INFLUENCE OF INSULIN RESISTANCE ON CONTRACTILE ACTIVITY-INDUCED ANABOLIC RESPONSE OF SKELETAL MUSCLE

A Dissertation

by

MATS INGE NILSSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Kinesiology

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ABSTRACT

Influence of Insulin Resistance on Contractile Activity-induced Anabolic Response of Skeletal Muscle. (December 2009)

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Although the long-term therapeutic benefits of exercise are indisputable, contractile activity may induce divergent adaptations in insulin-resistant vs. insulin-sensitive skeletal muscle. The purpose of this study was to elucidate if the anabolic response following resistance exercise (RE) is altered in myocellular sub-fractions in the face of insulin resistance. Lean (Fa/?) and obese (fa/fa) Zucker rats were assigned to sedentary and RE groups and engaged in either cage rest or four lower-body RE sessions over an 8-d period. Despite obese Zucker rats having significantly smaller hindlimb muscles when compared to age-matched lean rats, basal 24-h fractional synthesis rates (FSR) of mixed protein pools were near normal in distally located muscle groups (gastrocnemius, plantaris, and soleus) and even augmented in those located more proximally ($P < 0.05$; quadriceps). Although 2 x 2 ANOVA indicated a significant main effect of phenotype on mixed FSR in gastrocnemius and soleus ($P < 0.05$), phenotypic differences were partially accounted for by an exercise effect in the lean phenotype. Interestingly, obese rats exhibited a significant suppression of myofibrillar FSR compared to their lean

counterparts ($P < 0.05$; gastrocnemius), while synthesis rates of mitochondrial and cytosolic proteins were normal (gastrocnemius and quadriceps), suggesting a mechanism whereby translation of specific mRNA pools encoding for metabolic enzymes may be favored over other transcripts (e.g., contractile proteins) to cope with nutrient excess in the insulin-resistant state. Immunoblotting of the cytosolic fraction in gastrocnemius muscle indicated an augmented phosporylation of eIF4EBP1 (+ 9%) and $p70^{s6k}$ (+85%) in obese vs. lean rats, but a more potent baseline inhibition of polypeptide-chain elongation as evidenced by an increased phospho/total ratio of eEF2 (+78%) in the obese phenotype. Resistance exercise did not improve synthesis rates of myofibrillar, cytosolic, or mitochondrial proteins to the same extent in obese vs. lean rats, suggesting a desensitization to contractile-induced anabolic stimuli in the insulin-resistant state. We conclude that insulin resistance has diverse effects on protein metabolism, which may vary between muscle groups depending on fiber type distribution, location along the proximodistal body axis, and myocellular sub-fraction, and may blunt the anabolic response to voluntary resistance exercise.

DEDICATION

I dedicate this dissertation to my family: my father Jan Nilsson, my mother Vivi-Anne Nilsson, my brothers Peter and Joakim Nilsson, and lastly the love of my life, Darrelle Leeming, and our son Max Aedyn Nilsson.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to my committee chair, Dr. James D. Fluckey, for having the patience and the deep pockets to allow his graduate students to learn a multitude of laboratory procedures by trial and error. His complete understanding of the undeniable clumsiness of novice researchers [affectionately referred to as young "whippersnappers" by him], in combination with his in-depth knowledge of key experimental methodologies and laboratory equipment, have not only facilitated speedy reparations, but also significantly reduced his start-up package. On a more serious note, by recognizing my strengths and weaknesses and by tailoring his instruction to fit my personality and needs, Dr. Fluckey's mentoring skills resemble some of the world-class track and field coaches that I have had the pleasure to work with during my international career in athletics. His guidance and support have been indispensible for the successful completion of my PhD degree, which ultimately will enable me to start a new life with my family on the North-American continent. It has been a true pleasure being mentored by someone so selflessly devoted to self-actualizing others. Thank you!

Special recognition should be given to the members of my committee, but there is not enough space to adequately thank you individually. Prof. Lawler, Dr. Riechman, and Dr. Hogan: I am thankful for your astute mentorship, all the worthwhile suggestions for my dissertation studies, and our collaborative research efforts.

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CHAPTER I

INTRODUCTION

Epidemiology of Type 2 Diabetes

Although the genetic makeup of homo sapiens sapiens has remained relatively unchanged over the past 10.000 years, our culture has been transformed almost beyond recognition during the same time period, leading to a mismatch between our genetically controlled biology and the bio-environmental milieu (38). The discordance between modern lifestyle factors (physical activity levels and quality of diet) and our ancient genome has undoubtedly contributed to the epidemic-like rise in chronic degenerative diseases (CDD), which currently account for ~75% of all deaths in developed nations (38). Exact etiologies of these debilitating diseases are in many cases unknown, but alterations of candidate genes (single nucleotide polymorphisms or mutations) and/or inherited epigenetic modifications may confer an increased susceptibility upon which bio-environmental factors act to regulate disease progression (38).

This dissertation follows the style of *American Journal of Physiology Endocrinology and Metabolism*.

Since the industrial revolution, the incidence of obesity and type 2 diabetes (henceforth also referred to as diabetes and T2DM) has increased concomitantly with the global spread of technology and affluence. Excessive consumption of calorie-dense/nutrient-poor foods (i.e. Western-style diets) and physical inactivity are behaviors in stark contrast to the lifestyles led by our hunter-gatherer ancestors, and have caused ballooning waistlines all over the world. The World Health Organization currently estimates that over 1 billion adults are overweight (Body Mass Index [BMI] 25-29.99 kg/m^2) and at least 300 million of them are obese (BMI $\geq$30 kg/m^2). In 1995 and 2001, the global prevalence of diabetes was ~135 million and ~170 million, respectively, and this number is projected to be ~300 million in 2025 (82), with the greatest increases in the developing countries of Africa, Asia, and South America. The total number of deaths attributed annually to diabetes is around 2.9 million (134) and diabetes mellitus alone claims on average around 8% of total healthcare budgets in developed countries (135). Direct healthcare costs associated with the metabolic syndrome, a cluster of medical disorders commonly co-expressed with type 2 diabetes, currently dominate Western healthcare budgets (137).

The equivalent US statistics from 1999-2000 make equally grim reading considering that 14.4% of the population had impaired fasting glucose or diagnosed/undiagnosed diabetes [T2DM disease progression is defined in the next paragraph[1]] (19), 8.3% of the population was officially diagnosed with type 2 diabetes (19), and ~60% of the population was classified as either overweight or obese (46). Statistics from 1999-2002 indicate that ~65% of the US population was either

overweight or obese (62), and that the prevalence of obesity among US type 2 diabetics was 54.8% (20). The average weight gain over the last decade was estimated to be 0.45-0.90 kg/yr (66), corresponding to a total increase in body weight of 4.5 kg (5.5 lbs)-9kg (20 lbs) per American. Considering that for every kilogram gained in body mass there is a linear increase in risk of diabetes and cardiovascular comorbidities such as coronary heart disease (CHD), the health implications of excessive weight gain is potentially life threatening. In addition, conditions that predispose to overt diabetes, including impaired fasting glucose and glucose intolerance (and thus indirectly obesity), are not merely of academic interest, since, unless treated, ~7% of people with these problems will progress to type 2 diabetes every year (122). Approximately 1.3 million new US cases occur annually (i.e. incidence) and the total direct and indirect costs of treating diabetes, as estimated by the American Diabetes Association (ADA), exceed $130 billion per year.

[1]Stage of disease is determined by A) Fasting venous plasma glucose test (FG; administrated after at least 8 hours fasting) and/or B) Oral glucose tolerance test (OGTT; measured 2 hours post glucose load in venous blood) as established by WHO in 1998 and the American Diabetes Association in 2003 (1, 32). If the fasting glucose concentration is in the diagnostic range for diabetes, an OGTT is not required for diagnosis, but a confirmatory test should be performed due to intraindividual variations. Normal glucose regulation can only be verified using both FG and OGTT since subjects with fasting glucose values below diagnostic criteria may exhibit impaired glucose tolerance. Insulin resistance is defined as a reduced responsiveness of a cell, an organ, or the whole organism to insulin concentrations to which it is exposed (112) and is

generally present before any abnormalities in glucose regulation are detected. Insulin resistance is tightly coupled to plasma hyperinsulinemia with approximately twofold elevations in basal insulin concentrations in obese and/or type 2 diabetic patients (112).

A) FG: Normal glucose regulation (<100 mg/dL; <5.5 mmol/L) → impaired glucose regulation (100-125 mg/dL; 5.6-6.9 mmol/L) → diabetes mellitus (≥126 mg/dL; ≥7.0 mmol/L).
B) OGTT: Normal glucose regulation (<140 mg/dL; <7.8 mmol/L) → impaired glucose regulation (140-199 mg/dL; 7.8-11.0 mmol/L) → diabetes mellitus (≥200 mg/dL; ≥11.1 mmol/L).

Pathophysiology and Etiology of Type 2 Diabetes

The defining pathophysiologies (i.e. biological manifestations of a disease) of T2DM include a chronic elevation in blood sugar and plasma insulin levels (i.e. hyperglycemia and hyperinsulinemia, respectively), increased plasma amino acid levels (hyperaminoacidemia), and elevated plasma, intra-organ, and cellular lipid levels (dyslipidemia). Although cause and effect mechanisms still remain elusive, it is believed that disturbances in carbohydrate, lipid, and to a lesser extent protein metabolism lead to insulin resistance and ultimately type 2 diabetes. The comorbidities typically associated with T2DM, in addition to hyperglycemia and hyperinsulinemia, are collectively referred to as the metabolic syndrome, which is a cluster of pro-atherogenic conditions including hypertension, dyslipidemia, and abdominal obesity. It is estimated that 20% of

US adults suffer from the metabolic syndrome (51), and that these individuals are at a 3.5 times greater risk of death from CHD, as well as an increased risk of liver and kidney disease, and possibly cancer. All in all, type 2 diabetics have a two- to fourfold increased risk of developing cardiovascular complications and a threefold increased risk of suffering a mortal stroke compared to healthy individuals. Even the less benign "pre-diabetic" stages (impaired fasting glucose [IFG] and/or glucose intolerance) are strongly associated with cardiovascular disease (CVD) [including cerebrovascular and peripheral vascular disease], dyslipidemia, and various forms of cancer (61). Not surprisingly, micro- and macrovascular[2] complications cause 70% of deaths in this population (57), where retinopathy, nephropathy, and neuropathy (i.e. microvascular complications) account for the majority of morbidity and mortality (75). An estimated ~40-70% of all non-traumatic amputations world-wide are performed in diabetics due to ulcerations of the lower limb, and an astounding 1200 amputations are performed every week among the 16 million US diabetics (17). Less benign manifestations of T2DM include polyuria (frequent urination), polydipsia (increased thirst and fluid intake), polyphagia/hyperphagia (overeating), gastroparesis (delayed gastric emptying), depression, impotence, and blurred vision.

[2]Hypertension, atherosclerosis, arterial-wall stiffening/reduced compliance, coronary artery disease, endothelial dysfunction leading to increased permeability and decreased vasodilation (eNOS and tetrahydrobiopterin [BH4] levels decreased), arterial calcification, stenosis, thromboembolism, myocardial microangiopathy, cardiomyopathy, myocardial infarctions, aneurysms/hemorrhages/ruptures of

carotid arteries/vessels in brain, and peripheral vascular disease caused by ischemia, tissue damage, and inflammation.

Significant advances have been made in type 2 diabetes research over the last 100 years, but the etiology of the disease is ill-defined and cause-and-effect mechanisms remain elusive. Although there is a strong linear correlation between obesity and diabetes on an epidemiological level (20), and compelling evidence that disturbances in fat metabolism underlie many of the metabolic dysfunctions associated with the disease, the etiological reason(s) why most type 2 diabetics suffer from dyslipidemia and excessive adiposity remain unclear. Physical inactivity and consumption of calorie-dense/nutrient-poor foods are undeniably key factors in the development of the metabolic syndrome, but both environmental and inheritable factors may contribute to the advancement of the disease. It has been estimated that ~10% of type 2 diabetes cases are attributed to genetic factors (105), where single nucleotide polymorphisms (defined as occurring in >1% of the population), mutations (defined as occurring in <1% of the population), and/or epigenetic modifications of candidate genes collectively confer an increased susceptibility upon which environmental factors act to regulate disease progression (34). In other words, genes cock the gun and the environment pulls the trigger. There is indirect evidence that our genes are still programmed for the lifestyles associated with our historical ancestors (i.e. regular daily physical activity and consumption of the "stone-age diet"), and that the mismatch between the modern bioenvironment and the human genome sets the stage for the development of chronic degenerative diseases (38). The maladaptation between modern lifestyle factors and our

genetically controlled biochemistry have promoted a dramatic increase in incidence of chronic degenerative diseases since the industrial revolution (38). The effects of dietary factors and sedentary activity patterns account for about 365,000 deaths each year in the US and represent a leading contributor to death, second only to tobacco use (96).

Although the global cause of the exponential increase in chronic degenerative diseases in modern times has been identified (hence CDDs are commonly referred to as life style-related disorders), organ and cellular level mechanisms are generally poorly defined. In the case of overt type 2 diabetes, there is strong evidence that hyperglycemia is caused by a decreased insulin sensitivity [e.g., insulin resistance] of target cells in peripheral/splanchnic tissues [primarily fat, muscle and liver cells] and/or insufficient insulin production due to a gradually declining pancreatic β cell function (Fig. 1). Pancreatic failure is indeed the last stage in the progression of type 2 diabetes, but it is believed that impaired substrate clearance by skeletal muscle and adipose tissue, and to a lesser degree over-secretion of substrate by hepatocytes and portal adipocytes, are the immediate physiological causes of the clinical manifestation of the disease [i.e. hyperglycemia] (86). In addition to a reduced uptake/increased release of fatty acids by adipocytes and decreased glucose disposal in skeletal muscle, type 2 diabetes is characterized by an increased postabsorptive (basal; fasting) hepatic glucose production (HGP) and a reduced ability of insulin to suppress HGP (i.e. hepatic insulin resistance) (103). The glucose released by the liver can be derived from either glycogenolysis or gluconeogenesis. The mechanisms responsible for an increased hepatic gluconeogenesis in type 2 diabetics include hyperglucagonemia, increased circulating levels of

gluconeogenic precursors (lactate, alanine, glycerol), increased FFA oxidation, enhanced sensitivity to glucagon and decreased sensitivity to insulin. Although the majority of evidence indicates that a chronic elevation in gluconeogenesis is the major cause of the increase in HGP in type 2 diabetic subjects, it is likely that accelerated glycogenolysis also contributes.

In healthy individuals, increased gluconeogenesis and glucose release is compensated by decreased glycogenolysis, due to concomitant hyperinsulinemia, increased levels of glucose-6-phosphate, and lipolysis-derived ATP (inhibition of glycogen phosphorylase and activation of glycogen synthase), thereby maintaining hepatic glucose output at an optimal level (so called hepatic autoregulation) (85). In obese individuals, chronically elevated serum free fatty acids (FFAs) and high insulin levels lead to increased FFA uptake by the liver and increased synthesis of lipids, resulting in hepatic triglyceride (TG) accumulation, which is accompanied by hepatic insulin desensitization caused by lipid-mediated activation of protein kinase C (33). Thus, lipid-induced breakdown of hepatic autoregulation may underlie the increase in hepatic glucose output and ultimately whole-body hyperglycemia in type 2 diabetics.

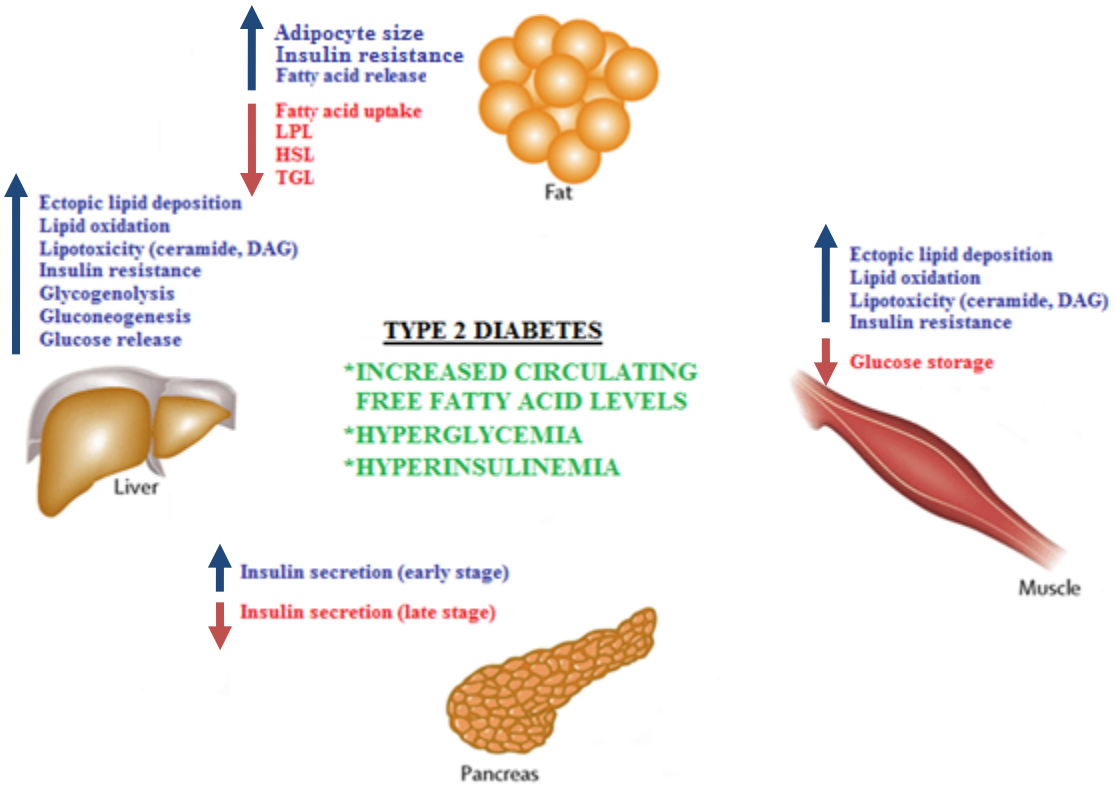


Fig. 1. The 'four horsemen' of diabetes (pancreas, adipose tissue, liver, and skeletal muscle). Type 2 diabetes is associated with insulin resistance of target cells in peripheral/splanchnic tissues, collectively causing hyperglycemia and increased circulating levels of free fatty acids. An impaired insulin action on adipose lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and triglyceride lipase (TGL) leads to an attenuated uptake but augmented release of non-esterified (free) fatty acids (FFA) into the blood stream. Chronically elevated plasma FFA levels lead to ectopic lipid deposition in the liver and augmented hepatic lipid oxidation followed by an increase in gluconeogenesis/glycogenolysis and release of glucose. A chronic increase in circulating FFAs also trigger intramuscular lipid accumulation and amplified lipid oxidation in skeletal muscles, which induces lipotoxicity (buildup of lipid intermediates such as ceramide and diacylglycerol [DAG]) and a reduction in glucose uptake by inhibiting insulin receptor signaling and GLUT-4 translocation. Pancreatic β-cells will initially augment insulin release in response to elevated plasma glucose levels in insulin-resistant individuals, but will gradually lose its ability to secrete the hypoglycemic hormone leading to overt type 2 diabetes and eventually β-cell failure.

