) and then centrifuged for thirty minutes at 13,000 RPM and at 4°C. Samples were quantified for protein concentration using the BioRad Protein Assay Kit (BioRad, Canada). Fifteen percent acrylamide gels for 4E-BP1 and 8 % acylamide gels for S6K1 and AKT were prepared by using the Bio-Rad electrophoresis equipment. Equal amounts of protein were loaded into each well. The gels were run at 120 V for two hours and then transferred onto PVDF membranes at 85 V for 3 hours. Nonspecific sites were blocked by incubation in 5% BSA in TBS-T and then incubated overnight at 4°C with primary antibody. For analysis of 4E-BP1, the primary antibody (#9644) was diluted 1:10,000 ratio in 5% BSA. For analysis of phosphorylated S6K1 (Thr 389), total S6K1, phosphorylated AKT (Thr 308) and total AKT, the primary antibodies (#9234, #9202, #9275, #9272 respectively) were diluted 1:1000 ratio in 5% BSA. All primary antibodies were purchased from Cell Signaling Technologies (New England Biolabs Canada). Secondary antibody (ab6721 Abcam Inc., U.S.A) was diluted 1:10,000 ratio in 5% BSA. Enhanced chemiluminescent detection (WBKLS0500 Millipore, Canada) and Kodak Image Station were used to visualize the bands. Results for 4E-BP1 are expressed as the fraction of γ (the most phosphorylated) to the total of α, β and γ. Phophorylated S6K1 and AKT were expressed relative to total S6K1 and total AKT respectively. Due to the large sample size, six different membranes were used for each of the above mentioned analyses. To control for variability between membranes, each sample was set relative to the Sham group that did not receive leucine on the same respective membrane.

Plasma insulin and Corticosterone determinations

Fed plasma samples collected on Day 26 were analyzed for corticosterone levels using a radioimmunoassay (#07120102 MP Biomedicals, USA). On Day 28, before administering the gavage to all the animals, a fasted blood sample was collected via tail nick to determine post fast insulin levels. Following the gavage another blood sample was collected from each animal to determine changes in insulin levels as a result of each animal's respective gavage. Levels were determined using a commercially available enzyme-linked immunosorbent assay kit (# 90060 Crystal Chem, USA) and read in a 96 well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek, USA).

Histochemistry

To identify skeletal muscle fibre type, a metachromatic myosin ATPase stain was performed on 10μm thick cross sections of the soleus, gastrocnemius and plantaris muscles using a modified Ogilvie and Feeback protocol (121). Sections were pre-incubated in an acidic buffer, pH = 4.40, to differentially inhibit myosin ATPases within the different fibre types. In this protocol using light microscopy, in gastrocnemius, type I fibres appear dark blue, type IIa appear white and type IIb and IId are not discernible from each other and classified as IIb/d. These fibres appear bluish-purple. The mixed gastrocnemius and soleus regions of each group were identified and a representative image of each muscle region were acquired for analysis. Over 200 fibres were counted per microscope image, per animal, to determine fibre type composition in a given area. Average fibre area was quantified based on the average area of ~21 fibres for each fibre type per image, with the experimenter being blinded to the groups (12). Quantification analysis was performed with Adobe Photoshop CS version 8.0 and reported in μm^2. All

images were acquired with a Nikon Eclipse 90i microscope and Q-Imaging MicroPublisher 3.3 RTV camera with Q-Capture software.

Data analyses

Carestream Molecular Imaging Software (Carestream Health, USA) was used to measure the density of S6K1, AKT and 4E-BP1 protein expressions. Statistical analyses were completed using Statistica 7.0 statistical software (StatSoft®, Tulsa, OK), with $P < 0.05$ as the criterion for statistical significance. All data are expressed as mean ± standard error of mean (SEM). Two-way factorial ANOVAs, followed by a Fisher post hoc test, where necessary, was used for the comparisons of blood glucose, body mass, insulin, AKT, 4E-BP1 and S6K1 levels. A one-way ANOVA was used to compare fasted blood glucose, running distances, corticosterone levels, muscle mass, fibre area and protein synthesis. Again, a Fisher post hoc test was performed where necessary.

Results

Blood Glucose and Body Weight Measurements

Whole blood glucose concentrations and body masses for the Sham, Diabetic and Diabetic Exercise groups over the course of the study are shown in Figure 1A. Seven days after their respective surgeries, the D and DE animals had markedly elevated blood glucose levels as compared to the Shams ($P<0.05$). Glycemia remained elevated in D and in DE compared to S throughout the remainder of the experimental period ($P<0.05$). Specifically, DE had significantly lower blood glucose levels than D on Day 10, 14, 17 and 21 (all, $P<0.05$). Fasted blood glucose concentrations, measured on day 28, were significantly elevated in D when compared to Sham (both $P<0.05$) (Figure 2).

Body masses for all animals are shown in Figure 1B. By Day 14, the D and DE animals were significantly smaller than the S animals and this attenuation in overall body mass was evident in both D and DE rats throughout the rest of the study ($p<0.05$). No significant differences in body masses were observed at any time in the study between the D and DE animals. Running distances for each animal averaged 2200 ± 97 meters per night in the DE group during the 3 weeks (Figure 3), except on the evening of the overnight fast where they increased to 4500 ± 1196 meters ($p<0.05$).

Measurements of Changes in Skeletal Muscle

Gastrocnemius and soleus muscle weights, at the time of sacrifice, for all animals are shown in Figure 4 and Figure 6, respectively. When absolute muscle mass was compared, both the D and DE animals had significantly smaller muscles than the S animals ($P<0.05$). DE animals had significantly greater gastrocnemius and soleus muscle mass than D ($P<0.05$). Because both diabetic groups were significantly smaller in mass than the S animals, gastrocnemius and soleus muscle mass was normalized for body weight. When normalized for body weight, in the gastrocnemius , the S animals still had a significantly greater muscle mass than both diabetic groups ($P<0.05$) and DE had a greater normalized muscle mass than D ($P<0.05$). In the soleus, when normalized for body weight, the S animals had significantly greater muscle mass than D ($P<0.05$) and not DE.

Histochemistry analysis (Figure 5 and Figure 7) revealed that diabetes results in reduced fibre size in both gastrocnemius and soleus muscle. In the gastrocnemius glycolytic or Type IIb and IId fibre sizes were significantly affected by diabetes as indicated by the significant difference between S and D animals in these fibres ($p<0.05$).

The effects of diabetes on oxidative fibres appears to be less pronounced in gastrocnemius as type I fibre and type IIa fibre sizes were not significantly different between groups. In soleus, however, oxidative fibres appear to be affected by diabetes as D animals have significantly smaller fibres when compared to S animals ($p<0.05$). Exercise helps fibre size regardless of fibre type as S and DE did not have significantly different fibre sizes in either gastrocnemius or soleus muscles.

Corticosterone and Insulin

Glucocorticoid hormones are known to affect protein synthesis in skeletal muscle by reducing rates of PS (20). Since both diabetes (102) and exercise (122) have been shown to independently increase glucocorticoid levels in rodents, basal corticosterone concentrations were measured. Both D and DE animals had significantly elevated corticosterone levels when compared to the S animals (Figure 8, $P<0.05$). Exercise, however, did not cause a significant difference in baseline corticosterone levels between the two diabetic groups.

Insulin levels were measured to confirm diabetic animals were hypoinsulinemic (Figure 9) and to measure observable changes as a consequence of oral leucine administration. Pre-gavage administration, insulin levels were not significantly different for any group of animals. Post-gavage, however, the S animals that received a placebo water gavage had a small but significant increase in insulin levels ($P<0.05$). This increase was not observed in the D and DE animals that also received a double distilled water gavage. Post-gavage, the S animals that received a leucine gavage experienced a significant increase in insulin levels ($P<0.05$). Again, this increase was not observed in the D and DE animals that also received a leucine gavage. In the S group, insulin levels

post gavage in animals that received a water gavage and in those that received a leucine gavage were not significantly different.

Protein Synthesis

Protein synthesis rates are shown in Figure 10. Sham animals had significantly higher levels of PS in the basal state (i.e. water gavage) when compared to D ($P<0.05$). There was no significant difference, however, between the DE and Sham basal PS rates. Direct comparisons between diabetic animals (D vs DE) revealed that exercise was effective in significantly increasing baseline rates of PS ($P<0.05$).

As expected, Sham animals that received leucine had significantly higher rates of PS than the Sham baseline animals ($P<0.05$). Moreover, DE animals that received leucine tended to have higher rates of PS than the DE baseline animals ($p = 0.06$). Leucine gavage did not stimulate a significant increase in PS rates in the D animals. When comparing animals that received a leucine gavage only, the Sham animals and DE both had significantly higher rates of PS compared to the D ($P<0.05$). Rates of leucine stimulated PS in Shams tended to be higher than in DE ($P =0.055$).

AKT and markers of (mTORC1) signalling

In the gastrocnemius, 4E-BP1 phosphorylation (Figure 11A) was measured for each group and was found to be significantly elevated in the Sham and DE animals after the administration of leucine compared with water gavage ($p<0.05$). Sham animals had an ~2- fold increase and the Diabetic Exercise animals had an ~1.5- fold increase as compared to water gavage animals in their respective group ($p<0.05$). S6K1 phosphorylation at threonine 389 was measured (Figure 11B) and was also found to be significantly elevated in the Sham and DE that were administered leucine compared to

placebo gavage ($p<0.05$). The Sham animals had a 9-fold increase and the Diabetic Exercise had a 4-fold increase ($p<0.05$). In contrast, D animals did not experience any significant changes in S6K1 phosphorylation following leucine gavage. Levels of AKT phosphorylation were measured at threonine 308 (Figure 13). No significant differences were observed between any of the groups of animals regardless of type of gavage. In the soleus, 4E-BP1 phosphorylation (Figure 12A) was measured for each group and it was found that leucine stimulated similar increases in 4E-BP1 phosphorylation in all groups.