Several theories regarding the etiology of insulin resistance have been proposed, all of which have support in literature. Prior do describing these theories it is important to mention that they are not mutually exclusive. According to the adipose tissue expandability hypothesis (52, 129), the capacity of an individual to expand their fat-mass to store lipid is a more important determinant of metabolic problems than absolute amount of adipose tissue. The failure to buffer lipid appropriately leads to elevated fatty acids and triglycerides, causing ectopic deposition of lipid (i.e. lipotoxicity) and insulin resistance in non-adipose organs such as liver, muscle, and pancreatic β-cells. The impaired adipose expandability hypothesis may explain the paradoxical finding that lipodystrophic individuals cannot accumulate fat, yet suffer from diabetes, dyslipidemia, and other metabolic complications. Further indirect support of this hypothesis can be seen in morbidly obese individuals who do not exhibit metabolic syndrome, indicating that they have yet to reach the point where they cannot expand their adipose tissue further. Another plausible theory pertains to an inherent or acquired mitochondrial deficiency (organelle number and/or function) causing a reduced capacity of organs to adjust lipid oxidation according to lipid bioavailability, which ultimately lead to ectopic fat deposition and lipid-induced toxicity in non-adipose tissues. Although not measuring substrate oxidation directly, Kelley and colleagues (2002) demonstrated that skeletal muscle mitochondria in type 2 diabetic and obese subjects are smaller and may exhibit impaired bioenergetic capacity compared to lean individuals (81). In contrast, more recent research in humans with T2DM and metabolic rodent models have suggested that impaired mitochondrial fatty acid oxidation does not account for an increased

intramuscular lipid content (15, 69). As a matter of fact, an elevated intramuscular lipid load, as observed in insulin-resistant rodent models (69), causes an elevation in fatty acid oxidation rates and improves mitochondrial density (69). It appears that when the rate of fatty acid transport across cytosolic and mitochondrial membranes exceeds the concurrently augmented capacity for mitochondrial oxidation (69), intramuscular lipids begin to accumulate and eventually cause damage in a time-dependent manner, possibly through oxidative stress (2, 12) Indeed, high-fat diet initially induces an increase in mitochondrial biogenesis and fatty acid oxidative capacity, with deteriorations in mitochondrial structure and function occurring first after several months of high-fat feeding (12). Consequently, mitochondrial dysfunction may be a consequence rather than a cause of cellular perturbations in substrate metabolism in T2DM (12). Lastly, the metabolic inflexibility theory suggests that T2DM is associated with a reduced ability to adapt glucose/lipid oxidation rates in response to changes in substrate availability (54). For example, an impaired capacity of oxidative tissues to adapt rates of lipid oxidation to lipid supply may ultimately lead to an increased triglyceride storage [ectopic lipid deposition] and insulin resistance. Metabolic flexibility to a high-carbohydrate meal, defined as an increase in RQ post feeding, has consistently been shown to be impaired in T2DM subjects during euglycemic-hyperinsulinemic clamp conditions compared to lean individuals (54). In addition, metabolic flexibility to a high-fat meal, defined as a drop in RQ, has previously been shown to be significantly different between diabetic and weight-matched non-diabetic subjects, with diabetics exhibiting a higher RQ following feeding (79). Although metabolic flexibility to long-term diets (fat or carbohydrate) does

not appear to be impaired in insulin-resistant individuals when assessed several days after the onset of feeding, the speed of adaptation appears to be compromised in this population, ultimately causing an increased accumulation of fat and possibly insulin resistance (54). Other plausible theories of insulin resistance are discussed in detail elsewhere (122), and include genetic factors (mutations or single nucleotide polymorphins of candidate genes), glucose toxicity, inadequate expansion of pancreatic β cells in response to increased insulin demand, and accumulation of amyloid (amylin) deposits in pancreatic islets.

Protein Metabolism and Type 2 Diabetes

Approximately 50% of whole body protein is synthesized and stored in skeletal muscle, which is an insulin-sensitive tissue that accounts for ~30-40% of total body weight (65), and is responsible for ~90% of glucose uptake after intravenous glucose administration (114) and ~25% after oral glucose load (80). Over 50% of skeletal muscle tissue (54.9% for males and 57.7% females) is located in the lower extremity (74), with much of this muscle positioned in the thighs and buttocks. Considering that skeletal muscle is one of the major sites of insulin resistance due to its importance in insulin-mediated glucose uptake, and that the lower body makes up >50% of total muscle mass, structural and functional maintenance of the larger muscle groups in the lower extremities may have clinical relevance for T2DM patients. Data from a recent NIH-sponsored multicenter randomized clinical trial indicate that type 2 diabetics have an altered tissue distribution with an excess of fat and lean tissue in the truncal region and a deficit of fat and lean tissue in the leg region (65). The clinical significance of a reduced

lean mass in the lower body is unknown, but a larger thigh and hip circumference, leading to a lower waist to hip ratio (WHR), has been shown to be associated with increased glucose tolerance on multiple occasions (116). Chowdhury and colleagues reported that differences in fasting glucose levels (FG) between age- and BMI-matched Indian and Swedish males (FG: 4.9 mmol/L and 4.5 mmol/L, respectively) were not due to differences in visceral fat, but rather a decreased muscle mass in the lower extremities of the former (24). Thus, despite exhibiting greater bodyweight, BMI, total fat mass, and total lean mass compared to healthy individuals, type 2 diabetics appear to have an altered lean mass distribution, which may act in parallel with insulin resistance to reduce glucose uptake and glycogen storage capacity of the lower body. Although the importance of maintaining lower body muscle mass compared to other muscle depots and the implications for whole body glucose tolerance in type 2 diabetics remain to be elucidated, indirect support of this notion may be provided by cross-sectional studies performed in the geriatric field. Age-related sarcopenia has repeatedly been shown to affect lower extremities more dramatically than upper extremities (74), and this loss parallels the progressive decline in glucose tolerance that begins in the third or fourth decade of life (30, 39).

Despite substantial alterations in glucose/lipid metabolism and reduced lower limb lean mass, perturbations in protein metabolism appear to be subtle in the human T2DM condition. Although significant methodological variations exist between studies (tracer choice, age, glycemic status, and gender of subject) making it difficult to draw any definite conclusions, assessments of protein synthesis and degradation rates in large

proximal muscle groups (e.g., quadriceps) have indicated an increased turnover or no difference between insulin-resistant and insulin-sensitive individuals (9, 60). Considering that type 2 diabetes-related complications such as peripheral neuropathy and microvascular dysfunction preferentially affect more distal regions of the limb (110), future research should aim to assess protein turnover in muscle groups such as gastrocnemius, plantaris, and soleus. Furthermore, despite subtle differences in the mixed protein pool, significant perturbations may still be present in cellular compartments of the muscle fiber that regulate substrate metabolism (mitochondria and cytosol) and energy utilization/muscle contraction (myofibrils). Glucose and lipid metabolism is ultimately dependent upon energy-demand, activation, and turnover of metabolic enzymes, and more research is needed to elucidate the individual contributions of each myocellular sub-fraction to the overall metabolic condition. Lastly, to fully understand the etiology of insulin resistance, efforts should be made to measure protein turnover over a 24-hour period without significantly perturbing circadian rhythms, sleep patterns, or normal nutrient intake.

Insulin-regulated glucose uptake, storage, and protein anabolism operate through the same pathway (Fig. 2), and it would make intuitive sense if impaired glucose handling in T2DM is associated with perturbations in protein anabolism. As described in previous paragraphs, recent research indicates that the balance between free fatty acid (FFA) availability, cellular uptake, and lipid oxidation is perturbed in the insulin-resistant state, which causes intramyocellular lipid accumulation and ultimately insulin resistance. The current theory on skeletal muscle insulin resistance is that an already

augmented lipid oxidation rate is unable to match an excess FFA uptake, which leads to an increased accumulation of lipid signaling intermediates (primarily diacylglycerol [DAG] and ceramide) and other lipid compounds (sphingolipids, eicosanoids, and phospholipids). These compounds interfere with insulin- and possibly contraction-mediated signal transduction by activating serine/threonine kinases such as PKCθ, thereby altering myocellular and whole-body glucose metabolism and potentially protein anabolism/breakdown. Perturbations in the association of IRS-1/2 to the insulin receptor and subsequent docking of phosphatidylinositol 3-kinase (PI3K) to IRS-1/2 appears to be prevalent in conditions of insulin resistance regardless of tissue type, leading to a decreased activation of protein kinase B (PKB/Akt). Akt-mediated inhibition of AS-160 [Akt-substrate 160; GTPase activating protein] is impaired in the insulin-resistant state, which maintains Rab proteins in GDP-bound states, and ultimately suppresses GLUT-4 translocation to the plasma membrane leading to reduced glucose uptake. In regards to skeletal muscle growth and insulin resistance, downstream signaling of Akt remains largely unelucidated. Although purely speculative, guanine nucleotide exchange factor (GEF) eIF2Bε may be partially inhibited (serines 535 and 539 phosphorylated) due to a decreased Akt-mediated GSK-3 repression [e.g., serine 21(GSK-3 α) and serine 9 (GSK-3β) becomes dephosphorylated]. Considering that obesity-induced insulin resistance is associated with an increased intramuscular branched chain amino acid concentration, at least in metabolic rodent models, a partial repression of eIF2Bε may be rescued by prevailing aminoacidemia and subsequent phosphorylation of serine 525. Nevertheless, due to an increased lipid-associated metabolic stress of the endoplasmic reiticulum,

activation and/or expression of PKR-like endoplasmic reticulum associated protein kinase (PERK) may be increased as a part of the unfolded protein response, which may lead to an inhibition of eukaryotic initiation factor eIF2 by keeping the α subunit phosphorylated (serine 51), ultimately preventing an interaction and GDP/GTP exchange between of eIF2 and eIF2Bε. This would reduce ternary complex formation, translation initiation, and potentially reduce myocellular protein synthesis rates. mTOR/raptor [mammalian target of rapamycin/regulatory associated protein of mTOR] dependent serine/threonine phosphorylations of ribosomal protein S6 kinase (p70S6k) and total p70S6k expression has previously been shown to be up-regulated in type 2 diabetes, perhaps as a result of BCAA-induced amino acid activation of mTOR. Phosphorylation state of the other major substrate of the mTOR/raptor complex, eukaryotic initiation factor 4E binding protein 1 (eIF4EBP1) would potentially be augmented due to aforementioned hyperaminoacidemia as well. This would in turn increase eIF4 complexation with eIF4G and eIF4A (eIF4F complex), which ultimately would lead to an increased 7-methyl-guanosine cap dependent mRNA translation. Considering that activation of downstream mTOR substrates p70S6K and eIF4EBP1 may be elevated in the insulin-resistant state, regulatory steps downstream of translation initiation must be inhibited (e.g., peptide-chain elongation), particularly if T2DM is associated with a suppression of global protein synthesis rates.

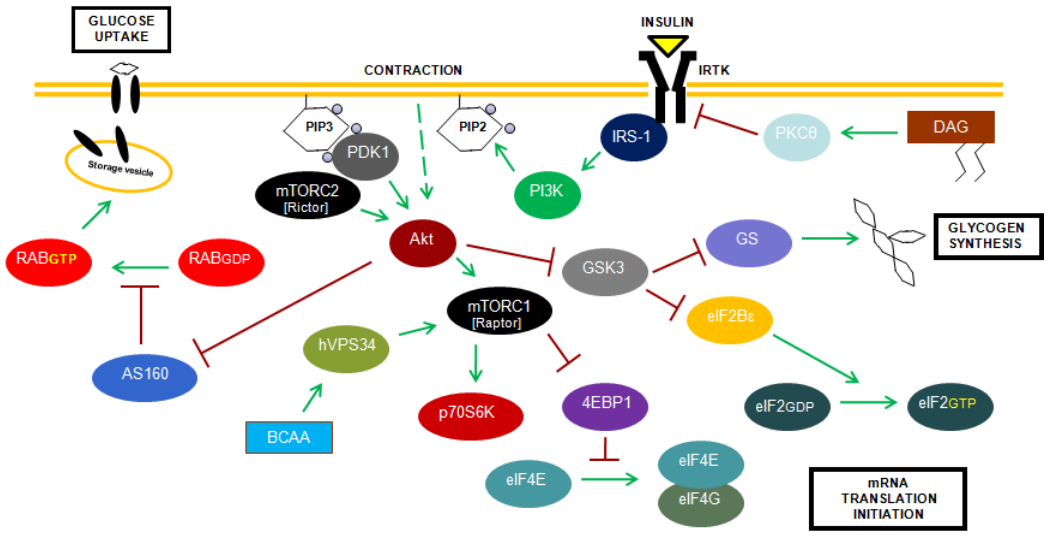


Fig. 2. Insulin-regulated glucose uptake, storage, and protein anabolism.

Exercise and Type 2 Diabetes

Similar to other life style-associated maladies, individuals who ultimately manifest overt T2DM pass through several clinical stages during the development of the disease, which may be reversed or halted with lifestyle modifications (diet and exercise) and/or pharmacological treatment. The bad news is that only ~50% of adults in the US engages in regular, leisure-time, physical activity [46.7% of women and 49.7% of men; (21)] and exercise adherence among clinical populations is generally poor outside clinical settings. Clearly, the development of cost-effective preventive and treatment strategies that increase long-term patient compliance must be considered of prime importance to reduce the economic burden and suffering associated with type 2 diabetes. Exercise, independent of caloric restriction and weight-loss, has proven to be an effective, low-cost, and non-pharmacological alternative to traditional diabetes medications (86). Regular physical activity has been shown to reduce the risk of

developing diabetes with 30-50% in healthy individuals (8), slow/reverse the progression of impaired insulin resistance in high-risk populations (31, 101, 126), improve glycemic control in overt type 2 diabetics (14), and confer additional protection against cardiovascular complications. Across all ranges of glycemic status, aerobically fit men have lower age-adjusted all-cause death rates than their less fit counterparts (83). In a Finnish heart disease risk factor study, men with maximal oxygen uptake (VO2max) of ≤ 25.8 ml /kg/min were more than four times as likely to develop diabetes than men with VO2max of ≥31.1 ml/kg/min after adjustment for BMI, baseline blood glucose, and other covariates (93). Several studies have confirmed an inverse correlation between aerobic fitness levels and incidence of cardiovascular events as well as relative risk for all-cause mortality in populations suffering from obesity, impaired fasting glucose, and/or overt diabetes (86, 131). Thus, there is compelling evidence that higher levels of cardiorespiratory fitness confers substantial protection against all-cause mortality, macrovascular disease, and type 2 diabetes in diverse populations with various degrees of insulin resistance (86).

Life style interventions (dietary modifications and exercise) have been shown to play a more substantial role in the prevention of type 2 diabetes in insulin-resistant individuals compared to traditionally used pharmacological agents (31). The American Diabetes Prevention Program reported that a lifestyle-modification program, with the goals of at least a 7 percent weight loss and at least 150 minutes of physical activity per week, reduced the incidence of diabetes by 58% and 27% compared to placebo and metformin, respectively, in an at-risk population (31). Although, metformin is one of the

most frequently prescribed drugs in the country overall and is the most popular anti-diabetic drug, recent research suggests that it may inhibit complex I of the electron transport system and impact aerobic capacity negatively (16).

A recent meta-analysis of 14 well-controlled randomized studies showed that 2 to 12 months of resistance and/or endurance exercise over a range of intensities improves glycemic control (~0.6% decreases in glycated hemoglobin; HbA_{1c}), reduces visceral and subcutaneous adipose tissue while maintaining bodyweight, decreases plasma triglycerides, and increases insulin sensitivity in type 2 diabetics (124). The aerobic exercise interventions, which, on average, consisted of three 53-min session per week of walking or cycling over an 18-wk period, were associated with a mean 0.67% reduction in HbA_{1c} levels, while resistance training (2-3 sets ranging from 10-20 repetitions at 50% of respondents' repetition maximum) yielded a comparable reduction of 0.64%. An exercise-induced reduction of 0.6% HbA_{1c} compares well with monotherapy of pharmacological agents such as biguanides, more specifically metformin, sulphonylureas, meglitinides, alpha-glucosidase inhibitors, DPP-4 inhibitors, and may be more effective than certain thiazolidinediones. Stratton et al. (2000) reported that a 1% rise in glycated hemoglobin represents a 21% increase in risk for any diabetes-related death, a 14% increased risk for myocardial infarction, and a 37% increased risk for microvascular complications (120). Although the decreases in HbA_{1c} through physical activity are substantial and are likely to markedly reduce the risk of diabetes-related complications, the optimal type, frequency, intensity, and duration of exercise for achieving these therapeutic goals are not known (124).

The American Diabetes Association, American Cancer Society, and American Heart Association advocate 0.5 hour/day of moderately intense exercise for the prevention of type 2 diabetes, CVD, and some types of cancer (40). Although raising the bar even higher may erode the motivation of chronically sedentary populations, there is strong evidence that intensity of exercise may be an important factor to enhance glucose homeostasis and ultimately prevent diabetes and CVD. Because long-term compliance remains a challenge for adult fitness and rehabilitation/intervention programs, which are traditionally based on aerobic endurance training, regular resistance exercise (RE) may provide a means for maintaining interest and increasing adherence. Nevertheless, studies in men suggest that vigorous exercise is associated with even greater reductions in risk of CVD than is moderate-intensity exercise (8). For example, each 1-MET (3.5 ml O_2/kg/min) increase in exercise intensity was associated with a 4% reduction in CHD risk independent of total exercise energy expenditure (123). Furthermore, it has been postulated that 2500-2800 kcal/wk (60-90 min/ day of moderate-intensity physical activity) may be required to maintain substantial loss of weight, which is an important goal in lifestyle modification programs for most type 2 diabetics. Exercise is often prescribed concurrently with caloric restriction in order to produce an energy deficit of 500-1000 kcal/day while maintaining lean mass and basal metabolic rate (66).

LaMonte and colleagues (2005) reviewed some of the acute and chronic exercise-induced muscle and systemic adaptations that enhance glucose homeostasis and confer protection against diabetes (86). Acute responses to exercise are immediate improvements in glucose uptake, transport, and/or disposal, but may be augmented with repeated bouts of exercise. Chronic adaptations to exercise include structural changes to the cardiovascular and musculoskeletal systems and require regular exposure to exercise to occur. Recent research has indicated that the acute anabolic response to contractile activity is blunted in the insulin resistant state. Although exercise has previously been shown to reduce whole-body glycosylated hemoglobin (HbA_{1c}) levels to a similar extent traditional diabetes medications (124), recent reports suggests that differences exist between normal and insulin-resistant skeletal muscle in their adaptation to mechanical loading (78, 95, 125), and that insulin resistance may blunt the anabolic response to resistance-type exercise (59, 77, 119).

CHAPTER II

CONTRACTILE ACTIVITY-INDUCED ANABOLIC RESPONSE IS ATTENUATED IN INSULIN-RESISTANT SKELETAL MUSCLE

Introduction

Since the industrial revolution, the incidence of life style-related diseases has increased concomitantly with the global spread of technology and affluence. Excessive consumption of calorie-dense/nutrient-poor foods and physical inactivity are behavioral patterns strongly associated with the development of obesity and type 2 diabetes (T2DM), which are rare conditions in cultures whose essential features mimic those of our hunter-gatherer ancestors (38). One of the first maladies associated with obesity is skeletal muscle insulin resistance, currently believed to be attributed to an increased accumulation of intracellular lipid metabolites (e.g., fatty acyl-CoA, diacylglycerol, and ceramide) that antagonize insulin action via activation of serine/threonine kinases and phosphorylation of IRS1 (67, 132, 141). Acquired or inherited mitochondrial deficiency, more specifically a reduction in the number or intrinsic function of the organelle, has been postulated to decrease the capacity to oxidize fat, causing a net lipid accumulation and insulin resistance in various metabolic disease conditions (92). This notion has been questioned lately (68), and recent research suggests that impaired mitochondrial fatty acid oxidation does not account for an increased intramuscular lipid content (15, 69). As a matter of fact, an increased intramuscular lipid load, as observed in insulin-resistant rodent models (69), causes an elevation in fatty acid oxidation rates and improves mitochondrial density (69).

Insulin resistance is associated with profound alterations in lipid and carbohydrate metabolism and has been linked to a down-regulation of Akt/mTOR growth signaling cascades (e.g. regulation of protein synthesis) in both humans and rodents. Although perturbations in basal state whole-body protein metabolism appear to be subtle in the human condition (9, 60), there are substantial methodological variations between studies (tracer choice, age, glycemic status, and gender of subject), making it difficult to draw any definite conclusions. Assessments of protein synthesis and degradation rates in large proximal muscle groups (e.g., quadriceps) in type 2 diabetics have indicated an increased turnover or no significant differences between insulin-resistant vs. insulin-sensitive skeletal muscle (9, 60). Considering that type 2 diabetes-related complications such as peripheral neuropathy and microvascular dysfunction preferentially affect more distal regions of the limb (110), future research should aim to assess protein turnover in muscle groups such as gastrocnemius, plantaris, and soleus. Furthermore, despite subtle differences in the mixed protein pool, significant perturbations may still be present in cellular compartments of the muscle fiber that regulate substrate metabolism (mitochondria and cytosol) and energy utilization/muscle contraction (myofibrils). Glucose and lipid metabolism is ultimately dependent upon energy-demand, activation, and turnover of metabolic enzymes, and more research is needed to elucidate the individual contributions of each myocellular sub-fraction to the overall metabolic condition.

At the turn of the 20th century, the economic burden of chronic diseases was estimated to be a mind-boggling 1 trillion dollars/year (13), with 35.3% of the US population exhibiting type 2 diabetes or impaired fasting glucose (25), 5.9-6.3% of the population had previously diagnosed T2DM (19, 25), and a staggering 65.7% of the population was classified as overweight or obese (62), including a 54.8% prevalence of obesity among type 2 diabetics (20). Clearly, the development of cost-effective interventions to delay or prevent the global obesity/diabetes pandemic must be considered of primary importance to improve health and relieve the burden of healthcare costs. Considering that type 2 diabetes is associated with a reduced glucose storage capacity, muscle weakness, and cardiovascular dysfunction, alternative intervention/treatment strategies should optimally augment muscle mass, strength, and oxidative capacity. Exercise, independent of caloric restriction and weight-loss, has proven to be an effective, low-cost, and non-pharmacological alternative to traditional diabetes medications. Because long-term compliance remains a challenge for adult fitness and rehabilitation/intervention programs, which are traditionally based on aerobic endurance training, regular resistance exercise (RE) may provide a means for maintaining interest and increasing adherence. Although resistance training has previously been shown to reduce whole-body glycosylated hemoglobin (HbA_{1c}) levels to a similar extent compared to chronic endurance exercise and traditional diabetes medications (124), recent reports suggests that differences exist between normal and insulin-resistant skeletal muscle in their adaptation to mechanical loading (78, 95, 125),

and that insulin resistance may blunt the anabolic response to resistance-type exercise (59, 77, 119).

The main purpose of the current study was to determine if skeletal muscle insulin resistance affects the 24-h anabolic response (cumulative plasticity) of cellular sub-fractions regulating metabolism (mitochondria and cytosol) and energy utilization (myofibrils). However, considering the paucity of information on insulin resistance and how it influences turnover rates of proteins in aforementioned compartments, a secondary goal was to characterize basal differences in protein synthesis rates between insulin-resistant and insulin-sensitive muscle. For these purposes we employed a novel primed-constant infusion method, centering on the use of deuterium oxide (^2H_2O) as a tracer [for review see (18, 35)], to assess 24-h cumulative protein synthesis of mitochondrial-, cytosolic-, and myofibrillar-rich fractions in proximal and distal muscle groups of sedentary and resistance exercised lean and obese Zucker rats. The primary event of obesity and insulin resistance is an increased food intake, irrespective of cause, making the obese Zucker rat, which carries a missense mutation in the leptin receptor gene, an almost ideal animal model for studying the effects of insulin resistance on protein synthesis. Similar to type 2 diabetic patients with metabolic syndrome, it exhibits hyperphagia (up to 19 wks of age), hyperinsulinemia, hyperleptinemia, hypertriglyceridemia, hypercholesterolemia, glucose intolerance, angiopathy, and neuropathy (99). The ^2H_2O method has a practical advantage in that animal or human subjects are not required to be in a post-absorptive state, which allows us to measure protein synthesis over long periods of time and ultimately get a more realistic assessment

of muscle anabolism in a free-living environment, thus, accounting for sleep, feeding, and 24-h fluctuations in hormone secretion. We report that cumulative fractional synthesis rates (FSR) of mixed proteins are suppressed in gastrocnemius and soleus muscles of obese Zucker rats, but significantly elevated in quadriceps muscle, indicating that the effects of insulin resistance on protein metabolism are diverse and dependent upon proximodistal location of the muscle group. We also provide evidence that protein synthesis rates are normal in myocellular compartments containing oxidative and glycolytic enzymes (e.g., mitochondria and cytosol) regardless of proximodistal location. Accordingly, the observed suppression in mixed protein synthesis rates in distal muscle groups are exclusively accounted for by a reduction in myofibrillar FSR, suggesting a preferential down-regulation of contractile proteins over metabolic enzymes. We surmise that rate-limiting steps in polypeptide elongation are more strongly inhibited in the obese phenotype, or that translational capacity and/or translational efficiency of specific mRNA pools are altered to cope with nutrient overload. Lastly, voluntary resistance exercise does not improve cytosolic, myofibrillar, or mitochondrial FSR to the same extent in obese vs. lean rats, which may be indicative of a blunted capacity to respond to contractile stimuli in the insulin-resistant state.

Methods

Animals

The obese Zucker rat exhibits similar dysfunctions in substrate metabolism that are present in type 2 diabetics with metabolic syndrome, making it a near ideal candidate for studying the effects of insulin resistance on protein turnover. Thus, thirty 4-mo-old male obese (fa/fa, Crl: ZUC-Lepr$^{fa/fa}$) and lean (Fa/?, Crl: ZUC-Lepr$^{Fa/?}$) Zucker rats (Charles River Laboratories, Wilmington, MA) were individually housed in a climate-controlled environment with a reversed 12:12-h light-dark cycle, having free access to water and standard commercial rat chow (Harlan 2016 Teklad Global) throughout the study period. Following 1 wk of acclimation, the rats were matched according to body composition (GE Lunar Prodigy DXA [small animal software]; total body-, lean-, fat-, and bone-mass) within each phenotype and thereafter grouped into sedentary (Fatty-SED [FS; n = 6], Lean-SED [LS; n = 8]) and resistance exercised (Fatty-RE [FE; n = 8], Lean-RE [LE; n = 8]) cohorts (Fig. 3). As expected, obese rats had a significantly higher % fat and bone mass compared to the lean phenotype ($P < 0.001$), while exhibiting significantly depressed % lean mass ($P < 0.001$). No differences in DXA-derived parameters were present between experimental conditions within phenotypes.

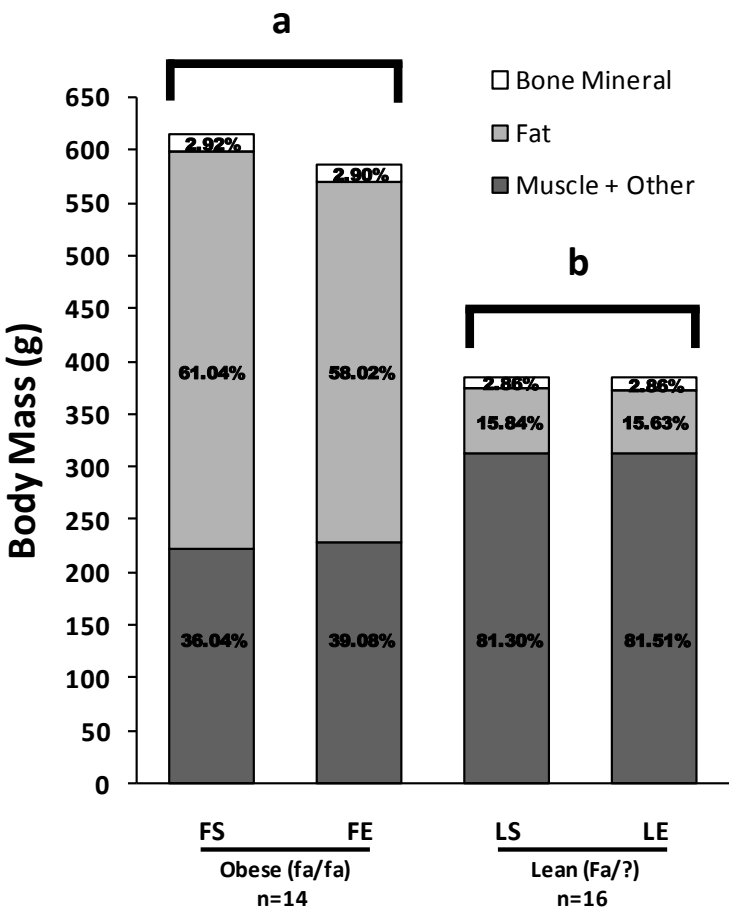


Fig. 3. Body composition of lean (Fa/?) and obese (fa/fa) Zucker rats (dual energy X-ray absorptiometry scans [DXA]). Animals (n = 30) were matched according to lean body mass within each phenotype and grouped into sedentary (lean sedentary, LS [n=8]; fat sedentary, FS [n=6]) and resistance exercised (lean resistance exercised, LE [n=8]; resistance exercised, FE [n=8]) cohorts. Total body mass, bone mineral content, or fat mass were significantly higher in obese vs. lean rats ($P < 0.001$), while muscle mass was significantly lower ($P < 0.001$). Bars/groups sharing the same letter are not significantly different in any of the aforementioned outcome parameters ($P > 0.05$). Values are expressed in grams (g; y-axis) and relative to total body mass (%, within bars), and presented as means ± standard error (SE).

Experimental protocol

For this study, we employed a well-established (42-45, 48, 49, 56, 63, 97, 133) voluntary resistance exercise (RE) paradigm to induce an anabolic response in protein sub-fractions of rat skeletal muscle. Although this 4-d progressive RE protocol can be considered "acute" since it does not result in appreciable changes of muscle mass, rates of protein synthesis in mixed gastrocnemius typically remain elevated 24-h post exercise (63). We have previously shown that this type of voluntary exercise increases bone mass in healthy rats (97, 133) and posterior crural muscle mass in insulin-deficient animals (44) if applied chronically. Despite a 'minimalistic approach' when it comes to the effective exercise duration (~2.5 min/session), the high work intensity (~75 reps/session at 105-175% BW) emphasizes muscular endurance and may promote mitochondrial adaptations. Since obesity-induced insulin resistance has been coupled to mitochondrial dysfunctions, an *in vivo* voluntary RE paradigm that induces mitochondrial growth may be favorable over other commonly used RE protocols when studying metabolic disorders.

We realize that direct electrical stimulation (E-STIM) of the lower limbs may provide better control over force production, however, the drive for muscle contraction does not begin in the brain in E-STIM protocols making them hard to compare to the voluntary lifting regimens performed by humans. Furthermore, the administration of anesthesia during simulated resistance exercise affects skeletal muscle contractility (72), compromises cardiopulmonary function (29), and may alter blood flow to and from working muscles, which may be particularly problematic when assessing protein turnover in metabolic models already exhibiting cardiovascular and neural dysfunctions. Compensatory hypertrophy models, such as synergistic ablation or synergistic denervation, are effective in inducing muscle growth by increasing satellite cell incorporation (myonuclei addition) into myofibers, but may not be reflective of the anabolic response to voluntary exercise (107).

Briefly, after a 1-wk acclimation period, RE cohorts were conditioned in a dark room to depress an illuminated bar in a Plexiglas exercise cage while wearing a Velcro vest adjustable for progressive increases in weights. Negative reinforcement via an electrical foot shock (< 2 mA, 60 Hz) was used to train the animals to perform the desired movement, which closely mimics a traditional leg squat as performed by humans, and requires full extension of hind legs and activation of associated muscle groups (mainly quadriceps and gastrocnemius complexes).

To account for possible confounding effects of negative reinforcement and handling on muscle metabolism, the sedentary cohorts wore the same Velcro vest and received the same average number of foot shocks as exercised animals, but without performing the actual exercise. In our experience, the obese rats mastered the movement pattern equally fast compared to their lean counterparts and did not require an altered absolute exercise intensity or increased shock-frequency. Following six operant conditioning (OC) sessions over 2 weeks, entrained rats of both phenotypes underwent a voluntary lower-body resistance exercise paradigm, consisting of four progressive sessions separated by either 48 h (RE1-RE3) or 72 h (RE3-RE4) [Fig. 4]. The RE cohorts performed the same number of sets and repetitions per weight (5 sets/session; 50 reps RE1, 68 reps RE2, 84 reps RE3, and 92 reps RE4;) and were allowed 2 seconds of rest between reps and 2 minutes of rest between sets. Weights were progressively increased throughout each session (30-230 g RE1; 80-230 g RE2; 80-230g RE3; and 80-280 g RE4) and the total amount lifted increased from 5500 g (RE1) to 15460 g (RE4). In accordance to previously published work using this exercise model in rats (42), the final bout (RE4) was completed 16 h prior to muscle harvest (4:30-5:00 PM).

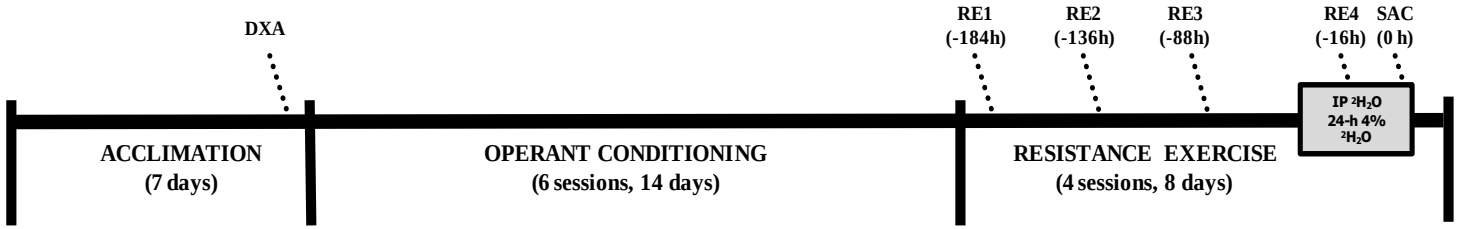


Fig. 4. Schematic display of the study design. After one week of acclimation, rats in RE groups were operantly conditioned to perform a squat-like movement while wearing a Velcro vest adjustable for progressive increases in weights. All entrained rats underwent the same resistance exercise paradigm with minimal negative reinforcement, which consisted of four progressive sessions separated by either 48 h (RE1-RE3) or 72 h (RE3-RE4). The final exercise bout (RE4) was completed 16 h prior to muscle harvest. To measure cumulative muscle protein synthesis, an intraperitonal 99.9% $^{2}H_2O$ bolus injection was administered 24 h prior to sacrifice, and 4% $^{2}H_2O$ was provided *ad libitum* in the drinking water throughout the last day.