Figures

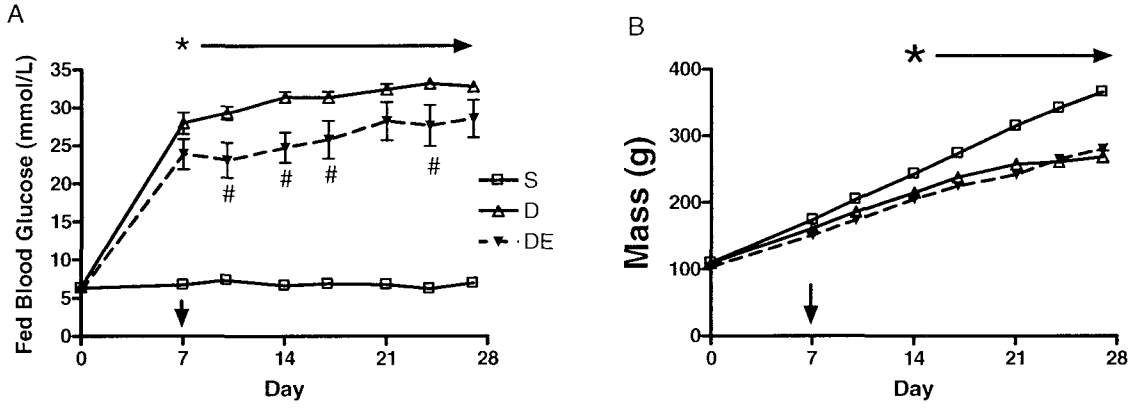


Figure 1:
Fed blood glucose levels and body masses in the Sham (S), Diabetic (D) and Diabetic exercise (DE) groups. (A)Blood glucose levels were significantly higher in the D and DE groups as compared to the S group beginning at Day 7 and throughout the rest of the protocol. The DE group had significantly lower blood glucose levels than the D group on Days 10, 14, 17 and 21. (B) Body mass was significantly lower in the D and DE groups versus the S group at Day 14 and throughout the rest of the protocol. The arrow indicates the time at which the running wheel was introduced to the DE group. * → Indicates the presence of a significant difference between the D and DE groups as compared to the S group at $P<0.05$. # indicates the presence of a significant difference between the DE and D groups at $P<0.05$; n= 10-12 per group

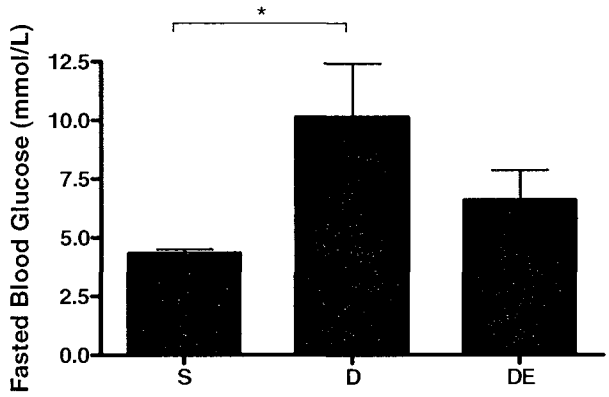


Figure 2:
Blood glucose levels in the S, D and DE groups following an 18 hour fast. Blood glucose levels were significantly higher in the D group when compared to the S group. No significant difference was found between the DE and S group. * Indicates the presence of a significant difference at P <0.05; n= 10-12 per group

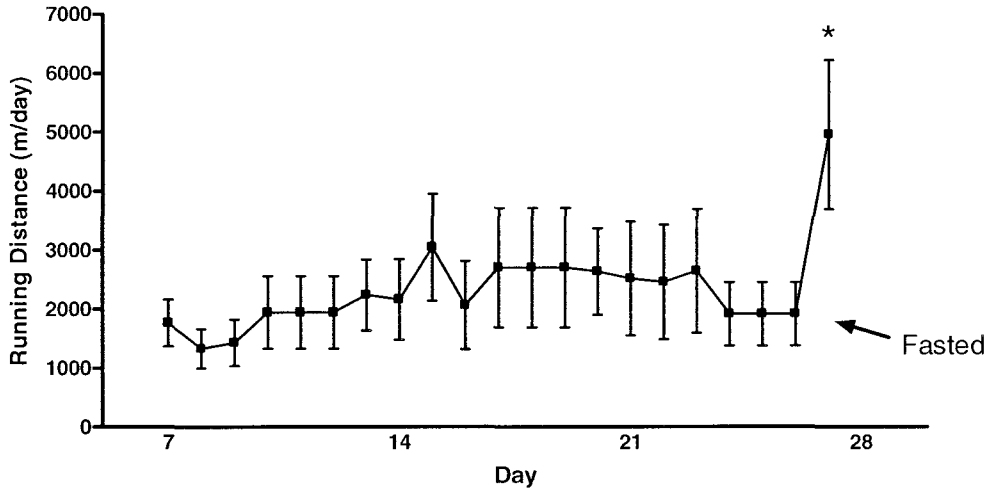


Figure 3:
Daily average running distances for animals in the DE group. Running distances for each animals averaged 2200 ± 97 meters per night in the DE group during the 3 weeks, except on the evening of the overnight fast where they increased to 4500 ± 1196 meters per night * Indicates the presence of a significant difference at P <0.05; n=12.

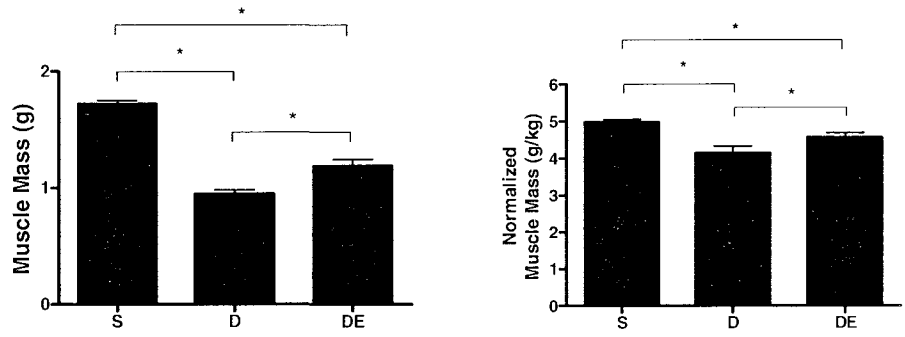


Figure 4:
Absolute and normalized gastrocnemius muscle mass in the S, D and DE groups. Absolute muscle mass was significantly lower in both the D and DE groups when compared to the S group. However, the DE group did have significantly more muscle mass than the D group. When normalized for body weight, the same results remained true. * Indicates the presence of a significant difference at P <0.05; n= 11-13 per group

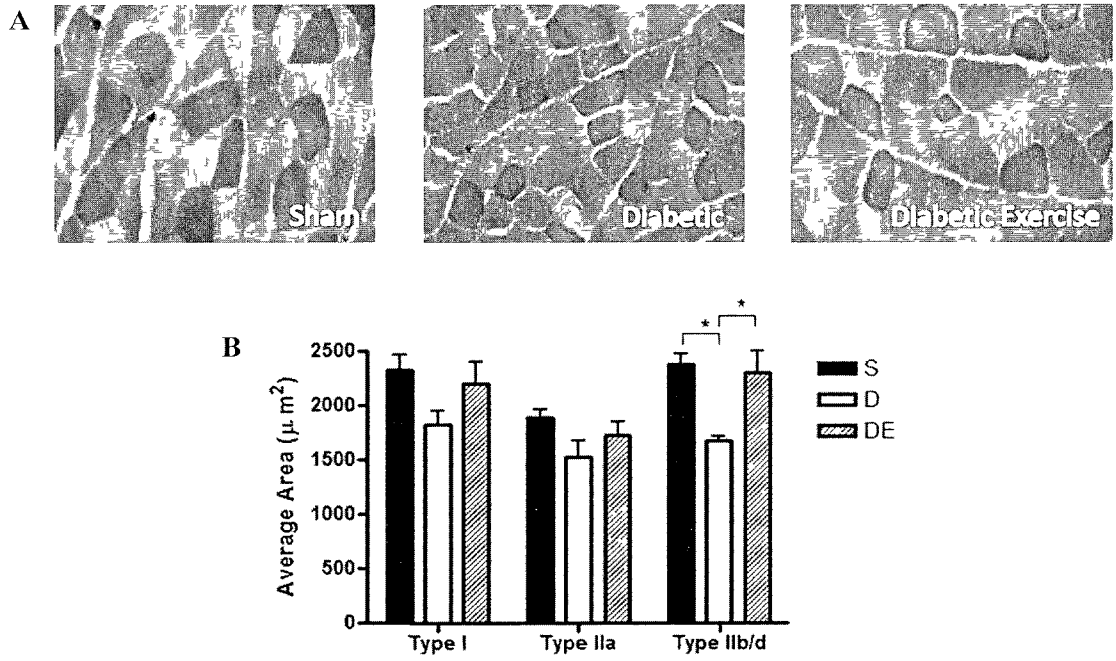


Figure 5:
Representative images of metachromatic fibre type and differences in fibre area in gastrocnemius of S, D and DE groups. A) Gastrocnemius muscle stained for metachromatic fibre type in S, D and DE. Note the visibly smaller fibres in D rats but not DE rats. Type I fibres appear dark blue, type IIa appear white and type IIb and IId are not discernible from each other and classified as IIb/d. These fibres appear bluish-purple. B) Examination of mixed gastrocnemius fibre area revealed that D rats had significantly smaller fibres in type IIb/d fibres compared to S and DE ($p < 0.05$). * Indicates the presence of a significant difference at P <0.05; n= 6-8 per group

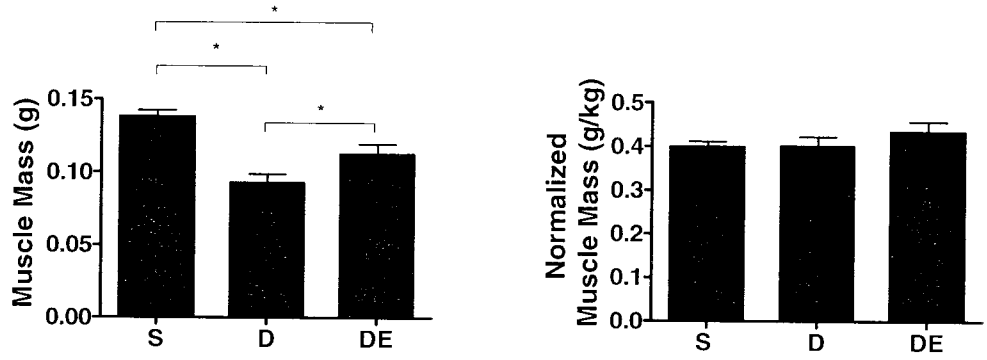
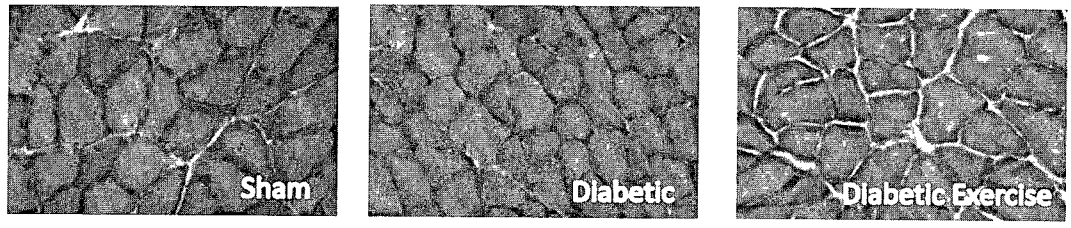


Figure 6:
Absolute and normalized soleus muscle mass and fibre area in the S, D and DE groups. Absolute muscle mass was significantly lower in both the D and DE groups when compared to the S group. However, the DE group did have significantly more muscle mass than the D group. When normalized for body weight, no significant differences were observed. (n= 11-13 per group)

A



B

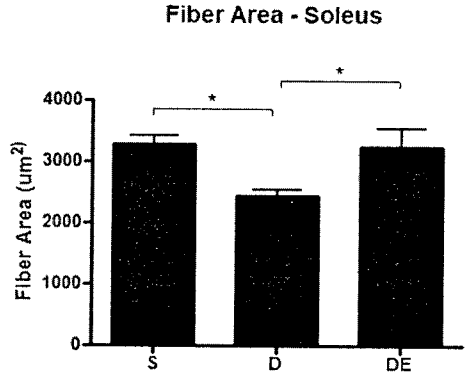


Figure 7:
Histochemistry of soleus muscle. A) Representative images taken at 10X Magnification, slides were 10 microns in thickness. B) Fibre area was significantly lower in the D groups but not DE group when compared to the S group. (n= 6-9 per group). * Indicates the presence of a significant difference at $P < 0.05$

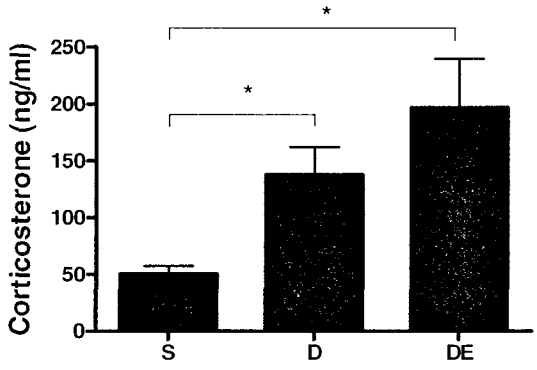


Figure 8:
Basal corticosterone concentration (0800) and differences in the S, D and DE groups. Basal corticosterone concentration was elevated in the D and DE groups as compared to the S group. Exercise in the DE group did not increase basal corticosterone concentrations when compared to the D group; n=7-9per group.