To measure cumulative protein synthesis in skeletal muscle of sedentary and trained cohorts, an intraperitoneal (IP) 99.9% $^{2}H_2O$ bolus injection (20ul/g BW) was administered to each animal 24 h prior to sacrifice (08:30-09:00 AM, 8 hours prior to last exercise session), and 4% $^{2}H_2O$ was provided *ad libitum* in the drinking water throughout the last day. Rat chow was withdrawn 4 hours prior to muscle harvest (04:30-5:00 AM). On the morning of sacrifice (08:30-09:00 AM), the 5-mo-old rats were anesthetized with ketamine hydrochloride (Ketaset; 37.5 mg/kg BW) and medetomidine (Domitor; 0.25 mg/kg BW) via IP route. Two mL of whole blood were collected by cardiac puncture followed by quick excision of quadriceps and posterior crural muscle groups (mixed gastrocnemius, soleus, and plantaris) prior to euthanasia. Fat, blood, and connective tissue were removed from muscles before snap-freezing in liquid nitrogen, pulverization, and storage at -80°C.

Measurement of ^{2}H enrichment in body water

As with all precursor-product labeling methods, the assessment of protein synthesis using heavy water requires reliable quantification of ^{2}H enrichment of the precursory pool (i.e. body water/plasma). ^{2}H-labeling of body water (E_{BW}) was measured by gas chromatography-mass spectroscopy (Agilent 7890 GC/5975 VL MSD) following 24-h isotopic exchange between ^{2}H-enriched plasma samples and acetone, a method allowing for as low as 0.008% ^{2}H enrichments of body water to be assayed (140). Briefly, 20 uL of calibration standards (0-5% $^{2}H_2O$, prepared by mixing naturally labelled water with 99.9% $^{2}H_2O$) or plasma samples were allowed to react for 24 hours at room-temperature with 2 uL of 10N NaOH and 4uL of a 5% (v/v) solution of acetone

in acetonitrile. After the 24-h incubation period, the solution was extracted by adding 0.5g of Na_2SO_4 followed by 600 ul of chloroform and vigorous vortex. A small aliquot (50-100 μL) was transferred to a GC/MS vial and automatically injected (1 μL) into an Agilent 7890A GC system, volatilized, and separated on a capillary column (HP-5ms, 30 m x 0.25 mm x 0.24 μm) using helium as the carrier gas (1 μL/min) at a 40:1 split ratio. The GC injector temperature was set at 220°C and the transfer line was held at 220°C. The initial temperature of the column program was 60°C followed by an increase of 20°C/min to 100°C, which was further increased to 220°C at a rate of 50°C/min and held constant for 1 min. Acetone eluted from the column ~1.7 min post injection and was ionized with an Agilent 5975C VL MSD operating in electron impact mode using an ionization energy of 70eV. Selective ion monitoring (SIM) of mass to charge ratios (*m/z*) 58 (M) and 59 (M+1) using a dwell time of 10 ms/ion were conducted after autotune. Peak abundances of ions 58 and 59 were extracted from chromatograms and M+1/M ratios were used to calculate % enrichment of body water based on a linear regression formula generated by deuterium oxide standards ($R^2 = 0.999$). ^{2}H-labeling of body water was readily detected in both phenotypes (3.04 ± 0.11%; mean ± SE), with an average of 3.05 ± 0.17% in sedentary groups and 3.04 ± 0.15% in resistance exercised groups. All plasma samples were measured twice (prepared on two separate occasions) and an average value of the two runs was used for all calculations.

Measurement of ^{2}H enrichment in skeletal muscle

Enrichments of mixed, myofibrillar-, cytosolic-, and mitochondrial-rich fractions in quadriceps, gastrocnemius, and plantaris/soleus (mixed only) were determined by measuring protein-bound ^{2}H-alanine (E_A) as described by Dufner et al. (36). Pulverized mixed muscle (30 mg) or isolated sub-fractions (obtained from 100 mg mixed muscle) were homogenized in 0.3 mL of a 10% (w/v) TCA solution and centrifuged at 800 x g for 15 min at 4°C. For each sample, the supernatant was discarded and the pellet was centrifuged and washed three additional times in TCA to remove unbound amino acids. After the wash steps, the protein-rich pellet was hydrolyzed for 24 hours in 6N HCl at 110°C (~0.13 mL/0.01g tissue; 400 μL for all fractions except mitochondria [200 μL]). An 100 μL aliquot of the hydrolysate (50 μL for mitochondria) was dried down for 1 hour at 110°C and thereafter derivitized with a 3:2:1 (vol:vol:vol) solution of methyl-8, methanol, and acetonitrile for 1 hour at 70°C (1 μL/1 μL hydrolysate). The resulting methyl-8 /^{2}H-alanine derivative was transferred to a GC/MS vial and analyzed with an Agilent 5975C VL MSD equipped with an Agilent 7890A GC system (HP-5ms capillary column, 30 m x 0.25 mm x 0.24 μm) to determine ^{2}H-labeling of protein-bound alanine. The initial temperature of the column program was set at 90°C and held for 5 min, increased by 5°C/min to 130°C, which was further increased at a rate of 40°C/min to 240 °C and held for 5 min, all steps performed at a constant helium flow of 1 mL/min. Peak abundances of ions 99 and 100 were extracted from chromatograms and M+1/M ratios were used to calculate % enrichment of protein-bound alanine using a regression formula generated by ^{2}H-alanine standards ($R^2 = 0.999$). ^{2}H-labeling of alanine were

readily detected in all sub-fractions using a 20:1 split ratio for mixed proteins, 10:1 split ratio for myofibrillar and cytosolic proteins, and 5:1 split ratio for mitochondrial proteins. All samples were analyzed three times, which included complete re-preparation of the frozen hydrolysate on each occasion, to account for variations in preps as well as GC/MS analysis. An average value was used for all FSR calculations.

Calculations

The fractional synthesis rates (FSR) of mixed, myofibrillar, cytosolic, and mitochondrial fractions were calculated using the equation

$$E_A \times [E_{BW} \times 3.7 \times t\ (h)]^{-1} \times 100$$

where E_A represents amount of protein-bound ^2H alanine (mole % excess), E_{BW} is the quantity of ^2H_2O in body water (mole % excess), and 3.7 represents the exchange of ^2H between body water and alanine [i.e. 3.7 of 4 carbon-bound hydrogens of alanine exchange with water, (36)]. This equation assumes that ^2H-labeling of body water equilibrates with free alanine more rapidly than alanine is incorporated into newly made protein and that protein synthesis is linear over the study (139).

In certain circumstances, the intrinsic ability of making proteins (FSR) may not accurately reflect differences when accounting for the total amount of proteins being made over a unit of time in the entire muscle (total or absolute FSR). Because muscle mass and sub-fractional protein contents/concentrations (i.e. protein pools) can differ markedly between healthy and diseased individuals, it is possible that one group with a reduced muscle mass but elevated FSR will have a lower rate of absolute FSR when compared to another group with slower FSR but a larger muscle mass. Previous studies

assessing protein turnover in the obese Zucker rat model using *in vitro* incubation (37), flooding dose (106), or 5-6 hours continuous infusion protocols (22, 130) have shown that calculated rates of absolute protein synthesis merely exaggerate differences in FSR since the obese phenotype exhibits a lower protein mass. However, considering the plausibility that the obese phenotype has an increased intrinsic ability of making certain proteins (as measured over 24-h with the ^2H_2O method), but a lower absolute FSR due to a reduced protein pool size, we adjusted FSR for sub-fractional protein contents (absolute FSR[3]). Additionally, potential differences in intramyocellular protein concentrations, which may affect total enrichment of protein-bound alanine, were accounted for by adjusting for sub-fractional protein concentrations (relative FSR[4]).

[3]Absolute FSR: Sub-fractional protein content x (E_A x [E_{BW} x 3.7 x t (h)]$^{-1}$ x 100)

[4]Relative FSR: Sub-fractional protein concentration x (E_A x [E_{BW} x 3.7 x t (h)]$^{-1}$ x 100)

Isolation of skeletal muscle sub-fractions

The differential centrifugation protocol for the isolation of subsarcolemmal mitochondria and cytosolic proteins was adapted from Rooyackers (108) and intermyofibrillar mitochondria and myofibrillar proteins from Wilkinson (136). Enrichments of sub fractions were confirmed with Western blotting using cytochrome c oxidase IV- [mitochondrial], α-actinin- [myofibrillar], and GAPDH- [cytosolic] specific antibodies. A recent study by the Endocrine Research Unit at Mayo Clinic College of Medicine reported a 75% purity of the sub-fraction containing subsarcolemmal mitochondria with a similar isolation procedure (73). The protocol used to isolate

contractile proteins and intermyofibrillar mitochondria has previously been confirmed to yield robust enrichments as evidenced by electron microscopy (136).

Briefly, 100 mg snap-frozen quadriceps/gastrocnemius muscle was thawed and gently homogenized with 50 slow and compressive strokes of a glass-pestle (i.e. Dounce homogenization technique) in ice-cold mitochondrial isolation buffer 1 (10 mM Hepes, 200 mM Sucrose, 50 mM Mannitol, 2 mM EDTA disodium salt, Sigma P8340 protease inhibitor cocktail, pH 7.4). The homogenate was centrifuged at 600 g for 10 min at 4°C, followed by careful removal of the supernatant (cytosolic proteins and subsarcolemmal mitochondria [SS]) from the pellet (myofibrillar, nuclear, and stromal proteins, intermyofibrillar mitochondria [IMF]). SS mitochondria were obtained by centrifugation of the supernatant at 10,000 x g for 10 min (4°C) and were stored in mitochondrial isolation buffer 2 (50 mM Hepes, 5 mM EGTA, 1 mM ATP, 100 mM KCl, 5 mM $MgSO_4$, Sigma P8340 protease inhibitor cocktail, pH 7.4) until combined with the IMF mitochondria for assessment of protein synthesis. The remaining supernatant, containing cytosolic proteins, was precipitated with 95% ethanol (50% [v/v] in total solution) during low-speed centrifugation at 700 x g for 10 min (4°C) and thereafter dried.

The pellet produced by the centrifugation of the original homogenate was suspended in mitochondrial isolation buffer 3 (100 mM KCl, 50 mM Tris, 5 mM MgCl2 hexahydrate, 1 mM EDTA disodium salt, 10 mM β-glycerophosphate disodium salt, 50 mM NaF, 1.5% BSA, Sigma P8340 protease inhibitor cocktail, pH 7.5) and centrifuged at 650 x g for 3 min (4°C). The resulting pellet was homogenized in the same buffer with a glass pestle using shear and compression to release the intermyofibrillar mitochondria,

followed by centrifugation at 650 g for 4 min at 4°C to collect the organelle-rich supernatant. IMF mitochondria were then pelleted by centrifugation at 10,000 x g for 10 min (4°C) and combined with the SS mitochondrial fraction. The mitochondrial-rich sub-fractions were washed in isolation buffer 2 and ethanol, followed by high-speed centrifugation and drying in preparation for protein synthesis measurements.

The myofibrillar-rich pellet was washed in mitochondrial isolation buffer 4 (same as buffer 3 but without BSA) and centrifuged at 1,200 x g for 10 min (4°C) twice, which was followed by the addition of 0.3 M NaOH and heating of the sample in a water bath for 30 minutes. The myofibrils and collagen were separated by low-speed centrifugation at 650 x g for 10 min, and the myofibrillar proteins (supernatant) were collected after precipitation with 1 M PCA during low-speed centrifugation, washed with ethanol, and dried.

Muscle protein concentration and content

Protein concentrations and total protein contents of mixed (i.e. total), myofibrillar-, cytosolic-, and mitochondrial-rich sub-fractions were assessed using a commercially available colorimetric protein assay and calculated as previously described by Frier and Locke (53). Briefly, mitochondrial isolation buffer 1 (250 uL for myofibrillar and cytosolic; 50 uL for mitochondria) were added to isolated sub-fractions, followed by determination of protein concentration by the BCA method (115). The homogenate volumes were multiplied by the concentrations to obtain the total amount of protein within each isolated sub-fraction. The resulting values were divided by the mass of the homogenized muscle (~75 mg for mitochondrial; ~25 mg for myofibrillar and

cytosolic) to yield protein-to-mass ratios (ug/mg wet mass), which were reported as sub-fractional protein concentrations. Lastly, protein-to-mass ratios were multiplied by the mass of the entire quadriceps/gastrocnemius to obtain total protein contents (mg/total wet mass).

Western blotting

Because muscle harvest was performed 16 h after the last exercise session to allow ample time for $^{2}H_2O$ enrichment of slow-turnover proteins (myofibrillar), and the window of opportunity to detect changes in Akt-mTOR signaling is ~1-3 h post acute RE, the main purpose of this study was to determine basal phenotypical differences in total expression of enzymes regulating growth/survival. Thus, if no main effect of RE or interaction between phenotype and RE was detected by 2 x 2 ANOVA, data were collapsed within phenotypes. Results from sedentary cohorts are presented alongside the collapsed data to allow for comparisons of animals in a truly unperturbed state (from an acute exercise perspective). Notwithstanding, evaluation of phosphorylation states were completed on proteins expected to be activated for many hours post RE, and all groups are included in graphs if results were significant.

Briefly, pulverized mixed gastrocnemius muscle (40 mg) was homogenized in 400 uL Norris buffer (25 mM Hepes, 5mM β-glycerophosphate, 200μM ATP, 25 mM Benzamidine, 2mM PMSF, 4mM EDTA, 10 mM $MgCl_2$, 100 mM NF, 10 mM Na_3VO_4, Sigma protease inhibitor cocktail P8340, 1% TritonX100, pH 7.4). Samples were thereafter vortexed at regular intervals in ice-cold conditions for 1 hour and centrifuged at 13,000 x g for 30 minutes to separate the cytosolic-rich (supernatant) and the

myofibrillar-rich (pellet) protein fractions. The pellets were discarded and a small aliquot of each supernatant was used to determine protein concentrations as previously described.

We used the traditional discontinuous Tris-Glycine buffer system developed by Ornstein and Davis (28, 100) and further perfected by Laemmli (84) to separate proteins using SDS-PAGE. Samples were placed in modified Laemmli buffer (250 mM Tris base, 8% SDS, 40% Glycerol, 0.004% Bromophenol Blue, 400 mM DTT) and heated for 10 minutes at 100°C for denaturation of native proteins into individual subunits. Depending upon abundance and molecular weight of the target antigen in rat skeletal muscle tissue, 30-100 ug of cytosolic protein were loaded in equal amounts onto 4-15% polyacrylamide gels (10 cm x 10 cm x 1.6 mm; 37.5:1 acrylamide/bisacrylamide ratio) and subjected to 1 hour electrophoresis in electrode buffer (25 mM Tris base, 19.2 mM Glycine, 0.1% SDS, pH 8.3) at 40 mA, holding voltage and power output constant (Thermo Scientific Owl P8DS System, Rochester, NY). Each experimental group was represented on all gels to minimize confounding effects of gel composition and electrophoresis condition.

Following electrophoresis, filter papers (Munktell Grade 1F) and nitrocellulose membranes (~55 cm^2, 0.2µm pore size, Amersham Biosciences) were soaked for 10 minutes in Otter buffer (49.6 mM Tris, 384 mM glycine, 20% methanol [v/v] and 0.01% SDS [w/v]) in preparation for semi-dry or wet transfers. Gels were sandwiched between wet filter paper, nitrocellulose, and another layer of filter paper, but were not pre-equilibrated in transfer buffer in order to avoid loss of proteins. A wet 16-h three-step

transfer (Thermo Scientific VEP-2 Mini Tank Electroblotting System, Rochester NY) or a or 40-min semi-dry transfer (Thermo Scientific Owl HEP-1 Semi Dry Electroblotting System, Rochester, NY) was used to transfer proteins onto the membranes. A progressive three-step wet transfer, consisting of 1 h at 1.3 mA/cm^2, 14 h at 3.8 mA/cm^2, and lastly 1 hr at 7.5 mA/cm^2, maximizes chances of eluting high-molecular weight proteins from the gel while maintaining binding of low molecular weight proteins to the membrane. Although a 40-min semi-dry transfer at 400 mA (~7.5 mA/cm^2) was sufficient for most molecular weight proteins, elution efficiency of proteins such as mTOR (289 kD) and raptor (150 kD) were significantly improved with the wet method. Loading consistency and transfer efficiency were verified with Ponceau S stain (0.1% Ponceau S [w/v], 5% acetic acid [v/v]) of the membranes and Coomassie blue staining (50% trichloroacetic acid [w/v], 0.1% Coomassie Brilliant Blue [w/v]) followed by destaining (50% methanol [v/v], 10% acetic acid [v/v]) of the gels.

Membranes were blocked for 1 hour in blocking buffer (20 mM Tris base, 5% dry milk [w/v], 8% NaCl [w/v], 100 mM NF, 10 mM Na_3VO_4), and thereafter incubated overnight at 4°C under gentle agitation in a heat-sealed plastic bag containing 1:1000 primary antibody/blocking buffer. Following a 15 min (3 x 5 min) wash-step in wash buffer (20 mM Tris base, 8% NaCl [w/v], 100 mM NF, 10 mM Na_3VO_4), membranes were incubated for 1 hour at room temperature with a 1:2000 secondary antibody/blocking buffer solution under continual agitation. After repeating the 15 min wash-step, membranes were incubated for 5 minutes in 10 mL of enhanced chemiluminescent substrate (ECL; 50% [v/v] Pierce Super Signal West Pico Luminol

Enhancer Solution, 50% [v/v] Super Signal West Pico Stable Peroxidase Solution) and protein bands were developed with a CCD camera, mounted in a FluorChem SP imaging system (Alpha Innotech, San Leandro, CA). Optical density was determined using AlphaEase FC software (Alpha Innotech), which was automatically set to subtract non-specific binding from densitometry values. All bands were analyzed three times and the average density values were normalized between membranes and expressed as arbitrary units. Expression of proteins regulating growth/survival and mRNA translation initiation/elongation were of prime interest and membranes were probed for protein kinase B (PKB; Akt), mammalian target of rapamycin (mTOR), regulatory associated protein of mTOR (Raptor), regulated in development and DNA damage responses 1 (REDD1), proline-rich Akt substrate (PRAS40), p70 ribosomal S6 kinase ($p70^{s6k}$), phospho-$p70^{s6k}$ [threonine 389], eukaryotic initiation factor 4E binding protein 1 (eIF4EBP1), phospho-eIF4EBP [threonine 37/46], eukaryotic elongation factor 2 (eEF2), phospho-eEF2 [threonine 56], eEF2K, extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2; P44/P42), eukaryotic initiation factor 2B epsilon (eIF2Bε), and atrogin-1/MAFbx. Immunodetection of phosphorylated antigens were completed before assessment of total protein. Membranes were stripped for 15 minutes at room-temperature (Restore Western Blot Stripping Buffer, Thermo Scientific) and reincubated with ECL to ensure no residual chemiluminescence. All primary (rabbit and mouse) and secondary antibodies (anti-rabbit and anti-mouse) were purchased from Cell Signaling (Beverly, MA, USA) except atrogin-1 (ECM Biosciences, Versailles, KY) and eIF2Bε, a kind gift from Scot Kimball, Pennsylvania State University.

Immunoprecipitation

Complexation of eIF4EBP1 and eIF4G with eIF4E in gastrocnemius muscle was measured using a standard immunoprecipitation technique based on the high binding affinity of bacterial membrane proteins A/G to immunoglobulins. Briefly, 100 µg cytosolic proteins in modified Norris buffer [total volume of ~70µL] (0.3% Chaps, 1 mM DTT, 25 mM Hepes, 5mM β-glycerophosphate, 200µM ATP, 25 mM Benzamidine, 2mM PMSF, 4mM EDTA, 10 mM $MgCl_2$, 100 mM NF, 10 mM Na_3VO_4, Sigma protease inhibitor cocktail P8340, pH 7.4) were gently agitated overnight at 4°C with 1 µL rabbit monoclonal eIF4E antibody (1:70 ratio; Cell Signaling). Collection of antigen/antibody complexes was achieved by adding 20 µL 50% protein A agarose bead slurry to each sample followed by 3-h incubation at 4°C. Samples were then centrifuged at 10,000 x g for 1 min at 4°C and pellets resuspended in 500 µL Norris buffer for a total of three times to remove unbound antibodies. The final pellet was denatured in 20 µL 4X Laemmli buffer (250 mM Tris base, 8% SDS, 40% Glycerol, 0.004% Bromophenol Blue, 400 mM DTT) by heating at 95°C for 10 minutes and thereafter centrifuged at 10,000 x g for 1 min. The resulting supernatant was subjected to electrophoresis using a 7.5% (eIF4G) and 15% (eIF4EBP1) polyacrylamide gel followed by semidry transfer to nitrocellulose membranes as previously described. The blots were then gently agitated overnight at 4°C with mouse monoclonal eIF4E (Santa Cruz Biotechnology, CA, USA), mouse monoclonal eIF4EBP (Santa Cruz), and rabbit polyclonal eIF4G (Cell Signaling) at a 1:200-1:1000 ratio. Lastly, membranes were incubated with anti-rabbit or anti-

mouse secondary antibodies (1:2000 ratio; Cell Signaling) for 1 hour at room temperature and developed with ECL.

Statistical analyses

The effects of phenotype (obese vs. lean) and physical activity (sedentary vs. resistance exercise [RE]) on expression and synthesis of proteins were assessed with 2 x 2 ANOVA using SigmaStat version 3.5. When significant F-ratios were present, a Student-Newman-Keuls post hoc procedure was used to evaluate differences among group means. If there was no main effect of RE or interaction between RE and phenotype using 2-way analysis of variance, Western blotting data were collapsed within phenotypes (Fat [F; n = 14 and Lean [L; n = 16]). To allow for comparisons of animals in truly unperturbed states (from an acute exercise perspective), differences between sedentary cohorts (FS vs. LS) were assessed for significance with independent t-tests and presented as inserts in the graphs depicting the collapsed data (for further rationale see methods). If homogeneity of data failed, analyses of variance were conducted on transformed values (square-root) and statistical comparisons between two independent group means were completed with Mann-Whitney-Wilcoxon rank sum tests. Significance was set at $P \leq 0.05$ and all data are presented as means ± SE.

Results

Wet mass, protein content, and protein concentration

As expected, quadriceps and gastrocnemius wet mass and total protein content, but not protein concentration, were significantly suppressed in the obese phenotype ($P < 0.05$), and acute resistance exercise (RE) did not significantly affect muscle weights or protein contents/concentrations in either cohort (Figs. 5-6). The ~22% difference in mixed protein content of gastrocnemius muscle between phenotypes ($P < 0.001$) was primarily attributed to ~25% lower myofibrillar protein content ($P < 0.001$; 176 ± 9 mg and 236 ± 9 mg in obese vs. lean rats, respectively) and secondarily ~15% lower cytosolic protein content ($P = 0.04$; 77 ± 5 mg and 91 ± 5 mg in obese vs. lean rats, respectively) in obese rats. Similarly, the ~35% difference in mixed protein content of quadriceps muscle between phenotypes ($P < 0.001$) was attributed to ~36% lower myofibrillar protein content ($P < 0.05$; 296 ± 44 mg and 461 ± 55 mg in obese vs. lean, respectively), ~35% lower cytosolic protein content ($P < 0.001$; 109 ± 11 mg and 167 ± 6 mg in obese vs. lean, respectively), and ~28% lower mitochondrial protein content ($P = 0.021$; 5.7 ± 0.41 mg and 7.9 ± 0.77 mg in obese vs. lean) in obese rats. Although total mitochondrial content was significantly suppressed in quadriceps muscle of obese rats, mitochondrial density/concentration was not significantly different between phenotypes ($P = 0.635$; 3.41 ± 0.22 μg/mg and 3.15 ± 0.36 μg/mg in obese vs. lean, respectively). The most interesting and novel finding in this study in regards to muscle composition was that total mitochondrial protein content in gastrocnemius muscle was not significantly different between phenotypes (2.73 ± 0.23 mg and 2.57 ± 0.23 mg in obese

vs. lean, respectively) due to a ~31% higher mitochondrial density/concentration in obese rats ($P = 0.01$; 1.86 ± μg/mg and 1.42 ± μg/mg in obese vs. lean, respectively). From a biological standpoint it makes sense that the obese phenotype, which exhibits a higher amount of intramuscular lipids due to an upregulation of plasmalemmal and mitochondrial FAT/CD36 in gastrocnemius muscle (69), has more mitochondria per unit muscle in order to handle an increased lipid load (6, 69). Wet weights of solues and plantaris were significantly suppressed in obese vs. lean rats ($P \leq 0.001$), but no significant differences were found between experimental conditions within phenotypes (Table 1).

In agreement with previous research, we show no differences in total protein concentration between phenotypes (37) and a significantly lower total protein content in obese rats (113). The relative contributions of myofibrillar (~55-65%), stromal (~10-15%), and cytosolic fractions (~20-30%) to the mixed protein pool compare well with what has been published previously in this model (87). Mitochondrial content generally ranges from 1 to 3% of total cellular volume (70) and corresponding values obtained in our study are at the lower end of this spectrum.

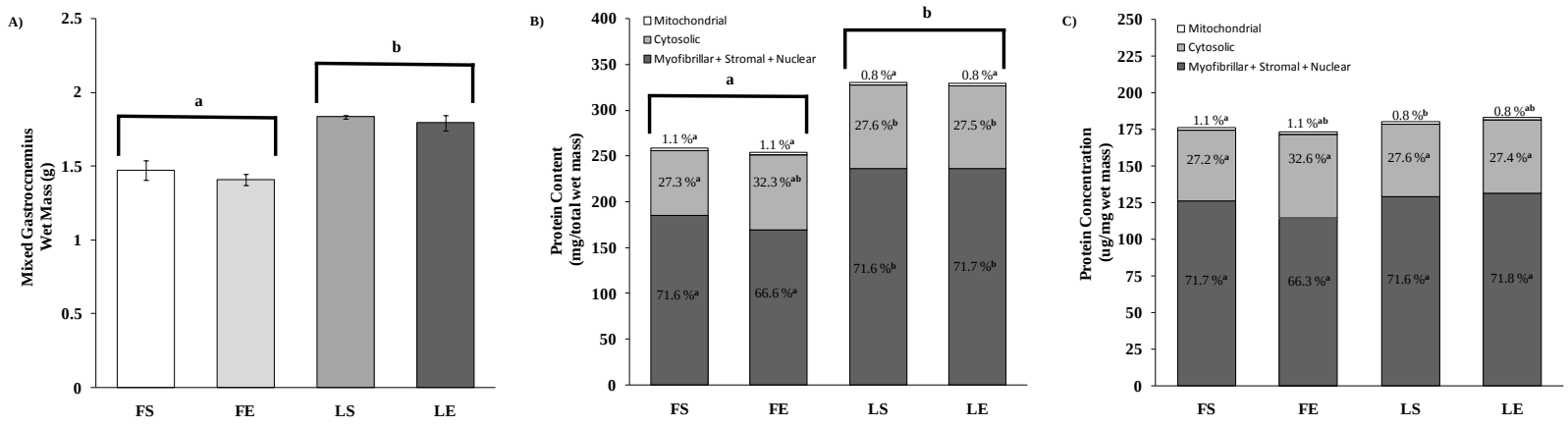


Fig. 5. Wet mass (Panel A), protein contents (Panel B), and protein concentrations (Panel C) of mixed gastrocnemius muscle. Panel A: Average wet mass of left and right gastrocnemius muscle. Main effect of phenotype ($P < 0.001$; 1.81 ± 0.03 g vs. 1.44 ± 0.03 g in lean vs. obese rats, respectively). Panel B: Mixed (total value), myofibrillar (bottom dark gray stack), sarcoplasmic (middle light gray stack), and mitochondrial (top white stack) protein contents. Main effect of phenotype on mixed ($P \leq 0.001$), myofibrillar ($P \leq 0.001$), and sarcoplasmic ($P = 0.04$) protein contents, but not mitochondrial ($P = 0.556$). Panel C: Mixed (total), myofibrillar (bottom), sarcoplasmic (middle), and mitochondrial (top) protein concentrations. Main effect of phenotype on mitochondrial protein concentrations ($P = 0.01$), but not mixed ($P = 0.417$), myofibrillar (0.194), or sarcoplasmic (0.552). As expected, two-way analyses of variance indicated no main effect of RE or interaction between RE and phenotype on any of the aforementioned outcome variables. Values (means ± standard error [SE]) are expressed in grams (g; Panel A), milligrams/total wet mass (mg; Panel B), micrograms/milligrams wet mass (µg/mg; Panel C), and relative to total protein contents/concentrations (%, within bars). Letters superscripted within bars denote significance in absolute values between experimental conditions according to SNK post hoc analysis or t-test ($P \leq 0.05$ if groups do not share the same letter).

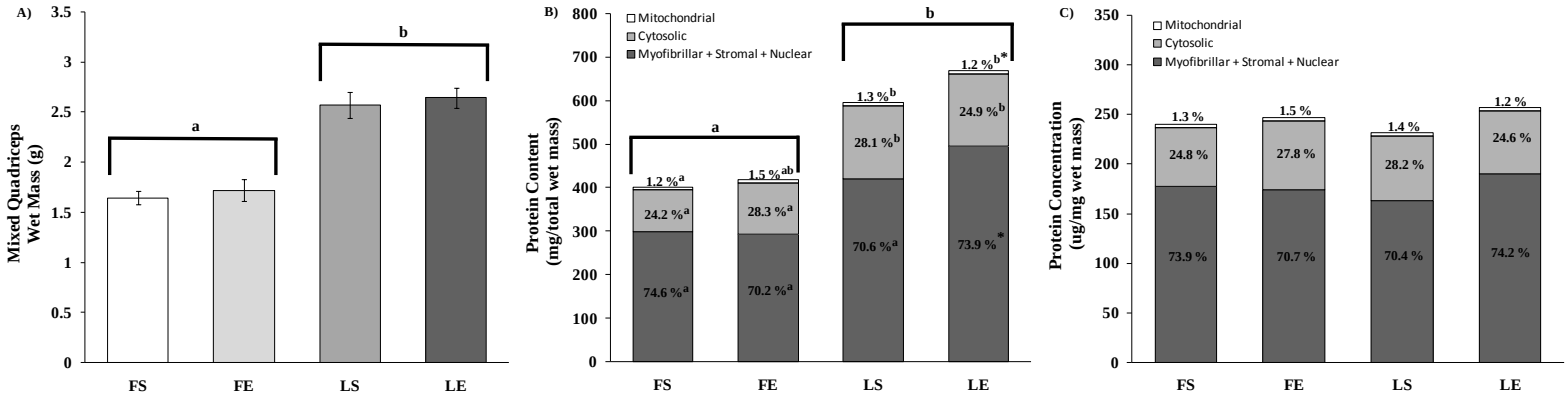


Fig. 6. Wet mass (Panel A), protein contents (Panel B), and protein concentrations (Panel C) of mixed quadriceps muscle. Panel A: Average wet mass of left and right quadriceps muscle. Main effect of phenotype ($P < 0.001$; 2.61 ± 0.08 g vs. 1.69 ± 0.07 g in lean vs. obese rats, respectively). Panel B: Mixed (total value), myofibrillar (bottom dark gray stack), sarcoplasmic (middle light gray stack), and mitochondrial (top white stack) protein contents. Main effect of phenotype on mixed ($P = 0.007$), myofibrillar ($P = 0.048$), mitochondrial ($P = 0.021$), and sarcoplasmic ($P < 0.001$) protein contents. Panel C: Mixed (total), myofibrillar (bottom), sarcoplasmic (middle), and mitochondrial (top) protein concentrations. No main effect of phenotype on mitochondrial ($P = 0.635$), mixed ($P = 0.643$), myofibrillar (0.982), or sarcoplasmic (0.989) protein concentrations. Two-way analyses of variance indicated no main effect of RE or interaction between RE and phenotype on any of the aforementioned outcome variables. Values (means ± standard error [SE]) are expressed in grams (g; Panel A), milligrams/total wet mass (mg; Panel B), micrograms/milligrams wet mass (µg/mg; Panel C), and relative to total protein contents/concentrations (%, within bars). Letters superscripted within bars denote significance in absolute values between experimental conditions according to SNK post hoc analysis or t-test ($P \leq 0.05$ if groups do not share the same letter). *Denotes borderline significant: Myofibrillar protein content, LE vs. FE ($P = 0.063$); mitochondrial protein content, LE vs. FS ($P = 0.076$).

Table 1. *Wet mass of soleus and plantaris*

	FS	FE	LS	LE	Phenotype	Activity	P*A
Wet Mass (g)							
Soleus	**0.183 ± 0.009^{ac}**	**0.174 ± 0.009^{a}**	**0.216 ± 0.007^{b}**	**0.198 ± 0.007^{bc}**	**P = 0.001**	**P = 0.108**	**P = 0.597**
Plantaris	**0.275 ± 0.02^{a}**	**0.279 ± 0.01^{a}**	**0.387 ± 0.01^{b}**	**0.364 ± 0.02^{b}**	**P < 0.001**	**P = 0.548**	**P = 0.359**

Values are means ± SE and expressed in grams (g). 2 x 2 ANOVA main effects (Phenotype, Activity) and interactions (P x A) are included to the far right in the table. Groups not sharing the same letter are significantly different as evaluated by SNK post hoc or t-tests ($P \leq 0.05$).

Cumulative fractional rates of protein synthesis (FSR)

We used heavy water (deuterium oxide, $^{2}H_2O$) to assess 24-h cumulative synthesis rates of mixed, mitochondrial (mito), cytosolic (cyto), and myofibrillar (myo) proteins in skeletal muscle of sedentary and resistance exercised lean and obese Zucker rats. Considering that uncertainties still exist regarding relative turnover rates of skeletal muscle sub-fractions, primarily attributed to considerable methodological variations between studies, comparisons between myocellular compartments should be viewed cautiously. In this study, the average synthesis rates of mixed (3.41%/d and 4.57%/d), myofibrillar (2.48%/d and 2.81%/d), and cytosolic (8.93%/d and 6.49%/d) sub-fractions in quadriceps and gastrocnemius muscle, respectively, are qualitatively similar to previous observations in humans and rats (i.e. Myo FSR < Mixed FSR < Cyto FSR). An approximate two- to three-fold difference between cytosolic and myofibrillar protein synthesis rates is viewed as normal in the postabsorptive state, and mixed FSR is typically 30-40% higher compared to myofibrillar FSR. In our hands, the average

synthesis rate of the two pools of mitochondria is ~5-15% higher compared to the mixed protein pool (4.00%/d vs. 3.41%/d and 4.76%/d vs. 4.57%/d) in quadriceps and gastrocnemius muscle, respectively.