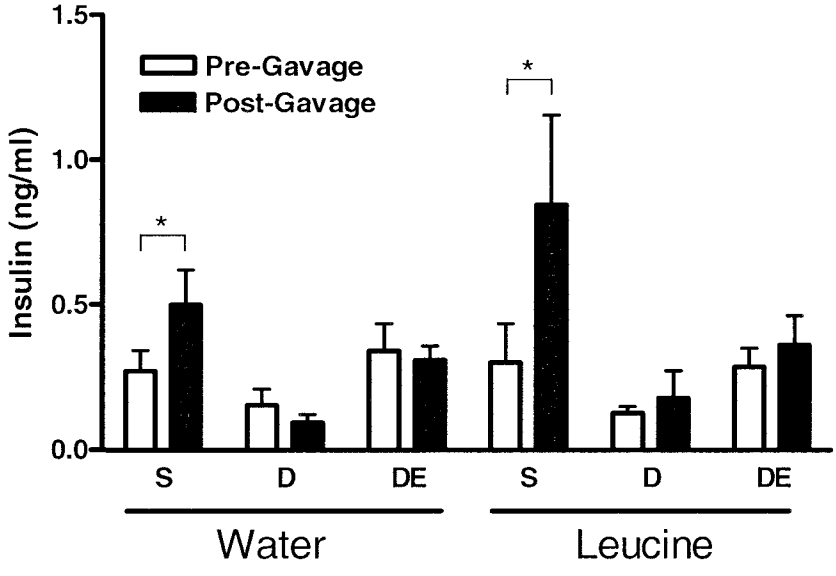


Figure 9:
Insulin concentrations after leucine and water gavage administrations in S, D and DE groups. Baseline insulin levels were not significantly different between any of the groups following an 18 hour fast. S animals that received water gavage had a small but significant increase in insulin levels. S animals that received a leucine gavage also had a significant increase in insulin levels. S post-water gavage levels and S post-leucine gavage levels were not statistically different. * Indicates the presence of a significant difference at $P < 0.05$; n=5-6 per treatment per group.

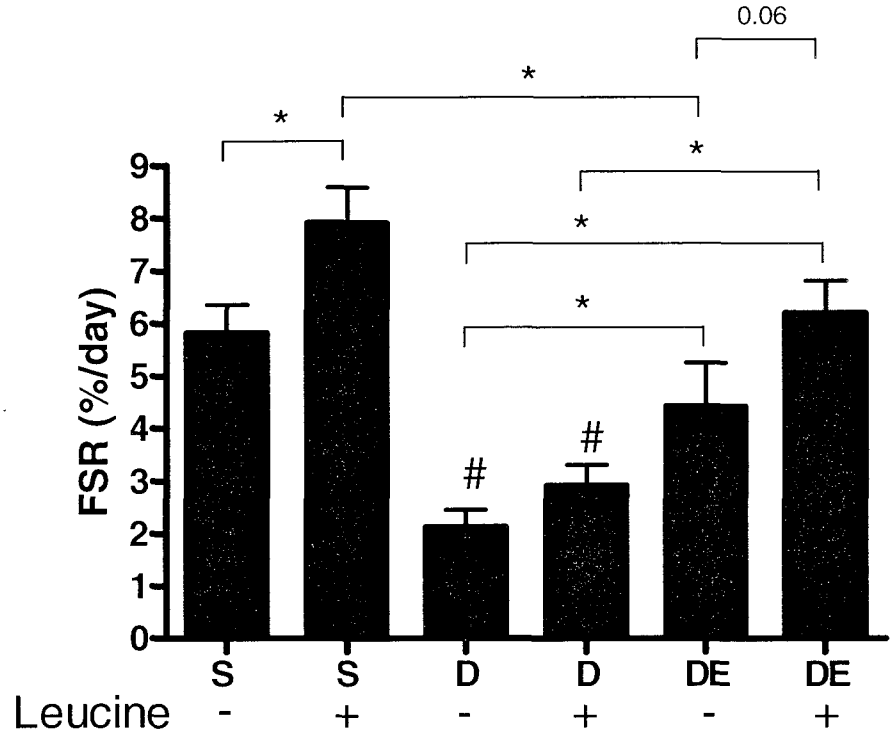


Figure 10:
Protein synthesis rates (PS) in gastrocnemius muscle following a leucine (+) or water (-) gavage administration in the S, D and DE groups. The DE (-) group had significantly elevated PS rates when compared to the D (-) group, suggesting that exercise had the effect of increasing baseline PS rates in diabetic animals. The DE (-) group and the S (-) group did not have a significant difference in PS rates. Leucine increased PS rates significantly in the S (+) group when compared to the S (-) group. The DE (+) group trended to have increased PS rates when compared to the DE (-) group (P=0.06). The DE (+) group did not have significantly different PS rates when compared to the S (+). * Indicates the presence of a significant difference at $P < 0.05$; # indicates a significant difference between D (-) and D (+) when compared to S (-); n=6-9per treatment per group.

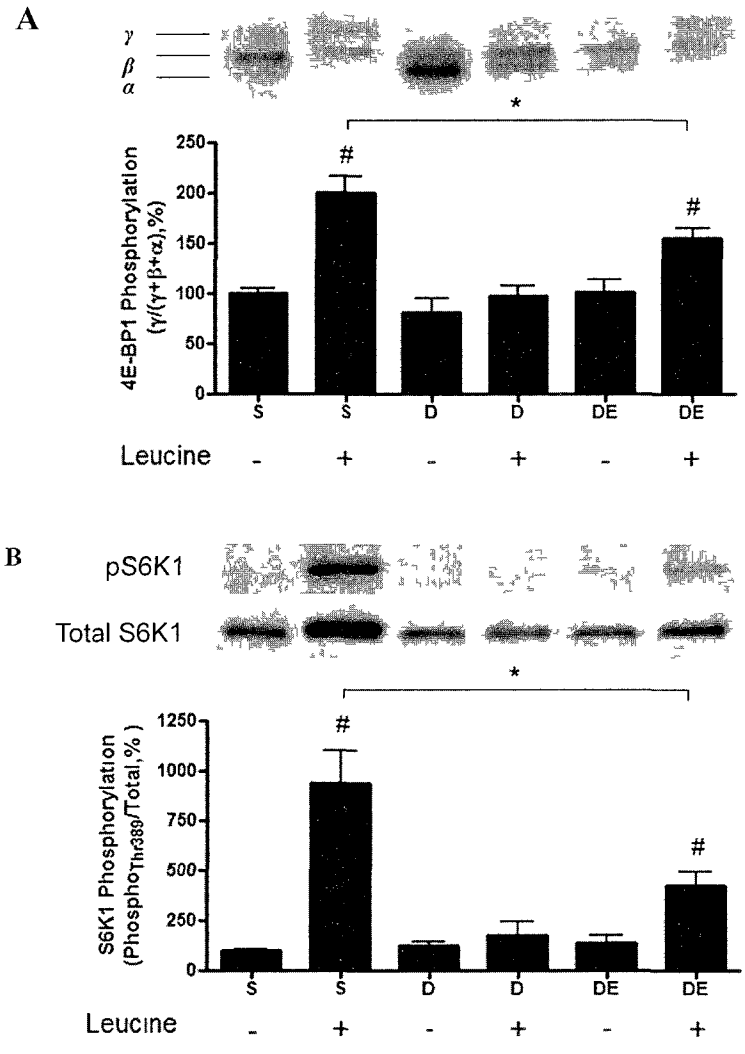


Figure 11:
Phosphorylation of (A) 4E-binding protein 1 (4E-BP1) and (B) S6K1 (threonine 389) following a leucine (+) or water (-) gavage in the gastrocnemius muscle of the S, D and DE groups. 4E-BP1 and S6K1 (threonine 389) phosphorylation are expressed as a percentage of the S (-) group. (A) There was a significant increase in 4E-BP1 phosphorylation in the S (+) group when compared to the S (-) group and a significant increase in the DE (+) group when compared to the S (-), D (-), D (+) and DE (-) groups. Compared to the S (+) and DE (+) groups, the S (+) group had significantly increased 4E-BP1 phosphorylation. Similar to 4E-BP1 phosphorylation, there was a significant increase in S6K1 (threonine 389) phosphorylation in the S (+) group when compared to the S (-) group and a significant increase in the DE (+) group when compared to the S (-), D (-), D (+) and DE (-) groups. Between the S (+) and DE (+) groups, the S (+) group had significantly increased S6K1 (threonine 389) phosphorylation. # indicates a significant difference when compared to respective basal gavage. * indicates a significant difference when between S (+) and DE (+). $P < 0.05$; n=6-7per treatment per group.

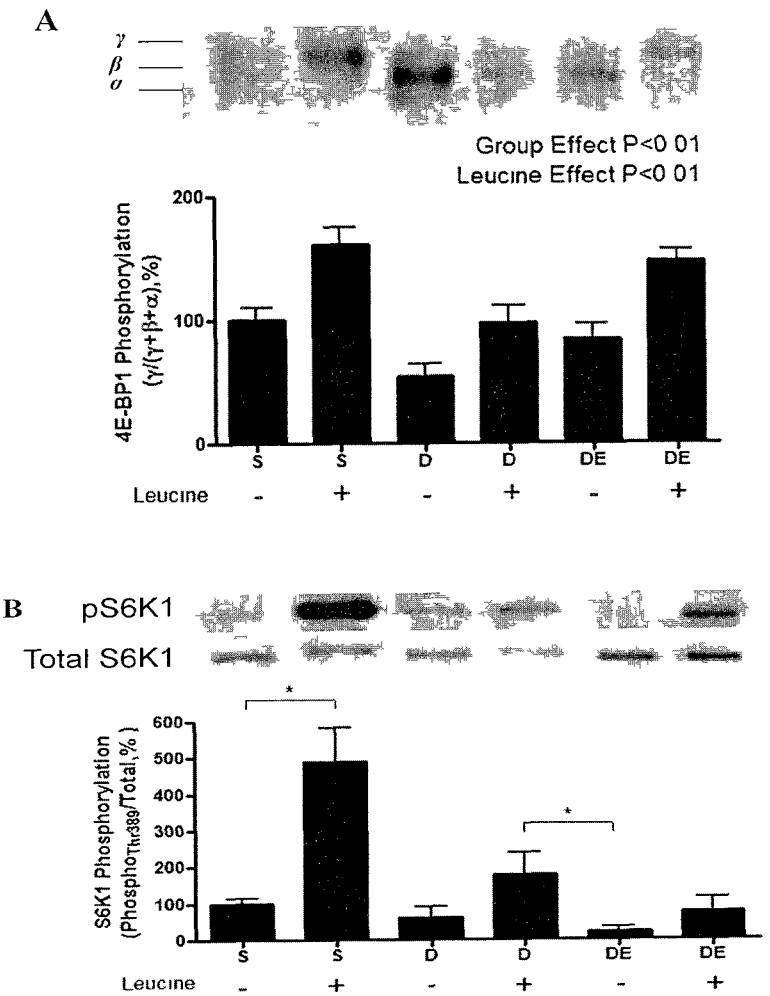


Figure 12:

Phosphorylation of (A) 4E-binding protein 1 (4E-BP1) and (B) S6K1 (threonine 389) following a leucine (+) or water (-) gavage in the soleus muscle of the S, D and DE groups 4E-BP1 and S6K1 phosphorylation are expressed as a percentage of the S (-) group (A) There was a significant effect of group and feeding in soleus, representing a similar response to leucine amongst all groups (B) There was a significant increase in S6K1 (threonine 389) phosphorylation in the S (+) group when compared to the S (-) group and a significant increase in the D (+) group when compared to the DE (-) group *Indicates the presence of a significant difference at P <0 05, n=6-7per treatment per group

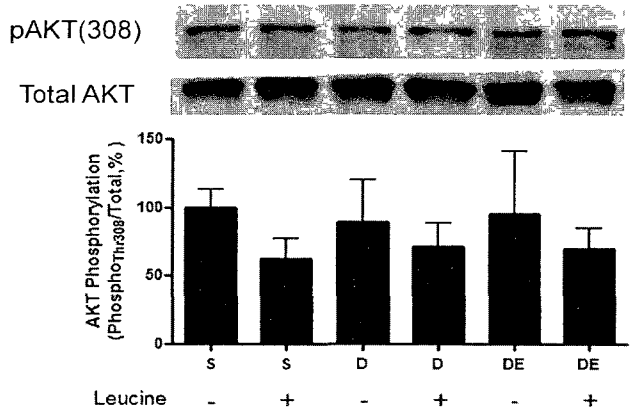


Figure 13:
Phosphorylation of AKT (threonine 308) following a leucine (+) or water (-) gavage in the gastrocnemius muscle of the S, D and DE groups. AKT (threonine 308) phosphorylation is expressed as a percentage of the S (-) group. There were no significant differences in AKT (threonine 308) phosphorylation between any of the groups regardless of type of gavage; n=6-7per group.

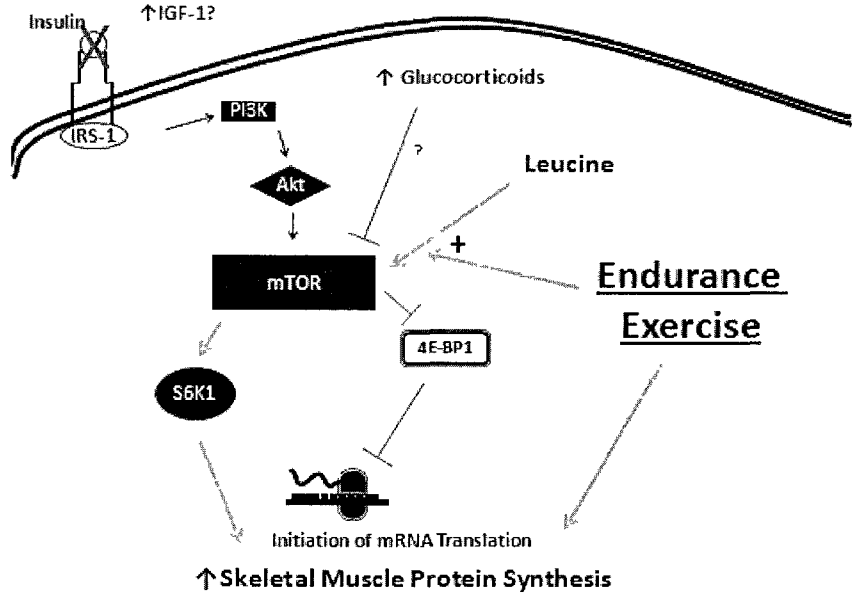


Figure 14:
Simplified scheme depicting possible mechanism of endurance exercise induced increases in skeletal muscle PS in a model of T1DM. In this model, endurance exercise increases PS independent of mTORC1 and insulin and has a permissive effect on the amino acid leucine's ability to activate mTORC1 signalling. Green arrows indicate activation; red solid lines indicate inhibition.

Discussion

The main findings of this study are that rates of PS are dramatically attenuated, both in the fasting state and in response to nutrient feeding (i.e. leucine gavage) in young T1DM rats under poor glycemic management. Furthermore, muscle growth is blunted and this occurs more readily in glycolytic fibres and to a lesser extent in oxidative fibres. Low volume endurance exercise largely normalizes these deficiencies. Although the effect of resistance exercise on skeletal muscle protein synthesis in a diabetic model has been studied to some degree (92, 93) and with limited success in rescuing muscle atrophy, this study is the first to investigate endurance exercise as an effective method of stimulating protein synthesis in T1DM animals. Importantly, we show that, in diabetic animals, endurance exercise has a permissive effect on leucine's ability to stimulate 4E-BP1 and S6K1 signalling and as a result helps stimulate skeletal muscle protein synthesis. The increased levels of PS and increased muscle mass experienced after regular endurance exercise, suggests that endurance exercise may help attenuate growth impairments in young skeletal muscle, which are caused by diabetes. These key findings suggest that physical activity is critical in young growing T1DM muscle, and is especially important during periods of hypoinsulinemia as it helps ensure optimal muscle growth is not jeopardized.

Under normal physiological conditions, skeletal muscle metabolism is tightly controlled by a fine balance between PS and degradation. Increases in PS stimulate muscle growth or hypertrophy while impairments result in muscle wasting (76, 123). In untreated diabetic animals, impairments in PS can have particularly profound effects on muscle growth. We show, similar to two recent papers by Gordon et al and Krause et al

(11, 12), that prolonged periods of uncontrolled diabetes result in significant detriments in skeletal muscle morphology in young animals. This atrophy of skeletal muscle is typically fibre type-specific. Slow-twitch muscles or type I fibres exhibit a small loss, while fast-twitch muscles (data not shown) and fatigable glycolytic (type IIB) fibres, exhibit more severe atrophy. These reductions in muscle suggest, therefore, that in paediatric populations, early detection and intervention are of critical importance to ensure optimal muscle growth is not jeopardized thereby affecting long term physical capacities. In paediatric populations, however, it is particularly difficult to maintain optimal insulin therapy and children are consequently more likely to experience prolonged periods of hypoinsulinemia (111). Regular endurance exercise can be an easily implemented strategy, independent of insulin, to increase PS levels thereby ensuring that during these periods of hypoinsulinemia muscle growth is protected from the negative effects of TIDM. It is well known that in untreated diabetic animals, low insulin levels blunt responses to nutritional stimuli, such as leucine, that would normally stimulate PS (86). In diabetic animals, adaptive physiological changes that occur through the introduction of endurance exercise may help restore leucine's ability to stimulate PS independent of insulin. Increased protein synthesis with leucine administration following exercise may provide another non-invasive strategy to protect skeletal muscle from the negative effects of TIDM.

Our key finding that endurance exercise can effectively stimulate PS and muscle mass growth independent of increased insulin/AKT signalling in diabetic animals is further evidence of the notion that physical activity can eliminate the requirement for insulin to increase muscle mass. This is consistent with previous work by Spangenburg et

al. (124) which showed, using IGF receptor mutant (MKR) mice, that growth factors, such as IGF-1/insulin, are not required for the activation of mTORC1 and muscle hypertrophy in response to synergistic ablation. In our study, we demonstrate for the first time that in a diabetic model not only can endurance exercise increase PS independent of insulin, but can also increase muscle mass. In gastrocnemius muscle, we show that fatigable glycolytic (type IIB) fibres exhibit the most severe atrophy and exercise helps protect muscle fibres from the negative environment of T1DM. Interestingly, in gastrocnemius of diabetic baseline animals we observed increases in protein synthesis independent of leucine (placebo gavage). This increased protein synthesis in diabetic rats that received a placebo gavage occurred in the absence of significant changes in basal 4E-BP1 or S6K1phosphorylation. This may be explained, in part, by the fact that diabetic skeletal muscle is in a sense in a state of atrophy due to chronically elevated levels of glucocorticoids that result from uncontrolled diabetes. To compensate alternate signalling pathways, other than 4EBP1 and S6K1 may be enhanced to increase PS. This is observed in, another state of atrophy, hind limb suspension. Hind limb suspension has been studied with exercise intervention and it would appear that resistance exercise increases skeletal muscle PS independent of changes in mTORC1 signalling when muscle is placed under this type of atrophic condition (125). Interestingly, although resistance training appears to increase PS in hind limb suspended muscle via insulin dependent mechanisms (92, 93), our study suggests that the effects of endurance training may be independent of this pathway, although this requires confirmation.

Unlike endurance exercise stimulated PS in the basal state, mTORC1 signalling may be the key regulator of nutrient stimulated PS. The leucine content of animal diets is

the predominant stimulator of PS (51) and an oral administration of leucine is frequently used in animal models to simulate protein in meal feeding (48) By administering a leucine gavage following a prolonged period of endurance exercise training in this study, we were able to measure how nutrition affects mTORC1 signalling following endurance exercise in diabetic muscle We found that endurance exercise had a permissive effect on leucine's ability to stimulate 4E-BP1 and S6K1 in diabetic animals to levels close to those found in sham animals given leucine Our finding of increased mTORC1 activation in DE rats suggests that exercise and protein feeding together help to offset much of the negative effects of hypoinsulinemia on protein synthesis associated with diabetes We suggest, therefore, that endurance exercise and the ingestion of leucine as a nutritional supplement and in a similar dose to that used in this study, together, provide an optimal means of increasing PS levels in diabetic animals to levels found in healthy animals independent of insulin

It should be noted, that in our study we show that growth of skeletal muscle mass improved despite no changes in growth as a whole, as evidenced by gross body mass (Figure 1B) The DE animals remained significantly smaller than the S animals and were not significantly different from D animals This can be in part due to the fact that insulin may be required for changes in protein synthesis and growth in tissues other than skeletal muscle Secondly, our study showed that endurance exercise had a permissive effect on leucine's ability to stimulate mTORC1 in diabetic muscle, we were unable to fully elucidate a mechanism to explain how this occurred Changes in blood flow and therefore amino acid delivery with exercise have been shown to help increase protein synthesis, whether this was the case in this study remain to be determined It should also

be noted that changes in IGF-1 levels following resistance exercise have been observed in moderately diabetic animals in the past (96, 126). In these moderately diabetic animals, changes in IGF-1 levels had proved to be part of the mechanism of the improved protein synthesis observed. We did not measure if insulin-like growth factor 1 (IGF-1) was increased in response to exercise in our diabetic animals. Finally, it may be suggested that the reductions in body mass accumulation observed in our diabetic animals is attributed to the removal of acinar cells belonging to the exocrine portion of the pancreas. This is not likely as it has been established in other studies that the digestive function of the pancreas is unaffected following a 90% partial pancreatectomy (127-129). In addition, the body mass reduction found in our pancreatectomy animals is consistent with the body mass reduction found in other non-pharmacological models of diabetes (12).