Distal muscle groups (gastrocnemius, soleus, and plantaris)

We found that the obese phenotype exhibited a mild, but significant, suppression of 24-h protein synthesis rates in mixed and myofibrillar sub-fractions (-11%, P = 0.015; -12%, P = 0.007, respectively) of gastrocnemius muscle, while cytosolic and mitochondrial FSR were not different from lean rats (Fig. 7*A*; 8*A,C,E*). In lieu of the fact that there was no statistical difference in mixed FSR between sedentary groups (P = 0.437; FS vs. LS), the observed phenotypical suppression was partly attributed to a modest exercise effect in the lean phenotype (P = 0.165; 4.62 ± 0.22 %/d and 5.00 ± 0.18 %/d in LS vs. LE, respectively). Myofibrillar FSR was significantly lower in obese vs. lean rats (2.64 ± 0.06 %/d and 2.99 ± 0.10 %/d in obese vs. lean rats, respectively), but there were no differences in this sub-fraction between exercised and sedentary cohorts. Similarly, mitochondrial FSR was not statistically different between exercised vs. sedentary obese rats (P = 0.349; 4.23 ± 0.28 %/d and 4.72 ± 0.34 %/d in FS vs. FE, respectively), but the 12% higher synthesis rate in the FE cohort indicates a mild exercise effect on this organelle in the obese phenotype. In contrast to obese rats, mitochondrial FSR was significantly higher in exercised vs. sedentary lean rats (+20%, P = 0.049; 4.41 ± 0.44 %/d and 5.54 ± 0.39 %/d in LS vs. LE, respectively), suggesting an increased mitochondrial sensitivity to contractile stimuli compared to the obese phenotype. Despite a blunted anabolic response to exercise in obese rats, there was no

significant difference in the ability of making mitochondrial proteins between phenotypes in the sedentary state (P = 0.738). Synthesis rates of mixed proteins in soleus, but not plantaris, were significantly lower in obese vs. lean rats (-7%, P = 0.044, Table 2), but sub-fractional synthesis rates were not explored in these muscle groups due to tissue quantity necessary for fractionation. Considering that there was no statistical difference between sedentary cohorts in lean vs. obese rats (P > 0.05), the observed main effect of phenotype was partially attributed to a modest exercise effect in lean rats (+6%, LE vs. LS). Our finding that FSR may be preferentially suppressed in predominately slow/red muscles over fast/white muscles supports previous findings by Fluckey and colleagues who reported a significantly lower FSR in red but not white gastrocnemius muscle in this rats strain (47).

As expected, when analyzing rate at which proteins were made in each sub-fraction on a whole muscle level (Fig. 7*B*; Fig. 8*B*,*D*,*F*), absolute FSR of mixed and myofibrillar proteins were significantly lower in the obese phenotype (-30%, P < 0.001; -34%, P < 0.001). However, when cytosolic and mitochondrial FSR were adjusted for total protein content (Fig. 8*DF*) and protein concentration (Fig. 8*DF, inserts*), no significant differences were detected between phenotypes. As a matter of fact, mitochondrial FSR adjusted to protein concentration was ~20% higher in obese vs. lean rats, but this did not reach statistical significance (P = 0.269). Because the intrinsic ability of synthesizing mitochondrial proteins was similar between phenotypes, a significantly higher mitochondrial protein concentration solely accounted for the elevated adjusted FSR in obese rats.

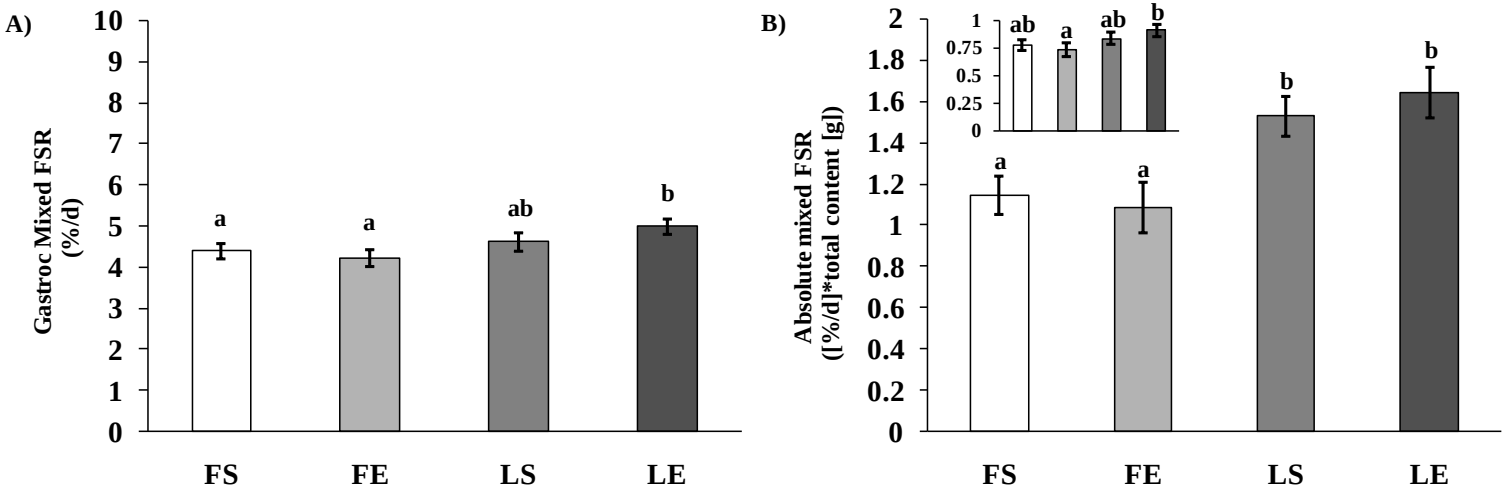


Fig. 7. Mixed protein synthesis rates of gastrocnemius muscle. Panel A: Intrinsic capacity of synthesizing mixed proteins (FSR) over a 24-h period. Main effect of phenotype ($P = 0.015$); FE vs. LE, $P = 0.006$; FE vs. LS, $P = 0.153$; FS vs. LE, $P = 0.049$; LS vs. LE, $P = 0.165$. Panel B: Protein synthesis over a 24-h period adjusted for mixed protein content (Absolute FSR). Main effect of phenotype ($P < 0.001$); FS vs. LS, $P = 0.022$; FS vs. LE, $P = 0.011$; FE vs. LS, $P = 0.006$; FE vs. LE, $P < 0.001$. Insert: 24-h FSR adjusted for mixed protein concentration. Main effect of phenotype ($P = 0.042$); FS vs. LE, $P = 0.126$; FE vs. LS, $P = 0.197$; FE vs. LE, $P = 0.025$. Two-way analyses of variance indicated no main effect of RE or interaction between RE and phenotype on any of the aforementioned outcome variables. Bars/groups sharing the same letter are not significantly different ($P > 0.05$) according to SNK post hoc analyses or t-tests. Data are expressed as %/d, with or without adjustments for protein content and protein concentration, and presented as means ± standard error (SE).

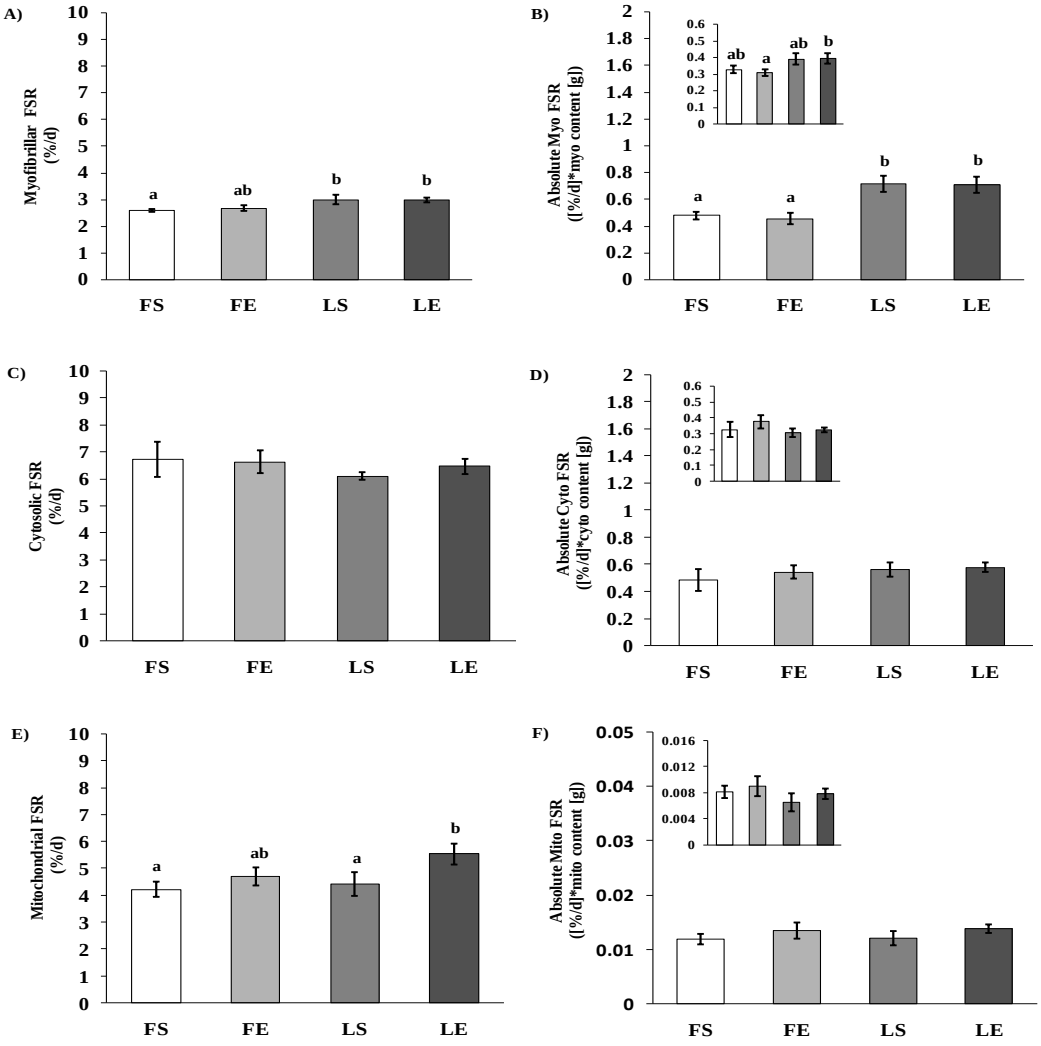


Fig. 8. Sub-fractional protein synthesis rates of gastrocnemius muscle. Panel A: MYO FSR. Main effect of phenotype (P = 0.007); FS vs. LS, P = 0.035; FS vs. LE, P = 0.008; FE vs. LS, P = 0.130, FE vs. LE, P = 0.072. Panel B: Absolute MYO FSR. Main effect of phenotype (P < 0.001); FS vs. LS, P = 0.004; FS vs. LE, P = 0.008; FE vs. LS, P = 0.005, FE vs. LE, P < 0.001. Insert: 24-h FSR adjusted for MYO protein concentration. Main effect of phenotype (P = 0.018); FS vs. LS, P = 0.156; FS vs. LE, P = 0.137; FE vs. LS, P = 0.066, FE vs. LE, P = 0.042. Panel C-D: Two-way ANOVA indicated no significant effects of phenotype, RE, or interactions (RE x phenotype) on CYTO outcome variables. Panel E: MITO FSR. Main effect of resistance exercise (P = 0.042) but not phenotype (P = 0.19); FS vs. FE, P = 0.349; FS vs. LE, P = 0.028; FE vs. LE, P = 0.111; LS vs. LE, P = 0.042. Panel F: No effects of phenotype or RE on adjusted MITO FSR. Bars/groups sharing the same letter are not significantly different (P > 0.05) according to SNK post hoc analyses or t-tests. Data are expressed as %/d, with or without adjustments for protein content and protein concentration, and presented as means ± standard error (SE).

Table 2. *Fractional protein synthesis rates (FSR) of soleus and plantaris*

	FS	FE	LS	LE	Phenotype	Activity	P*A
FSR (%/d)							
Soleus	**6.38 ± 0.16^{ab}**	**6.23 ± 0.23^{a}**	**6.62 ± 0.14^{ab}**	**7.00 ± 0.34^{b}**	**P = 0.044**	**P = 0.635**	**P = 0.281**
Plantaris	**4.72 ± 0.39**	**4.42 ± 0.25**	**4.97 ± 0.52**	**4.75 ± 0.54**	**P = 0.505**	**P = 0.573**	**P = 0.916**

Values are means ± SE and expressed as percent per hour (%/h). 2 x 2 ANOVA main effects (Phenotype, Activity) and interactions (P x A) are included to the far right in the table. Groups not sharing the same letter are significantly different as evaluated by SNK post hoc or t-tests ($P \leq 0.05$).

Proximal muscle groups (quadriceps)

In contrast to gastrocnemius and soleus, 24-h protein synthesis rates of mixed proteins in quadriceps muscle were significantly higher in obese vs. lean rats (+14%, P = 0.007, Fig. 9*A*), which in part was due to a mildly elevated FSR in the cytosolic fraction of the obese phenotype (+8%, P = 0.221, Fig. 10*C*). No significant differences were found between phenotypes in the intrinsic capacity to synthesize myofibrillar (P = 0.517, Fig. 10*A*) or mitochondrial proteins (P = 0.996, Fig. 10*E*). Myofibrillar (P = 0.062; 2.14 ± 0.14 %/d and 2.98 ± 0.42 %/d in LS vs. LE, respectively) and cytosolic (P = 0.042; 7.95 ± 0.51 %/d and 9.32 ± 0.55 %/d in LS vs. LE, respectively) FSR were 40% and 17% higher, respectively, in exercised vs. sedentary lean rats.

The exercise response was blunted in the obese phenotype with non-significantly higher myofibrillar ($P = 0.667$; 2.31 ± 0.23 %/d and 2.46 ± 0.25 %/d in FS vs. FE, respectively) and cytosolic ($P = 0.366$; 8.90 ± 0.47 %/d and 9.55 ± 0.55 %/d in FS vs. FE, respectively) FSR in exercised vs. sedentary obese rats. Interestingly, a diminished exercise response in obese rats may in part be due to an elevated basal state, at least in the cytosolic sub-fraction, as exercise levels were similar between phenotypes.

When analyzing the rate of proteins made in each sub-fraction on a whole muscle level, absolute FSR of mixed, myofibrillar, cytosolic, and mitochondrial proteins in quadriceps were significantly suppressed in the obese phenotype ($P < 0.05$; Fig. 9*B*; Fig. 10*B,D,F*). No significant differences were detected between phenotypes or experimental conditions when FSR was adjusted for mixed (Fig. 9*B, insert*) or sub-fractional protein concentrations (Fig. 10*B,D,F, inserts*). Similar to gastrocnemius muscle, mitochondrial FSR adjusted to protein concentration was elevated ~16% in quadriceps of obese vs. lean rats, but this did not reach statistical significance ($P = 0.29$).

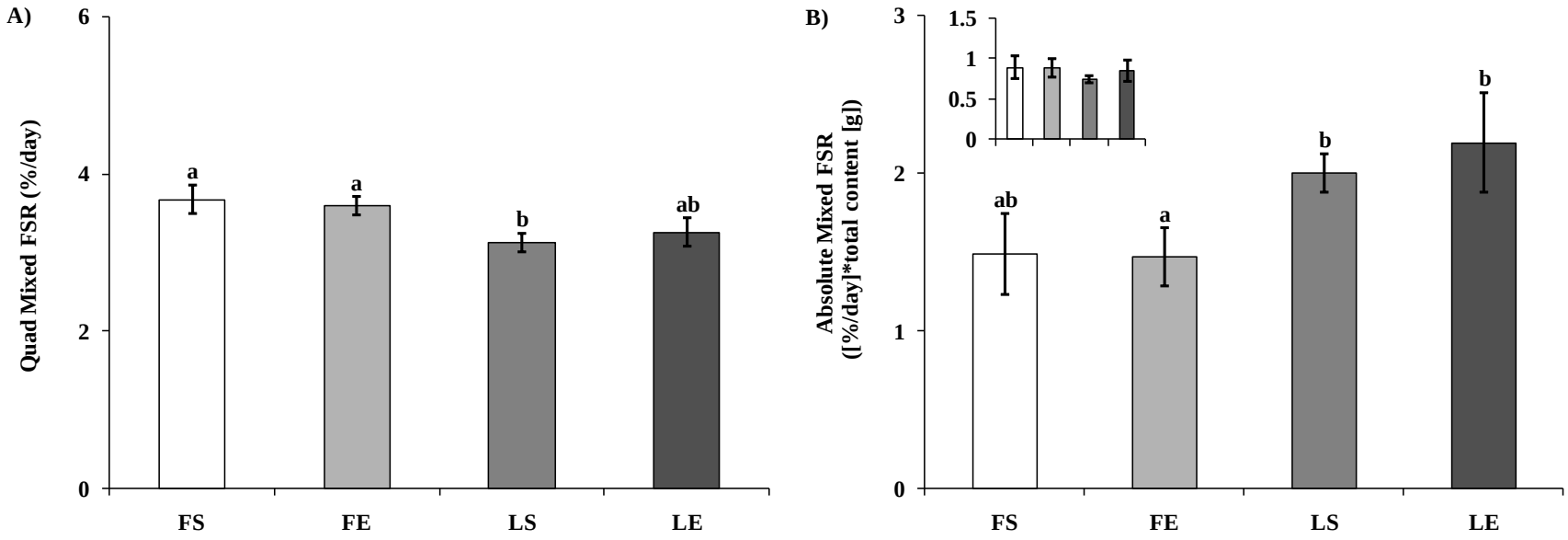


Fig. 9. Mixed protein synthesis rates of quadriceps muscle. Panel A: Intrinsic capacity of synthesizing mixed proteins (FSR) over a 24-h period. Main effect of phenotype (P = 0.007); FS vs. LS, P = 0.019; FS vs. LE, P = 0.133; FE vs. LS, P = 0.014; FE vs. LE, P = 0.112. Panel B: Protein synthesis over a 24-h period adjusted for mixed protein content (Absolute FSR). Main effect of phenotype (P = 0.02); FS vs. LS, P = 0.176; FS vs. LE, P = 0.132; FE vs. LS, P = 0.056; FE vs. LE, P = 0.038. Insert: 24-h FSR adjusted for mixed protein concentration. No main effect of phenotype (P = 0.462). Two-way analyses of variance indicated no main effect of RE or interaction between RE and phenotype on any of the aforementioned outcome variables. Bars/groups sharing the same letter are not significantly different (P > 0.05) according to SNK post hoc analyses or t-tests. Data are expressed as %/d, with or without adjustments for protein content and protein concentration, and presented as means ± standard error (SE).

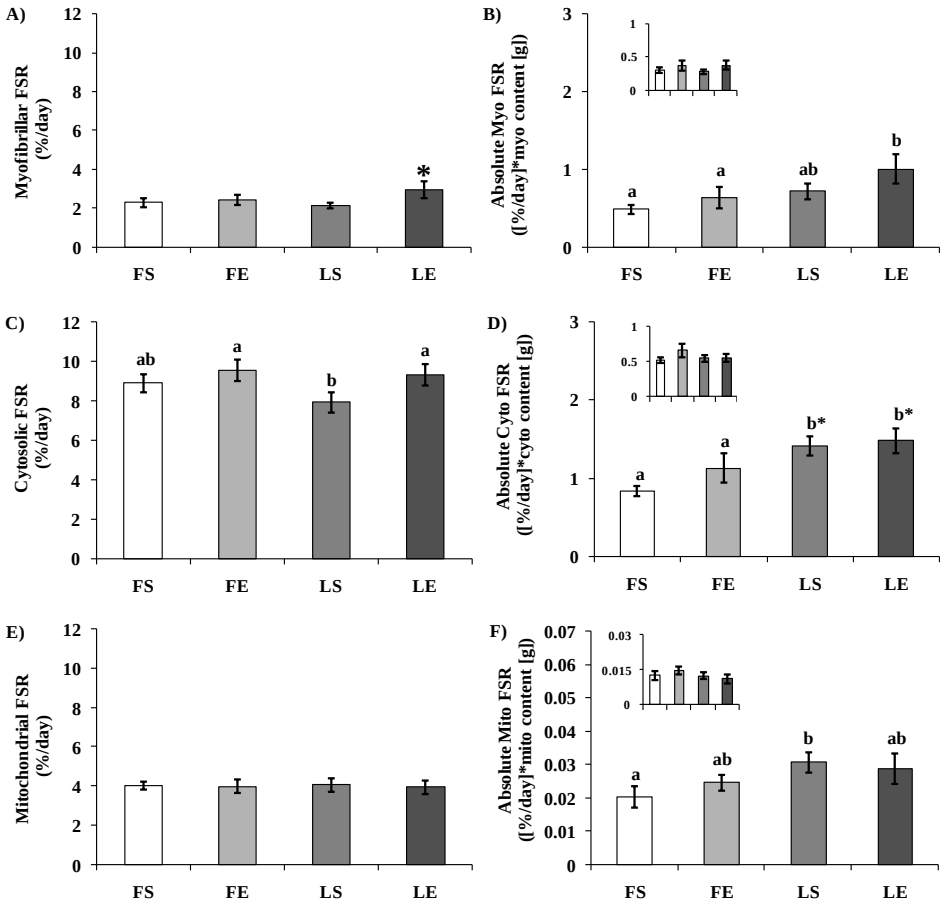


Fig. 10. Sub-fractional protein synthesis rates of quadriceps muscle. Panel A: MYO FSR. Borderline main effect of RE (P = 0.081); LS vs. LE, P = 0.062. Panel B: Absolute MYO FSR. Main effect of phenotype (P = 0.034) and borderline main effect of RE (P = 0.11); FS vs. LE, P = 0.043; FE vs. LE, P = 0.05; LS vs. LE, P = 0.129. Insert: 24-h FSR adjusted for MYO protein concentration. Two-way ANOVA indicated no effects of phenotype (P = 0.856), RE (P = 0.196), or interaction (RE x phenotype; P = 0.791). Panel C: CYTO FSR. Main effect of RE (P = 0.042); LS vs. LE, P = 0.037; FE vs. LS, P = 0.052. Panel D: Absolute CYTO FSR. Main effect of phenotype (P = 0.007); FS vs. LS, P = 0.023; FS vs. LE, P = 0.01; FE vs. LS, P = 0.065; FE vs. LE, P = 0.1. Insert: 24-h FSR adjusted for CYTO protein concentration. Two-way ANOVA indicated no significant effects of phenotype (P = 0.606), RE (P = 0.27), or interaction (RE x phenotype; P = 0.332). Panel E: MITO FSR. No significant effects of phenotype, RE, or interaction. Panel F: Absolute MITO FSR. Main effect of phenotype (P = 0.037); FS vs. LS, P = 0.043; FS vs. LE, P = 0.147; FE vs. LS, P = 0.105. Insert: 24-h FSR adjusted for MITO protein concentration. No significant effects of phenotype, RE, or interaction. Bars/groups sharing the same letter are not significantly different (P > 0.05) according to SNK post hoc analyses or t-tests. *Denotes borderline different (see text caption). Data are expressed as %/d, with or without adjustments for protein content and protein concentration, and presented as means ± standard error (SE).

Expression and regulation of growth/survival signals (Akt-mTOR pathway)

Considering that acute RE is less likely to induce permanent adaptations in skeletal muscle compared to resistance training, and that muscle harvest occurred 16 hours after the last exercise session, we did not expect major differences in expression or activity of anabolic signals between sedentary and resistance-exercised groups. As anticipated, we did not find a higher expression/activity of the typical regulators of protein synthesis [Akt-mTOR- $p70^{s6k}$] in gastrocnemius muscle when assessed 16 hours post RE (Fig. 11*A-C*; collapsed data), despite elevated 24-h mixed and mitochondrial FSR in the exercised lean cohort. The most notable exception was a more robust phosphorylation of eIF4EBP1 in obese (+ 54%) and lean (+17%) RE groups compared to sedentary controls (Fig. 12*B*), but no statistical differences in eIF4E·eIF4EBP1 or eIF4E·eIF4G association between experimental conditions were found (Fig. 13). As a result, Western blotting data from sedentary and exercised groups were collapsed within each phenotype (Fatty [F] vs. Lean [L]) unless there was a main effect of RE or interaction between phenotype and RE.

Obese rats exhibited a lower expression of key anabolic proteins including Akt (-16%, P = 0.074), mTOR (-26%, P = 0.058), and Raptor (-24%, P = 0.013), but no differences in P44/P42, eIF2Bε, and eIF2α were detected (Fig. 11*A-F*). Despite a lower cytosolic abundance of these important upstream mediators of muscle growth, the activation of downstream targets regulating mRNA translation initiation were not compromised compared to lean rats, as evidenced by a more robust phosphorylation of mTOR substrates eIF4EBP1(+28%, Fig. 12*B*), the major binding protein regulating

eIF4E activity and 5'cap-dependent translation, and $p70^{s6k}$ (+162%, Fig. 14*B*), the main enzyme responsible for phosphorylating the S6 protein in the 40S ribosomal subunit. A tendency for a decreased association between eIF4E and eIF4EBP1 was noted in the obese phenotype (-28%, obese vs. lean), but this did not reach statistical significance (P = 0.417, Fig. 13). In lieu of the fact that we observed a more robust phosphorylation of down-stream initiators of mRNA translation, we decided to measure the abundance/activity of key regulatory proteins in peptide-chain elongation in an attempt to explain the observed suppression in mixed and myofibrillar protein synthesis rates in the obese phenotype. Collapsed data did not indicate a lower expression of eukaryotic elongation factor 2 (eEF2) or a more potent inhibition of eEF2 (Thr 56) in obese vs. lean rats (Fig. 15*A-D*). However, when sedentary groups were analyzed separately, we found that the obese phenotype exhibited a stronger deactivation of peptide-chain elongation compared to lean rats, as evidenced by 87% higher phospho/total ratio of eEF2 (Fig. 15*C, insert*). We did not detect any significant phenotypical differences in expression of eEF2-inihibitory factor eEF2K or the negative mTOR regulators PRAS40 and REDD1 (Fig. 16*A-C*). Albeit not directly affecting protein synthesis *per se*, atrogin-1 (E3 ligase of the ubiquitin-proteasome proteolytic pathway) expression was significantly higher in the sedentary obese group compared to its lean counterpart (Fig. 16*D, insert*), indicating that other mechanisms, such as protein degradation, may contribute to the skeletal muscle atrophy observed in the obese phenotype.

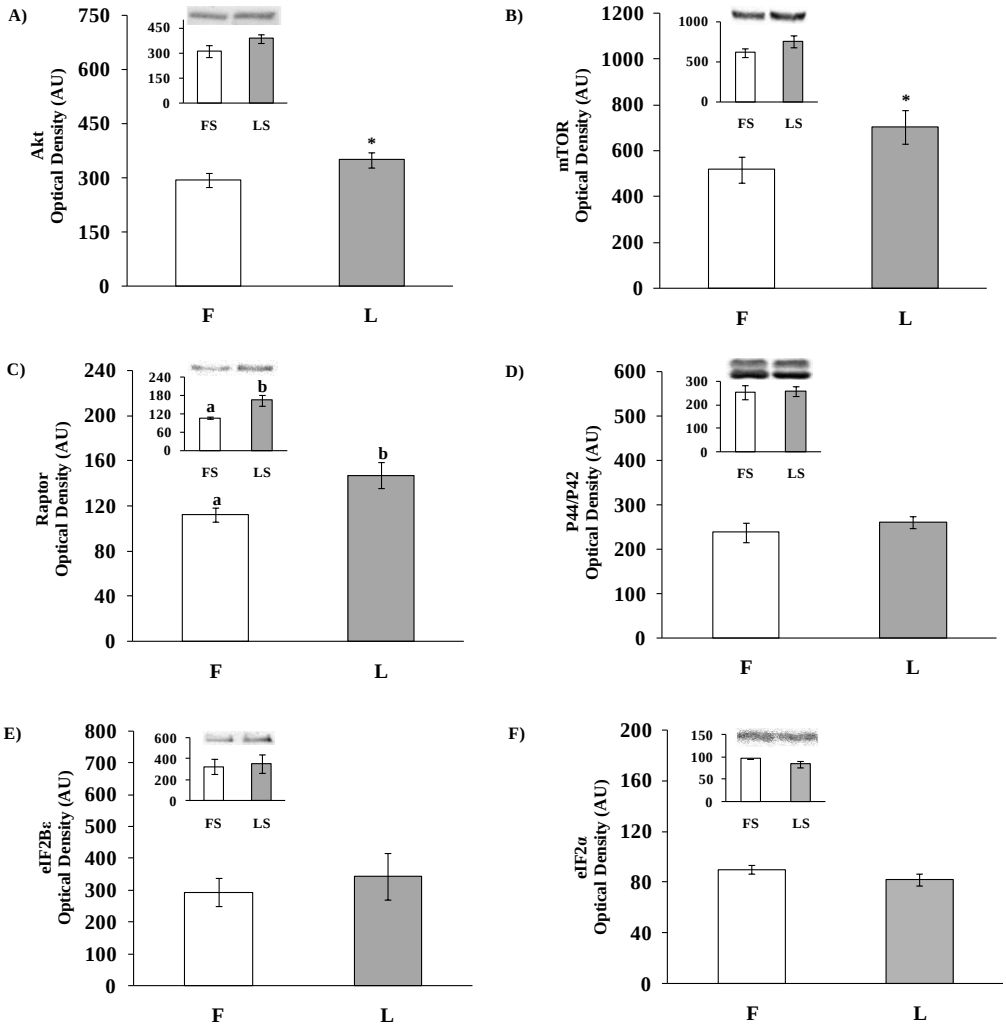
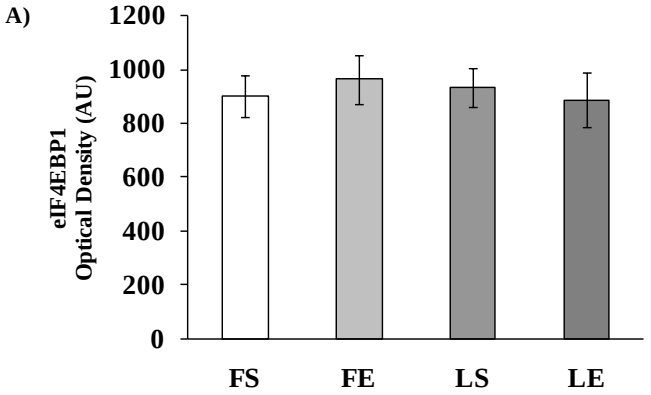
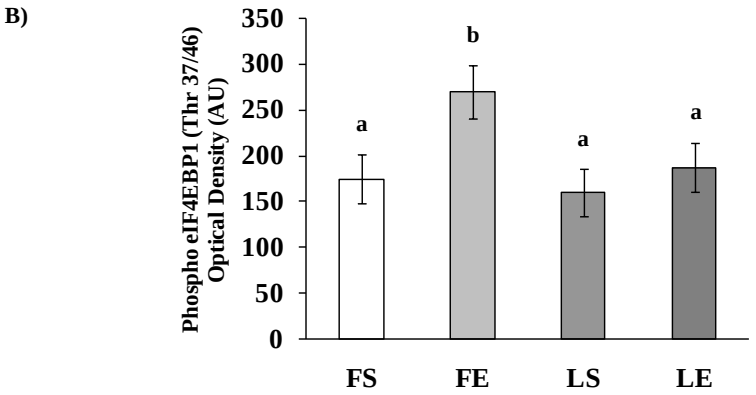
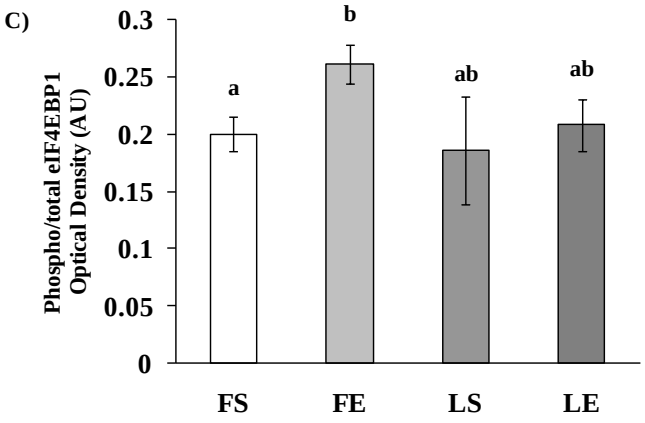


Fig. 11. Expression of proteins regulating growth in gastrocemius muscle. If no main effect of RE or interaction between RE and phenotype were detected by 2-way ANOVA, data were collapsed within each phenotype and presented as Fatty (F) vs. Lean (L). The sedentary conditions represent unperturbed states from an exercise standpoint and are included as inserts. Panel A-C: The obese phenotype exhibited borderline lower Akt and mTOR expression (P = 0.077 and P =0.058, respectively), and significantly lower Raptor expression (F vs. L, P = 0.013; FS vs. LS, P =0.006) compared to its lean counterpart. No differences were detected in expression of proteins considered to be independent of traditional mTOR signaling, including ERK1/ERK2 (Panel D), eIF2Bε (Panel E), and eIF2α (Panel F). Optical density values are means ± SE and expressed in arbitrary units. Groups that do not share the same letter are significantly different ($P \leq 0.05$) as evaluated by two-way ANOVA (F vs. L, main effect of phenotype), SNK post analyses (FS vs. LS, Raptor expression), or independent t-tests (all other comparisons). * Denotes borderline difference between phenotypes (see text caption).







D)

	FS	FE	LS	LE	Phenotype	Activity	P*A
eIF4EBP1	901 ± 77 n = 5	963 ± 92 n = 7	932 ± 72 n = 6	887 ± 99 n = 6	P = 0.805	P = 0.921	P = 0.550
peIF4EBP1	175 ± 27[a] n = 4	269 ± 29[b] n = 4	160 ± 26[a] n = 4	187 ± 26[a] n = 4	P = 0.098	P = 0.043	P = 0.234
Phos./Tot.	0.20 ± 0.02[a] n = 4	0.26 ± 0.02[b] n = 4	0.19 ± 0.05[ab] n = 4	0.21 ± 0.02[ab] n = 4	P = 0.259	P = 0.170	P = 0.506

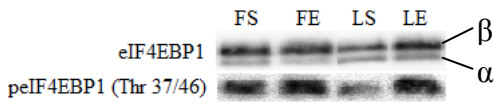


Fig. 12. Expression of total, phospho-specific, and phospho/total ratio eIF4EBP1 in gastrocnemius muscle. The number of animals used for each blot is indicated in the table along with 2 x 2 ANOVA main effects (Phenotype, Activity) and interactions (P x A) (Panel D). A representative blot of each experimental condition is included below the table. Optical density values are means ± SE and expressed in arbitrary units. Groups not sharing the same letter are significantly different as evaluated by SNK post hoc or t-tests ($P \leq 0.05$).