Conclusions:

In summary, we found that low volume endurance exercise is an effective means to stimulate protein synthesis in diabetic animals independent of insulin treatment. In addition, we show that following exercise training leucine supplementation may be a powerful means to increase PS in insulin deficient diabetic animals. This is a particularly important finding for paediatric diabetic populations as they are particularly sensitive to the growth impairments associated with hypoinsulinemia and yet are also more likely to experience periods of hypoinsulinemia. This finding also provides further support for the possible use of endurance exercise and a nutritional supplement, such as leucine, to increase PS rates in models of TIDM and other diseases associated with muscle wasting.

End of Manuscript

DISCUSSION AND FUTURE DIRECTIONS 5.0

Previous work conducted by our laboratory has shown that atrophy or impaired growth of skeletal muscle in uncontrolled T1DM is typically fibre type-specific. Slow-twitch or type I fibres have been shown to exhibit a minimal loss, while fast-twitch fibres, especially the fatigable glycolytic (type IIB) fibres, have been shown to exhibit the most severe atrophy (11, 12, 25-27). The histochemistry work conducted in this manuscript confirmed this finding in gasctrocnemius muscle. We proceeded to further explore this idea by looking at muscles composed of predominantly oxidative fibres and glycolytic fibres, soleus and epitrochlearis respectively. We showed that following a period of four weeks of uncontrolled diabetes the soleus muscle, although smaller in absolute size, was not significantly smaller when corrected for body weight then sham animals (Figure 6). The same was not true for the epitrochlearis muscle (Supplemental Figure 5). The gastrocnemius muscle used in the analysis for the manuscript, although being of a mixed fibre composition appeared to act more like the glycolytic epitrochlearis muscle.

As mentioned in the Literature Review, in the study by Flaim et al. it was shown that in diabetic muscle, rates of protein synthesis are decreased. This study concluded that in muscle groups composed of glycolytic fibres there are severe reductions in protein synthesis, while in the soleus, an oxidative muscle composed primarily of type I fibres, the reduction in protein synthesis compared to healthy controls is almost negligible (34). We measured changes in mTORC1 in the different muscles following the administration of a leucine gavage. This was done to identify whether differences in the way mTORC1 was activated could explain why the muscles were growing differently and why Flaim et al. observed different rates of protein synthesis. Interestingly, we observed that in the

soleus, 4E-BP1 levels were increased significantly in sedentary diabetic animals following a leucine gavage (Figure 12A). Similarly, S6K1in the soleus trended to be elevated in sedentary diabetic animals as well (Figure 12B). In the glycolytic epitrochlearis no changes in either 4E-BP1 or S6K1 were observed following the administration of a leucine gavage in diabetic animals (Supplemental Figure 7). These findings suggest that in soleus muscle mTORC1 signalling in response to leucine is not blunted as severely as it is in muscles composed of glycolytic fibres. Although, these findings provide some insight into the reason why oxidative skeletal muscle is less susceptible to the diabetic environment, it remains largely incomplete. Further investigation is needed to determine how endurance exercise and leucine affect protein synthesis in these muscles of different fibre types. A better understanding of how these muscles of different fibre types are regulated may provide insight on the optimal treatment of diabetic myopathy.

Interestingly, in measuring changes in phosphorylation of 4E-BP1, we commonly found increases in total 4E-BP1 levels in diabetic muscle (Supplemental Figure 1). This finding has not been reported before in the literature because generally it is levels of phosphorylation of 4E-BP1 that are reported. Phosphorylation of 4E-BP1 is particularly important because it results in the dissociation of 4E-BP1 from eIF4E and subsequent formations of the eIF4E-eIF4G complex, which allows mRNA to bind with the ribosome and initiate mRNA translation (43). Whether, increased levels of total 4E-BP1 are associated with inhibitions in protein synthesis in diabetes remains to be determined. Analysis of 4E-BP1 mRNA would need to be confirmed in order to help substantiate this idea in diabetic muscle.

The volitional wheel running model of endurance exercise used in this manuscript provided effective results in increasing protein synthesis in skeletal muscle. This method is preferred for this type of study because it minimizes the levels of stress imposed on the animal models when compared to more aggressive methods such as treadmill running. In diabetic animals, where we know glucocorticoids are substantially elevated (Supplemental Figure 2), it is possible that increased stress caused by invasive exercise protocols may inhibit the positive effects associated with endurance exercise. Although volitional wheel running causes less stress than invasive exercise methods, volitional wheel running is also associated with higher levels of variability between animals which can skew results. For this reason, different forms of endurance exercise should be evaluated. An important model of exercise to examine is chronic muscle stimulation in vivo, which has been used in other studies as a means to mimic endurance exercise (130). With chronic stimulation, variability in exercise amounts between animals could be eliminated and with single leg stimulation, each animal could serve as its own respective control providing better comparative results. Showing increased protein synthesis using this method of exercise may strengthen the notion that endurance exercise can induce positive changes in protein synthesis in diabetic animals.

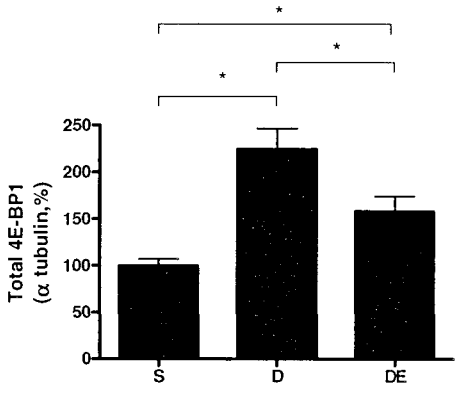
Further studies on the effects of endurance exercise on skeletal muscle should also measure how protein degradation is affected by endurance exercise in diabetic models. In our study, we attempted some preliminary work on degradation as a result of diabetes. We looked at ubiquitinated protein levels in both soleus and epitrochlearis muscles (Supplemental Figures 4 and 6 respectively). We found that degradation appeared to be significantly elevated in the soleus muscle as a result of diabetes, and found that the

epitrochlearis muscle trended to have elevated levels of protein ubiquitination. It should be noted that these measurements were taken following an approximate eighteen hour fasting period and it has been shown that a fasting alone can significantly increase protein ubiquitination. A better understanding of how endurance exercise and nutrition affect protein degradation in the fed state would help provide further insight into the mechanisms of diabetes on muscle loss and the effects of endurance exercise in restoring muscle mass.

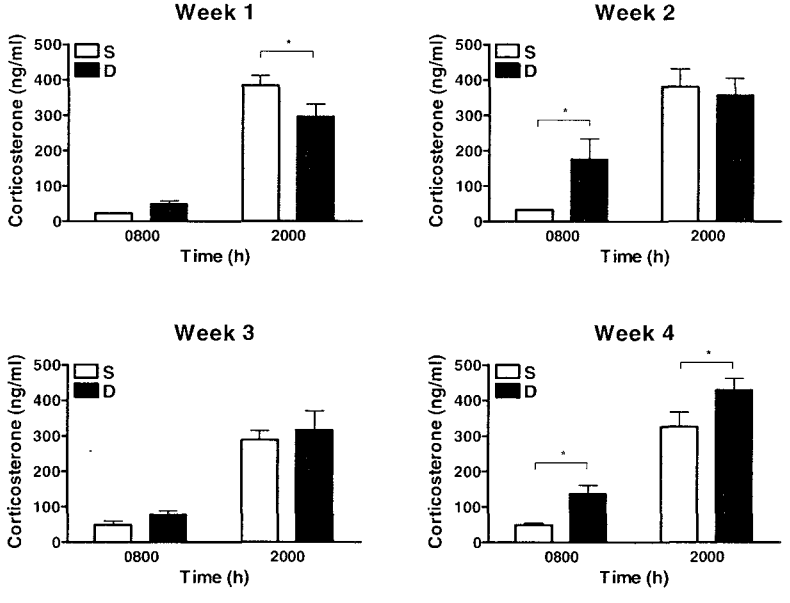
Our study provides evidence that an acute administration of leucine following endurance exercise further stimulates mTORC1 signalling and protein synthesis in skeletal muscle. Our study cannot, however, confirm that this increase in protein synthesis as a result of acute leucine administration would result in increased skeletal muscle mass. Future studies using chronic leucine supplementation with endurance exercise could help address this question. One way to do this is to use the method developed by Lynch et al. Lynch et al.'s model of chronic leucine supplementation involved adding leucine to the drinking water of healthy ad libitum-fed rats for a period of 12 days. This study examined the effects of this chronic leucine supplementation on protein synthesis in the adipose tissue, kidney, heart, liver, and skeletal muscle of the rats (131). This form of leucine supplementation was accompanied by increased rates of protein synthesis in the adipose tissue, liver, and skeletal muscle. These results suggest that chronic leucine supplementation results in the stimulation of protein synthesis in several body tissues (131, 132), suggesting that this type of intervention may have beneficial effects on skeletal muscle size and whole body growth in diabetic rodents.

Finally, in conjunction with endurance exercise and leucine supplementation, the effects of hormone replacement therapies should also be studied in these models. Restoring insulin levels with exogenous insulin is the first obvious method. This would help show whether endurance exercise and leucine supplementation can fully restore whole body growth in conjunction with increased skeletal muscle size. A second possible method which may be considered is the use of leptin therapy. This intervention, although unconventional, has been shown to have positive effects on growth in diabetic animals. Specifically, recent work by Unger et al. has proposed the idea of leptin therapy in insulin-deficient type I diabetes (133, 134). In an initial paper presented in the Journal of Proceedings of the National Academy of Sciences, Unger's research group showed leptin's ability to not only improve glucose parameters in diabetic rats, but also to cause reduced detriment in size. This was evidenced by increased length and mass in hyperleptinemia diabetic animals. Unger's research group explained these findings in part by the up-regulation of IGF-1 expression in plasma and increased IGF-1 receptor phosphorylation in muscle of hyperleptinemia animals. These findings suggest that leptin reverses the catabolic consequences of a complete insulin deficiency, potentially by enhancing the actions of IGF-1 on skeletal muscle. Despite these observations, no measures of skeletal muscle protein synthesis or mTORC1 signalling were studied. Future studies should examine the effects of leptin on mTORC1 signalling and protein synthesis in diabetic animals as this may provide an insulin independent method to correct diabetic impairments.

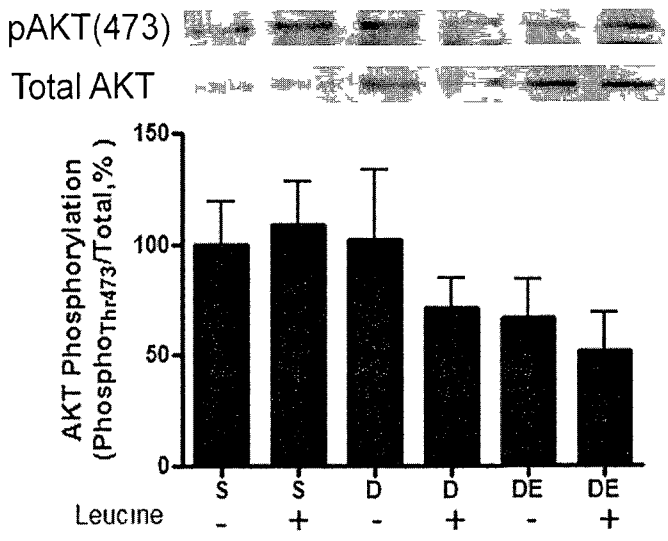
SUPPLEMENTAL WORK 6.0



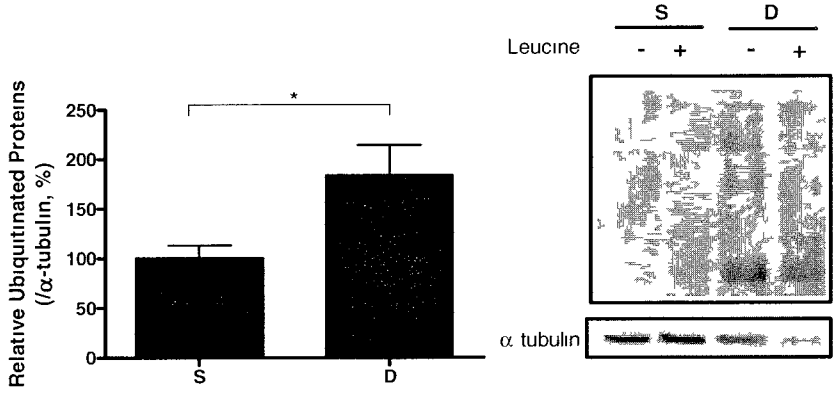
Supplemental Figure 1:
Total 4E-binding protein 1 in the gastrocnemius muscle of the S, D and DE groups. There was a significant increase in total 4E-BP1 levels in D and DE groups when compared to the S. D group and a significant when compared to the DE. There was also a significant difference between the S and groups * Indicates the presence of a significant difference at P <0.05; n=12-14 per group.



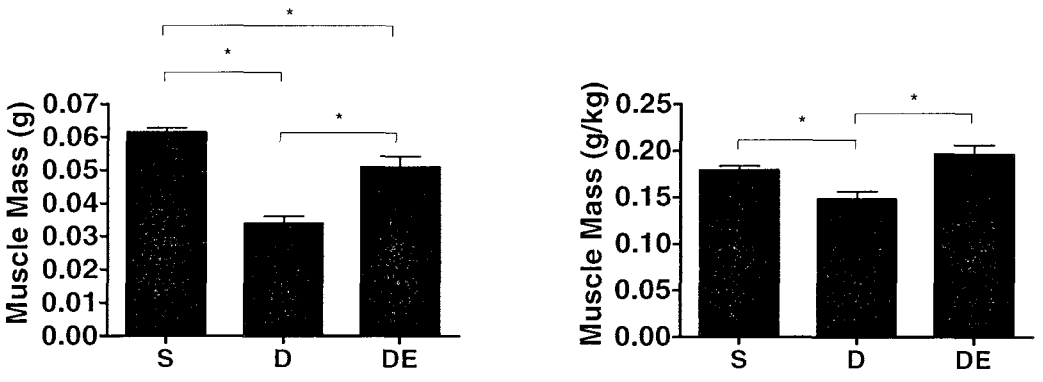
Supplemental Figure 2:
Corticosterone profile in the S and D groups over the 4 week treatment period (Basal-0800 and Peak-2000). Basal corticosterone concentration was elevated in D groups as compared to the S group during weeks 2 and 4. Peak corticosterone concentrations when were only significantly elevated after four weeks of hyperglycemia in D group as compared to the S group. * Indicates the presence of a significant difference at P <0.05; n=7-9per group.



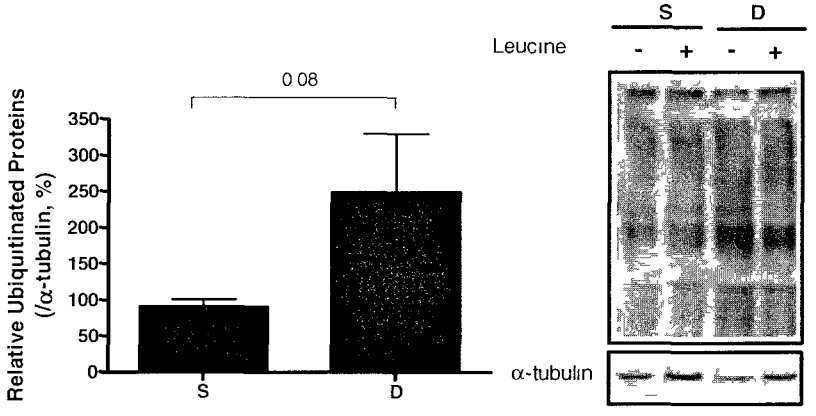
Supplemental Figure 3:
AKT phosphorylation (threonine 473) following a leucine (+) or water (-) gavage in the soleus muscle of the S, D and DE groups AKT (threonine 473) phosphorylation expressed as a percentage of the S (-) group There were no significant differences in AKT phosphorylation between any of the groups regardless of type of gavage, n=6-7 per treatment per group



Supplemental Figure 4:
Total ubiquitinated proteins in soleus muscle of S and D groups There was a significant increase in total ubiquitinated proteins between S and D animals * Indicates the presence of a significant difference at P <0 05, n=10-12per group

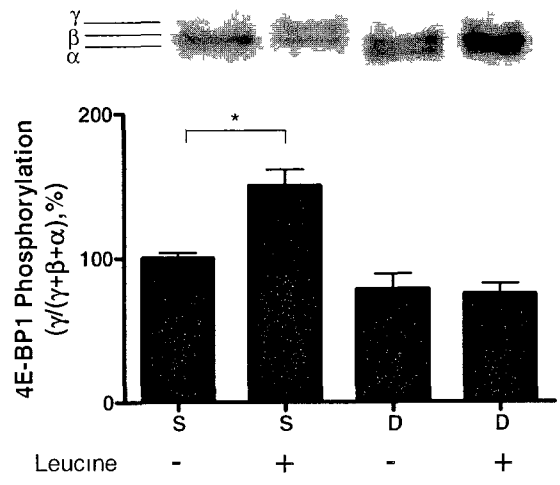


Supplemental Figure 5:
Absolute and normalized epitrochlearis muscle mass in the S, D and DE groups. Absolute muscle mass was significantly lower in both the D and DE groups when compared to the S group. However, the DE group did have significantly more muscle mass than the D group. When normalized for body weight, the same results remained true. * Indicates the presence of a significant difference at P <0.05; n= 11-13 per group

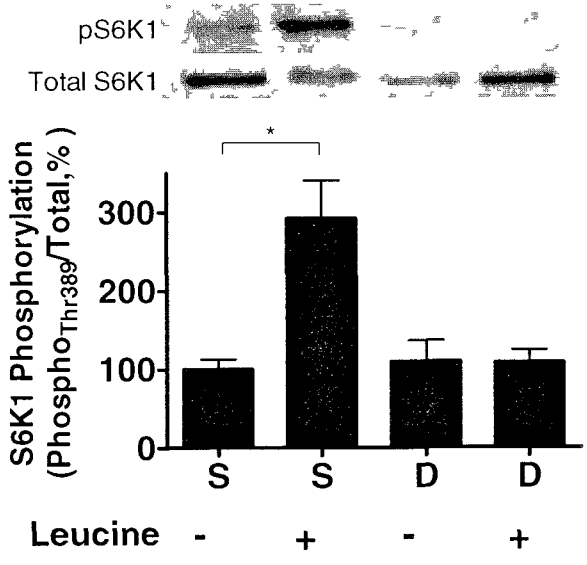


Supplemental Figure 6:
Total ubiquitinated proteins in epitrochlearis muscle of S and D groups. There was a trend of increased total ubiquitinated proteins between S and D animals (p=0.08); n=10-12per group.

A



B



Supplemental Figure 7:
Phosphorylation of (A) 4E-binding protein 1 (4E-BP1) and (B) S6K1 (threonine 389) following a leucine (+) or water (-) gavage in the epitrochlearis muscle of the S and D groups. 4E-BP1 and S6K1 (threonine 389) phosphorylation are expressed as a percentage of the S (-) group. (A) There was a significant increase in 4E-BP1 phosphorylation in the S (+) group when compared to the S (-) group. (B) Similar to 4E-BP1 phosphorylation, there was a significant increase in S6K1 (threonine 389) phosphorylation in the S (+) group when compared to the S (-) group (C) * Indicates the presence of a significant difference at $P < 0.05$; n=6-7per group.

REFERENCES 7.0

1. Brownlee M: The pathobiology of diabetic complications: A unifying mechanism. *Diabetes.* 54:1615-1625, 2005

2. Andersen H, Jakobsen J: Diabetes mellitus. *Curr Opin Neurol.* 10:376-380, 1997

3. Andersen H, Poulsen PL, Mogensen CE, Jakobsen J: Isokinetic muscle strength in long-term IDDM patients in relation to diabetic complications. *Diabetes.* 45:440-445, 1996

4. Andersen H, Gadeberg PC, Brock B, Jakobsen J: Muscular atrophy in diabetic neuropathy: A stereological magnetic resonance imaging study. *Diabetologia.* 40:1062-1069, 1997

5. Andersen H, Gjerstad MD, Jakobsen J: Atrophy of foot muscles: A measure of diabetic neuropathy. *Diabetes Care.* 27:2382-2385, 2004

6. Andersen H, Schmitz O, Nielsen S: Decreased isometric muscle strength after acute hyperglycaemia in type 1 diabetic patients. *Diabet Med.* 22:1401-1407, 2005

7. Caso G, McNurlan MA: Effect of insulin on whole body protein metabolism in children with type 1 diabetes. *Curr Opin Clin Nutr Metab Care.* 13:93-96, 2010

8. Charlton M, Nair KS: Protein metabolism in insulin-dependent diabetes mellitus. *J Nutr.* 128:323S-327S, 1998

9. Hawke TJ, Garry DJ: Myogenic satellite cells: Physiology to molecular biology. *J Appl Physiol.* 91:534-551, 2001

10. Bassel-Duby R, Olson EN: Signaling pathways in skeletal muscle remodeling. *Annu Rev Biochem.* 75:19-37, 2006

11. Gordon CS, Hawke TJ, Krause MP, Serino AS, Campbell JE, Cafarelli E, Adegoke OA, Riddell MC: Impaired growth and force production in skeletal muscles of young partially pancreatectomized rats: A model of adolescent type 1 diabetic myopathy. , In Revision

12. Krause MP, Riddell MC, Gordon CS, Imam SA, Cafarelli E, Hawke TJ: Diabetic myopathy differs between Ins2Akita+/- and streptozotocin-induced type 1 diabetic models. *J Appl Physiol.* 106:1650-1659, 2009

13. Reske-Nielsen E, Harmsen A, Vorre P: Ultrastructure of muscle biopsies in recent, short-term and long-term juvenile diabetes. *Acta Neurol Scand.* 55:345-362, 1977

14. Jakobsen J, Reske-Nielsen E: Diffuse muscle fiber atrophy in newly diagnosed diabetes. *Clin Neuropathol.* 5:73-77, 1986

15. Caprio S, Cline G, Boulware S, Permanente C, Shulman GI, Sherwin RS, Tamborlane WV: Effects of puberty and diabetes on metabolism of insulin-sensitive fuels. *Am J Physiol.* 266:E885-91, 1994

16. Sandri M: Signaling in muscle atrophy and hypertrophy. *Physiology (Bethesda).* 23:160-170, 2008

17. Braziuniene I, Garlick J, Mileva I, Desikan V, Wilson TA, McNurlan M: Effect of insulin with oral nutrients on whole-body protein metabolism in growing pubertal children with type 1 diabetes. *Pediatr Res.* 65:109-112, 2009

18. Bennet WM, Connacher AA, Jung RT, Stehle P, Rennie MJ: Effects of insulin and amino acids on leg protein turnover in IDDM patients. *Diabetes.* 40:499-508, 1991

19. Godil MA, Wilson TA, Garlick PJ, McNurlan MA: Effect of insulin with concurrent amino acid infusion on protein metabolism in rapidly growing pubertal children with type 1 diabetes. *Pediatr Res.* 58:229-234, 2005

20. Kimball SR, Jefferson LS: Control of translation initiation through integration of signals generated by hormones, nutrients and exercise. *J Biol Chem.* , 2010

21. Miyazaki M, Esser KA: Cellular mechanisms regulating protein synthesis and skeletal muscle hypertrophy in animals. *J Appl Physiol.* 106:1367-1373, 2009

22. Nygren J, Nair KS: Differential regulation of protein dynamics in splanchnic and skeletal muscle beds by insulin and amino acids in healthy human subjects. *Diabetes.* 52:1377-1385, 2003

23. Fujita S, Rasmussen BB, Cadenas JG, Grady JJ, Volpi E: Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability. *Am J Physiol Endocrinol Metab.* 291:E745-54, 2006

24. Hillier TA, Fryburg DA, Jahn LA, Barrett EJ: Extreme hyperinsulinemia unmasks insulin's effect to stimulate protein synthesis in the human forearm. *Am J Physiol.* 274:E1067-74, 1998

25. Armstrong RB, Gollnick PD, Ianuzzo CD: Histochemical properties of skeletal muscle fibers in streptozotocin-diabetic rats. *Cell Tissue Res.* 162:387-394, 1975

26. Cotter MA, Cameron NE, Robertson S, Ewing I: Polyol pathway-related skeletal muscle contractile and morphological abnormalities in diabetic rats. *Exp Physiol.* 78:139-155, 1993

27. Snow LM, Sanchez OA, McLoon LK, Serfass RC, Thompson LV: Myosin heavy chain isoform immunolabelling in diabetic rats with peripheral neuropathy. *Acta Histochem.* 107:221-229, 2005

28. Effects of type 1 diabetes mellitus on skeletal muscle: Clinical observations and physiological mechanisms[article online], In Press. Available from

29. Johnston AP, Campbell JE, Found JG, Riddell MC, Hawke TJ: Streptozotocin induces G2 arrest in skeletal muscle myoblasts and impairs muscle growth in vivo. *Am J Physiol Cell Physiol.* 292:C1033-40, 2007

30. Sanchez OA, Snow LM, Lowe DA, Serfass RC, Thompson LV: Effects of endurance exercise-training on single-fiber contractile properties of insulin-treated streptozotocin-induced diabetic rats. *J Appl Physiol.* 99:472-478, 2005

31. Cameron NE, Cotter MA, Robertson S: Changes in skeletal muscle contractile properties in streptozocin-induced diabetic rats and role of polyol pathway and hypoinsulinemia. *Diabetes.* 39:460-465, 1990

32. Paulus SF, Grossie J: Skeletal muscle in alloxan diabetes. A comparison of isometric contractions in fast and slow muscle. *Diabetes.* 32:1035-1039, 1983

33. McGuire M, Dumbleton M, MacDermott M, Bradford A: Contractile and electrical properties of sternohyoid muscle in streptozotocin diabetic rats. *Clin Exp Pharmacol Physiol.* 28:184-187, 2001

34. Flaim KE, Copenhaver ME, Jefferson LS: Effects of diabetes on protein synthesis in fast- and slow-twitch rat skeletal muscle. *Am J Physiol.* 239:E88-95, 1980

35. Karinch AM, Kimball SR, Vary TC, Jefferson LS: Regulation of eukaryotic initiation factor-2B activity in muscle of diabetic rats. *Am J Physiol.* 264:E101-8, 1993

36. Pain VM, Garlick PJ: Effect of streptozotocin diabetes and insulin treatment on the rate of protein synthesis in tissues of the rat in vivo. *J Biol Chem.* 249:4510-4514, 1974

37. Jefferson LS, Koehler JO, Morgan HE: Effect of insulin on protein synthesis in skeletal muscle of an isolated perfused preparation of rat hemicorpus. *Proc Natl Acad Sci U S A.* 69:816-820, 1972

38. Drummond MJ, Dreyer HC, Fry CS, Glynn EL, Rasmussen BB: Nutritional and contractile regulation of human skeletal muscle protein synthesis and mTORC1 signalling. *J Appl Physiol.* 106:1374-1384, 2009

39. Shah OJ, Anthony JC, Kimball SR, Jefferson LS: 4E-BP1 and S6K1: Translational integration sites for nutritional and hormonal information in muscle. *Am J Physiol Endocrinol Metab.* 279:E715-29, 2000

40. Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N: Amino acid regulation of TOR complex 1. *Am J Physiol Endocrinol Metab.* 296:E592-602, 2009

41. Bolster DR, Jefferson LS, Kimball SR: Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signalling. *Proc Nutr Soc.* 63:351-356, 2004

42. Haghighat A, Mader S, Pause A, Sonenberg N: Repression of cap-dependent translation by 4E-binding protein 1: Competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.* 14:5701-5709, 1995

43. Lin TA, Kong X, Haystead TA, Pause A, Belsham G, Sonenberg N, Lawrence JC,Jr: PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science.* 266:653-656, 1994

44. Vary TC, Jefferson LS, Kimball SR: Insulin fails to stimulate muscle protein synthesis in sepsis despite unimpaired signalling to 4E-BP1 and S6K1. *Am J Physiol Endocrinol Metab.* 281:E1045-53, 2001

45. Proud CG: Regulation of mammalian translation factors by nutrients. *Eur J Biochem.* 269:5338-5349, 2002

46. Ayuso MI, Hernandez-Jimenez M, Martin ME, Salinas M, Alcazar A: A new hierarchical phosphorylation pathway of the translational repressor eIF4E-binding protein 1 (4E-BP1) in ischemia-reperfusion stress. *J Biol Chem.* , 2010

47. Sonenberg N, Pause A: Signal transduction. protein synthesis and oncogenesis meet again. *Science.* 314:428-429, 2006

48. Vary TC, Lynch CJ: Nutrient signalling components controlling protein synthesis in striated muscle. *J Nutr.* 137:1835-1843, 2007

49. Yoshizawa F, Kimball SR, Vary TC, Jefferson LS: Effect of dietary protein on translation initiation in rat skeletal muscle and liver. *Am J Physiol.* 275:E814-20, 1998

50. Proud CG: mTOR-mediated regulation of translation factors by amino acids. *Biochem Biophys Res Commun.* 313:429-436, 2004

51. Norton LE, Layman DK, Bunpo P, Anthony TG, Brana DV, Garlick PJ: The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signalling in rats. *J Nutr.* 139:1103-1109, 2009

52. Kimball SR, Jefferson LS: Regulation of global and specific mRNA translation by oral administration of branched-chain amino acids. *Biochem Biophys Res Commun.* 313:423-427, 2004

53. Anthony JC, Anthony TG, Kimball SR, Vary TC, Jefferson LS: Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr.* 130:139-145, 2000

54. Anthony JC, Anthony TG, Kimball SR, Jefferson LS: Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J Nutr.* 131:856S-860S, 2001

55. Anthony JC, Lang CH, Crozier SJ, Anthony TG, MacLean DA, Kimball SR, Jefferson LS: Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. *Am J Physiol Endocrinol Metab.* 282:E1092-101, 2002

56. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR: Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr.* 130:2413-2419, 2000

57. Crozier SJ, Kimball SR, Emmert SW, Anthony JC, Jefferson LS: Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr.* 135:376-382, 2005

58. Gulati P, Gaspers LD, Dann SG, Joaquin M, Nobukuni T, Natt F, Kozma SC, Thomas AP, Thomas G: Amino acids activate mTOR complex 1 via Ca2+/CaM signalling to hVps34. *Cell Metab.* 7:456-465, 2008

59. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM: The rag GTPases bind raptor and mediate amino acid signalling to mTORC1. *Science.* 320:1496-1501, 2008

60. Meijer AJ, Codogno P: Nutrient sensing: TOR's ragtime. *Nat Cell Biol.* 10:881-883, 2008

61. Findlay GM, Yan L, Procter J, Mieulet V, Lamb RF: A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem J.* 403:13-20, 2007

62. Goberdhan DC, Ogmundsdottir MH, Kazi S, Reynolds B, Visvalingam SM, Wilson C, Boyd CA: Amino acid sensing and mTOR regulation: Inside or out? *Biochem Soc Trans.* 37:248-252, 2009

63. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley LC, Finan PM, Murphy LO: Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell.* 136:521-534, 2009

64. Yan L, Mieulet V, Burgess D, Findlay GM, Sully K, Procter J, Goris J, Janssens V, Morrice NA, Lamb RF: PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signalling to mTOR. *Mol Cell.* 37:633-642, 2010

65. Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR, Jefferson LS: Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. *J Physiol.* 553:213-220, 2003

66. Bolster DR, Kimball SR, Jefferson LS: Translational control mechanisms modulate skeletal muscle gene expression during hypertrophy. *Exerc Sport Sci Rev.* 31:111-116, 2003

67. Norton LE, Layman DK: Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. *J Nutr.* 136:533S-537S, 2006

68. Anthony JC, Anthony TG, Layman DK: Leucine supplementation enhances skeletal muscle recovery in rats following exercise. *J Nutr.* 129:1102-1106, 1999

69. Anthony TG, McDaniel BJ, Knoll P, Bunpo P, Paul GL, McNurlan MA: Feeding meals containing soy or whey protein after exercise stimulates protein synthesis and translation initiation in the skeletal muscle of male rats. *J Nutr.* 137:357-362, 2007

70. Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS: Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. *Am J Physiol.* 274:C406-14, 1998

71. Williamson DL, Kubica N, Kimball SR, Jefferson LS: Exercise-induced alterations in extracellular signal-regulated kinase 1/2 and mammalian target of rapamycin (mTOR) signalling to regulatory mechanisms of mRNA translation in mouse muscle. *J Physiol.* 573:497-510, 2006

72. Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ, Wackerhage H: Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signalling can explain specific

adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J.* 19:786-788, 2005

73. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD: Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol.* 3:1014-1019, 2001

74. Baar K, Esser K: Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol.* 276:C120-7, 1999

75. Hornberger TA, Sukhija KB, Chien S: Regulation of mTOR by mechanically induced signalling events in skeletal muscle. *Cell Cycle.* 5:1391-1396, 2006

76. Phillips SM: Physiologic and molecular bases of muscle hypertrophy and atrophy: Impact of resistance exercise on human skeletal muscle (protein and exercise dose effects). *Appl Physiol Nutr Metab.* 34:403-410, 2009

77. Spangenburg EE: Changes in muscle mass with mechanical load: Possible cellular mechanisms. *Appl Physiol Nutr Metab.* 34:328-335, 2009

78. Paul GL, Rokusek JT, Dykstra GL, Boileau RA, Layman DK: Oat, wheat or corn cereal ingestion before exercise alters metabolism in humans. *J Nutr.* 126:1372-1381, 1996

79. Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS: Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. *Am J Physiol.* 274:C406-14, 1998

80. MacKenzie MG, Hamilton DL, Murray JT, Taylor PM, Baar K: mVps34 is activated following high-resistance contractions. *J Physiol.* 587:253-260, 2009

81. Phillips SM: Protein requirements and supplementation in strength sports. *Nutrition.* 20:689-695, 2004

82. Wullschleger S, Loewith R, Hall MN: TOR signalling in growth and metabolism. *Cell.* 124:471-484, 2006

83. Pacy PJ, Nair KS, Ford C, Halliday D: Failure of insulin infusion to stimulate fractional muscle protein synthesis in type I diabetic patients. anabolic effect of insulin and decreased proteolysis. *Diabetes.* 38:618-624, 1989

84. Pain VM, Albertse EC, Garlick PJ: Protein metabolism in skeletal muscle, diaphragm, and heart of diabetic rats. *Am J Physiol.* 245:E604-10, 1983

85. Bennet WM, Connacher AA, Smith K, Jung RT, Rennie MJ: Inability to stimulate skeletal muscle or whole body protein synthesis in type 1 (insulin-dependent) diabetic patients by insulin-plus-glucose during amino acid infusion: Studies of incorporation and turnover of tracer L-[1-13C]leucine. *Diabetologia.* 33:43-51, 1990

86. Anthony JC, Reiter AK, Anthony TG, Crozier SJ, Lang CH, MacLean DA, Kimball SR, Jefferson LS: Orally administered leucine enhances protein synthesis in skeletal

muscle of diabetic rats in the absence of increases in 4E-BP1 or S6K1 phosphorylation. *Diabetes.* 51:928-936, 2002

87. Goldberg AL: Influence of insulin and contractile activity on muscle size and protein balance. *Diabetes.* 28 Suppl 1:18-24, 1979

88. Goldberg AL: Role of insulin in work-induced growth of skeletal muscle. *Endocrinology.* 83:1071-1073, 1968

89. Farrell PA, Fedele MJ, Hernandez J, Fluckey JD, Miller JL,3rd, Lang CH, Vary TC, Kimball SR, Jefferson LS: Hypertrophy of skeletal muscle in diabetic rats in response to chronic resistance exercise. *J Appl Physiol.* 87:1075-1082, 1999

90. Farrell PA, Fedele MJ, Vary TC, Kimball SR, Jefferson LS: Effects of intensity of acute-resistance exercise on rates of protein synthesis in moderately diabetic rats. *J Appl Physiol.* 85:2291-2297, 1998

91. Farrell PA, Fedele MJ, Vary TC, Kimball SR, Lang CH, Jefferson LS: Regulation of protein synthesis after acute resistance exercise in diabetic rats. *Am J Physiol.* 276:E721-7, 1999

92. Kostyak JC, Kimball SR, Jefferson LS, Farrell PA: Severe diabetes inhibits resistance exercise-induced increase in eukaryotic initiation factor 2B activity. *J Appl Physiol.* 91:79-84, 2001

93. Fedele MJ, Hernandez JM, Lang CH, Vary TC, Kimball SR, Jefferson LS, Farrell PA: Severe diabetes prohibits elevations in muscle protein synthesis after acute resistance exercise in rats. *J Appl Physiol.* 88:102-108, 2000

94. Bark TH, McNurlan MA, Lang CH, Garlick PJ: Increased protein synthesis after acute IGF-I or insulin infusion is localized to muscle in mice. *Am J Physiol.* 275:E118-23, 1998

95. Adams GR, Haddad F: The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy. *J Appl Physiol.* 81:2509-2516, 1996

96. Fedele MJ, Lang CH, Farrell PA: Immunization against IGF-I prevents increases in protein synthesis in diabetic rats after resistance exercise. *Am J Physiol Endocrinol Metab.* 280:E877-85, 2001

97. Albright A, Franz M, Hornsby G, Kriska A, Marrero D, Ullrich I, Verity LS: American college of sports medicine position stand. exercise and type 2 diabetes. *Med Sci Sports Exerc.* 32:1345-1360, 2000

98. Gute D, Fraga C, Laughlin MH, Amann JF: Regional changes in capillary supply in skeletal muscle of high-intensity endurance-trained rats. *J Appl Physiol.* 81:619-626, 1996

99. Cameron NE, Cotter MA, Robertson S: Angiotensin converting enzyme inhibition prevents development of muscle and nerve dysfunction and stimulates angiogenesis in streptozotocin-diabetic rats. *Diabetologia.* 35:12-18, 1992

100. Tipton KD, Wolfe RR: Exercise-induced changes in protein metabolism. *Acta Physiol Scand.* 162:377-387, 1998

101. Eliakim A, Moromisato M, Moromisato D, Brasel JA, Roberts C,Jr, Cooper DM: Increase in muscle IGF-I protein but not IGF-I mRNA after 5 days of endurance training in young rats. *Am J Physiol.* 273:R1557-61, 1997

102. Chan O, Inouye K, Riddell MC, Vranic M, Matthews SG: Diabetes and the hypothalamo-pituitary-adrenal (HPA) axis. *Minerva Endocrinol.* 28:87-102, 2003

103. Bullock GR, Carter EE, Elliott P, Peters RF, Simpson P, White AM: Relative changes in the function of muscle ribosomes and mitochondria during the early phase of steroid-induced catabolism. *Biochem J.* 127:881-892, 1972

104. Goldberg AL, Goodman HM: Relationship between cortisone and muscle work in determining muscle size. *J Physiol.* 200:667-675, 1969

105. Rannels SR, Jefferson LS: Effects of glucocorticoids on muscle protein turnover in perfused rat hemicorpus. *Am J Physiol.* 238:E564-72, 1980

106. Shah OJ, Anthony JC, Kimball SR, Jefferson LS: Glucocorticoids oppose translational control by leucine in skeletal muscle. *Am J Physiol Endocrinol Metab.* 279:E1185-90, 2000

107. Wang H, Kubica N, Ellisen LW, Jefferson LS, Kimball SR: Dexamethasone represses signalling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1. *J Biol Chem.* 281:39128-39134, 2006

108. Shah OJ, Iniguez-Lluhi JA, Romanelli A, Kimball SR, Jefferson LS: The activated glucocorticoid receptor modulates presumptive autoregulation of ribosomal protein S6 protein kinase, p70 S6K. *J Biol Chem.* 277:2525-2533, 2002

109. Kimball SR, Do AN, Kutzler L, Cavener DR, Jefferson LS: Rapid turnover of the mTOR complex 1 (mTORC1) repressor REDD1 and activation of mTORC1 signalling following inhibition of protein synthesis. *J Biol Chem.* 283:3465-3475, 2008

110. McGhee NK, Jefferson LS, Kimball SR: Elevated corticosterone associated with food deprivation upregulates expression in rat skeletal muscle of the mTORC1 repressor, REDD1. *J Nutr.* 139:828-834, 2009

111. Hamilton J, Daneman D: Deteriorating diabetes control during adolescence: Physiological or psychosocial? *J Pediatr Endocrinol Metab.* 15:115-126, 2002

112. Little JP, Phillips SM: Resistance exercise and nutrition to counteract muscle wasting. *Appl Physiol Nutr Metab.* 34:817-828, 2009

113. Munoz KA, Aannestad A, Tischler ME, Henriksen EJ: Skeletal muscle protein content and synthesis after voluntary running and subsequent unweighting. *Metabolism.* 43:994-999, 1994

114. Nagao K, Bannai M, Seki S, Kawai N, Mori M, Takahashi M: Voluntary wheel running is beneficial to the amino acid profile of lysine-deficient rats. *Am J Physiol Endocrinol Metab.* 298:E1170-8, 2010

115. Stipanuk MH: Leucine and protein synthesis: MTOR and beyond. *Nutr Rev.* 65:122-129, 2007

116. Kimball SR, Jefferson LS: Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *J Nutr.* 136:227S-31S, 2006

117. Bonner-Weir S, Trent DF, Weir GC: Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. *J Clin Invest.* 71:1544-1553, 1983

118. Brown AP, Dinger N, Levine BS: Stress produced by gavage administration in the rat. *Contemp Top Lab Anim Sci.* 39:17-21, 2000

119. Garlick PJ, McNurlan MA, Preedy VR: A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3H]phenylalanine. *Biochem J.* 192:719-723, 1980

120. Suzuki O, Yagi K: A fluorometric assay for beta-phenylethylamine in rat brain. *Anal Biochem.* 75:192-200, 1976

121. Ogilvie RW, Feeback DL: A metachromatic dye-ATPase method for the simultaneous identification of skeletal muscle fiber types I, IIA, IIB and IIC. *Stain Technol.* 65:231-241, 1990

122. Fediuc S, Campbell JE, Riddell MC: Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in sprague-dawley rats. *J Appl Physiol.* 100:1867-1875, 2006

123. Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW: Control of the size of the human muscle mass. *Annu Rev Physiol.* 66:799-828, 2004

124. Spangenburg EE, Le Roith D, Ward CW, Bodine SC: A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy. *J Physiol.* 586:283-291, 2008

125. Fluckey JD, Dupont-Versteegden EE, Knox M, Gaddy D, Tesch PA, Peterson CA: Insulin facilitation of muscle protein synthesis following resistance exercise in hindlimb-suspended rats is independent of a rapamycin-sensitive pathway. *Am J Physiol Endocrinol Metab.* 287:E1070-5, 2004

126. Fedele MJ, Vary TC, Farrell PA: Selected contribution: IGF-I antibody prevents increases in protein synthesis in epitrochlearis muscles from refed, diabetic rats. *J Appl Physiol.* 90:1166-73; discussion 1165, 2001

127. Yasugi H, Mizumoto R, Sakurai H, Honjo I: Changes in carbohydrate metabolism and endocrine function of remnant pancreas after major pancreatic resection. *Am J Surg.* 132:577-580, 1976

128. Sommer H: Functional recovery of the exocrine pancreas in rats after partial resection. *Eur Surg Res.* 19:318-322, 1987

129. Mizumoto R, Kawarada Y, Goshima H, Tamaki H, Sekoguchi T, Tomikawa I: Carbohydrate metabolism and endocrine function in the pancreas remnant after major pancreatic resection. *Am J Surg.* 143:237-243, 1982

130. Ljubicic V, Adhihetty PJ, Hood DA: Application of animal models: Chronic electrical stimulation-induced contractile activity. *Can J Appl Physiol.* 30:625-643, 2005

131. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC: Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. *Am J Physiol Endocrinol Metab.* 283:E824-35, 2002

132. Donato J,Jr, Pedrosa RG, Cruzat VF, Pires IS, Tirapegui J: Effects of leucine supplementation on the body composition and protein status of rats submitted to food restriction. *Nutrition.* 22:520-527, 2006

133. Wang MY, Chen L, Clark GO, Lee Y, Stevens RD, Ilkayeva OR, Wenner BR, Bain JR, Charron MJ, Newgard CB, Unger RH: Leptin therapy in insulin-deficient type I diabetes. *Proc Natl Acad Sci U S A.* 107:4813-4819, 2010

134. Yu X, Park BH, Wang MY, Wang ZV, Unger RH: Making insulin-deficient type 1 diabetic rodents thrive without insulin. *Proc Natl Acad Sci U S A.* 105:14070-14075, 2008