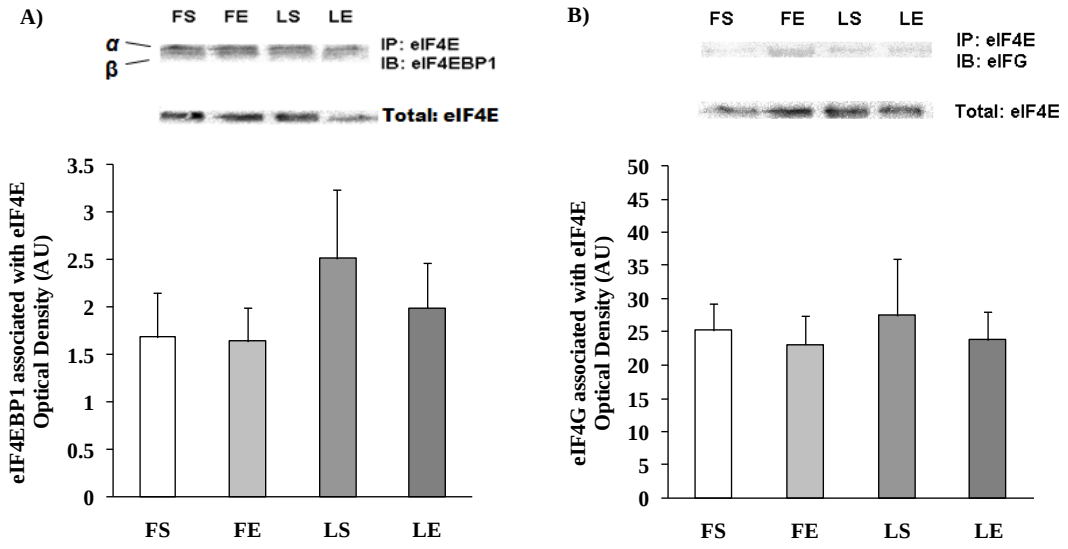
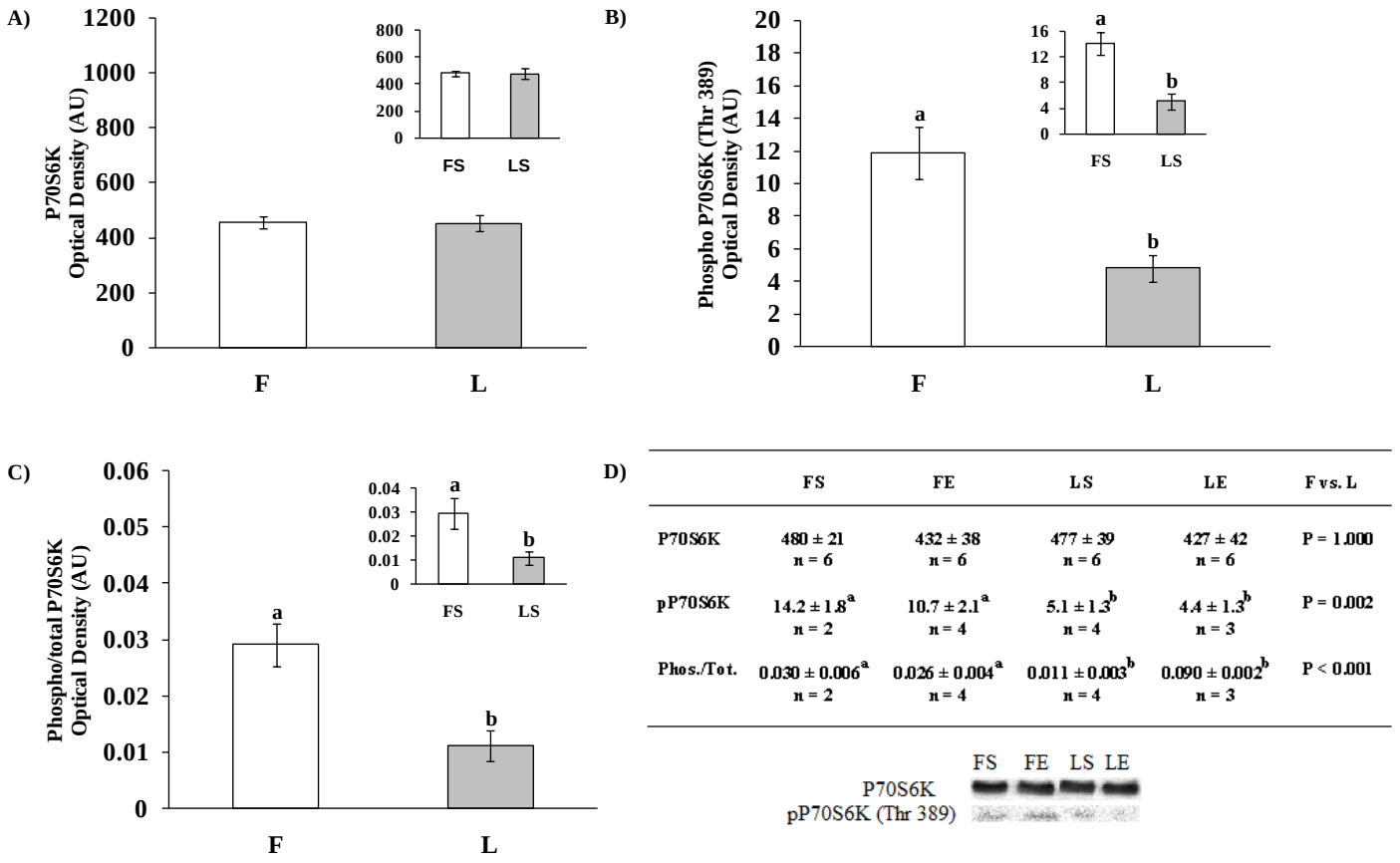
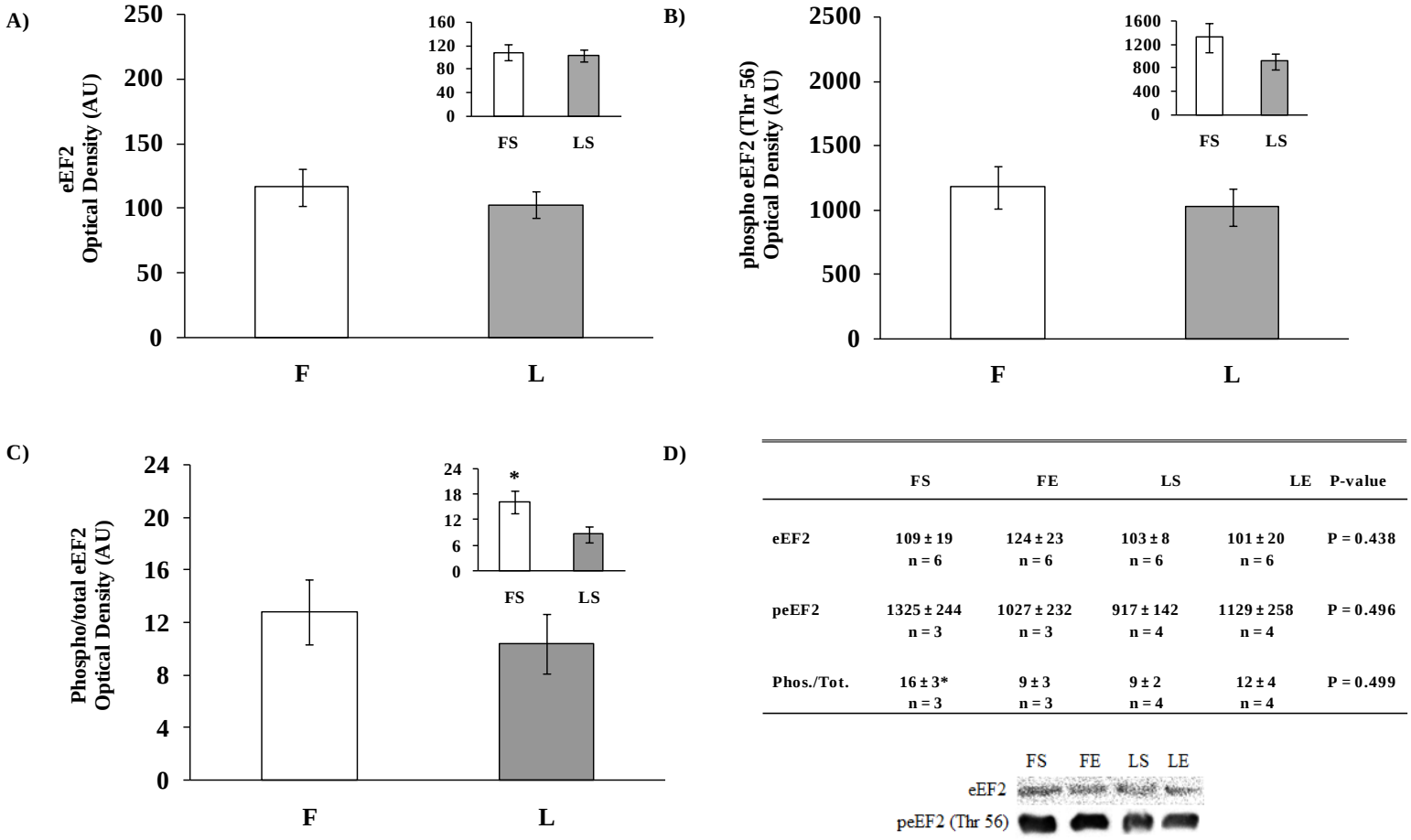


Fig. 13. Association of eIF4EBP1 (Panel A) and eIF4G (Panel B) with eIF4E in gastrocnemius muscle. Immunoprecipitation (IP) of eIF4E was followed by electrophoresis and quantification of amount of eIF4EBP1 and eIF4G bound to eIF4E by immunoblotting (IB). A representative Western blot is included above the figures (n = 4 in each group). Optical density values are means ± SE and expressed in arbitrary units. No significant differences were found between groups ($P > 0.05$).



	FS	FE	LS	LE	F vs. L
P70S6K	480 ± 21 n = 6	432 ± 38 n = 6	477 ± 39 n = 6	427 ± 42 n = 6	P = 1.000
pP70S6K	14.2 ± 1.8^{a} n = 2	10.7 ± 2.1^{a} n = 4	5.1 ± 1.3^{b} n = 4	4.4 ± 1.3^{b} n = 3	P = 0.002
Phos./Tot.	0.030 ± 0.006^{a} n = 2	0.026 ± 0.004^{a} n = 4	0.011 ± 0.003^{b} n = 4	0.090 ± 0.002^{b} n = 3	P < 0.001

Fig. 14. Expression of total, phospho-specific, and phospho/total ratio $p70^{s6k}$ in gastrocnemius muscle. The number of animals used for each blot is indicated in the table along with significance values obtained from 2 x 2 ANOVA [F vs. L, main effect of phenotype] (Panel D). A representative blot of each experimental condition is included below the table. Optical density values are means ± SE and expressed in arbitrary units. Groups not sharing the same letter are significantly different ($P \leq 0.05$).



	FS	FE	LS	LE	P-value
eEF2	109 ± 19 n = 6	124 ± 23 n = 6	103 ± 8 n = 6	101 ± 20 n = 6	P = 0.438
peEF2	1325 ± 244 n = 3	1027 ± 232 n = 3	917 ± 142 n = 4	1129 ± 258 n = 4	P = 0.496
Phos./Tot.	16 ± 3* n = 3	9 ± 3 n = 3	9 ± 2 n = 4	12 ± 4 n = 4	P = 0.499

Fig. 15. Expression of total, phospho-specific, and phospho/total ratio of eEF2 in gastrocnemius muscle. The number of animals used for each blot is indicated in the table along with significance values obtained from 2 x 2 ANOVA (F vs. L, main effect of phenotype) (Panel D). A representative blot of each experimental condition is included below the table. Optical density values are means ± SE and expressed in arbitrary units. *FS vs. LS borderline different as evaluated by independent t-test (P = 0.06).

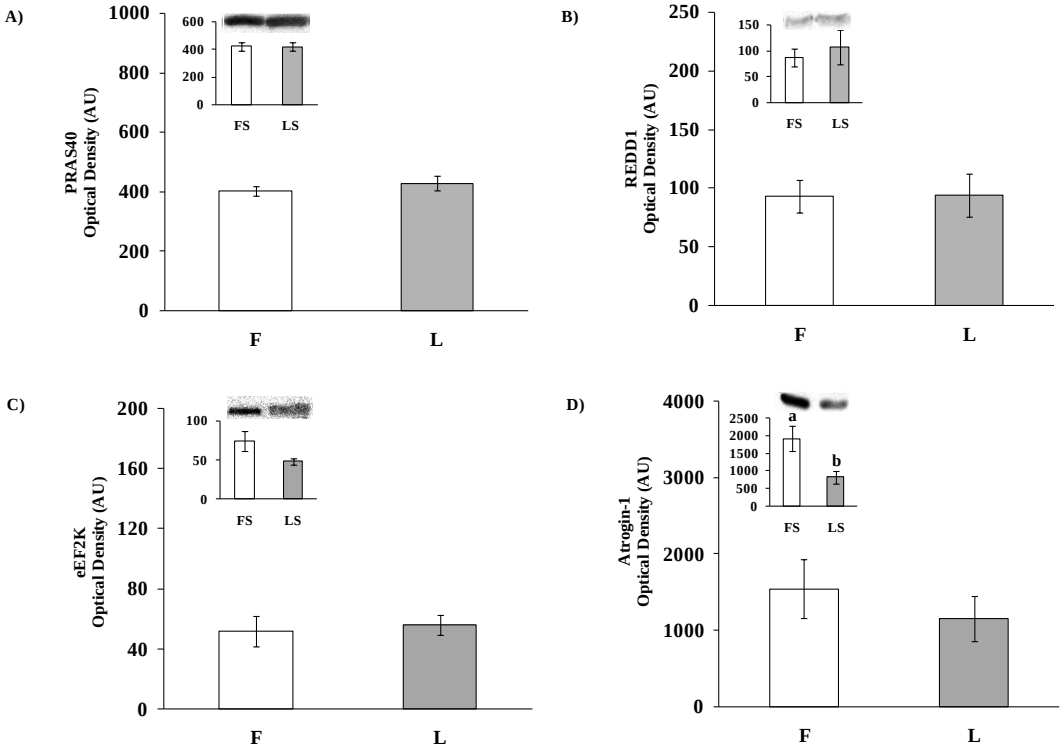


Fig. 16. Expression of inhibitors of growth in gastrocnemius muscle. If no main effect of RE or interaction between RE and phenotype were detected by 2-way ANOVA, data were collapsed within each phenotype and presented as Fatty (F) vs. Lean (L). No phenotypical differences were detected in expression of proteins inhibiting mTOR signaling, including PRAS40 (Panel A) and REDD1 (Panel B). Basal expression of AMPK-activated eFE2K, an inhibitor of eukaryotic elongation factor 2, was borderline different between sedentary groups ($P = 0.133$). Although not directly involved in protein synthesis, atrogin-1 expression was significantly elevated in sedentary obese rats ($P = 0.029$). Optical density values are means ± SE and expressed in arbitrary units. Groups that do not share the same letter are significantly different ($P \leq 0.05$) as evaluated by independent t-tests.

Discussion

The primary aim of this study was to determine if insulin resistance alters the adaptive response of sub-cellular compartments regulating substrate metabolism and energy utilization in skeletal muscle following contractile activity. To achieve this goal, we employed a well-established resistance exercise (RE) model in diabetic rats (44) in conjunction with a novel precursor-product labeling technique that centers on the use of heavy water (deuterium oxide, ^2H_2O) to measure protein turnover. The attractiveness of ^2H_2O as a metabolic label lies in its proven utility for assessing long-term biosynthesis of macromolecules in a variety of species under free-living conditions (18), including obese rodent models (3). We extended previous research by measuring 24-h (cumulative) protein synthesis rates of mixed, mitochondrial, cytosolic, and myofibrillar sub-fractions in skeletal muscle of lean and obese Zucker rats following resistance exercise. To our knowledge, the current study is the first to address whether or not insulin resistance alters the cumulative anabolic response to voluntary resistance exercise in sub-fractions of skeletal muscle.

Insulin resistance is typically associated with mitochondrial dysfunction and the current RE paradigm was designed to promote muscular endurance as well as protein anabolism (see methods). We were partially successful in achieving this goal since mitochondrial FSR in gastrocnemius was significantly augmented in the lean phenotype (+20%), which was accompanied by a modest increase in cytosolic and mixed protein synthesis rates (+6% and +8%, respectively), but no change in myofibrillar FSR (-0.4%). We did, however, observe a robust amplification of myofibrillar and cytosolic synthesis

rates in quadriceps muscle of lean rats (+40% and +17%, respectively), which strongly suggests that our RE protocol indeed was anabolic. Interestingly, the exercise-induced anabolic response was altered in gastrocnemius of the obese phenotype, but this was primarily due to a blunted increase in mitochondrial FSR (+12%), with no significant changes in cytosolic, myofibrillar, or mixed FSR (-1%, + 3%, -4%, respectively). These results suggest that there is a fraction-specific resistance to contractile stimuli in this muscle group. In addition, obese rats were unable to augment synthesis rates of myofibrillar and cytosolic proteins in quadriceps to the same extent as lean rats, further supporting the notion of a reduced sensitivity to contractile activity-induced stimuli in the insulin-resistant state. These findings are partly supported by studies that have utilized compensatory loading models, insulin, or nutrients to promote protein synthesis in insulin-resistant humans and rodent models, although diverging results exist (9, 50, 59, 60, 94, 102, 121). Muscle growth was recently shown to be blunted in soleus muscle (84% SO fibers) of obese Zucker rats following synergistic ablation of gastrocnemius and plantaris (59). In contrast, a near identical hypertrophic response was demonstrated in plantaris muscle (94% FG/FOG fibers) of obese rats compared to their lean counterparts following synergistic denervation of soleus and gastrocnemius (102), indicating that the hypertrophic response in this rat strain may be muscle group- and/or fiber type-specific. Nevertheless, compensatory loading models induce significant increases in satellite cell proliferation, and it is widely debated whether or not satellite cell incorporation into myofibers is obligatory for muscle growth to occur following voluntary exercise (107). Therefore, it is difficult to compare the results of the current

study to previous research since different means were used to induce anabolism and to assess protein turnover.

The most novel finding, in regards to the plasticity of myocellular sub-fractions following resistance exercise in gastrocnemius muscle, is that mitochondria appear to be desensitized to contractile activity in the insulin-resistant state. Long-standing metabolic disease is associated with morphological changes of mitochondria in humans (81) and obese Zucker rats (6), which potentially could alter the organelle's typical adaptive response to exercise (71). Menshikova (95) and Toledo (125) reported that chronic endurance training preferentially augments mitochondrial biogenesis in obese and type 2 diabetic individuals by increasing surface area of the inner mitochondrial membrane (cristae), rather than inducing mitochondrial proliferation (mtDNA content), as typically seen in healthy subjects. Whether or not this differential adaptation in the insulin-resistant state is attributed to an attenuated acute anabolic response, in terms of synthesis or import of mitochondrial proteins, is less clear. Since mitochondrial FSR, as measured in our study, represents ^{2}H-alanine incorporation in both nuclear- and mtDNA-encoded proteins over a 24-h period, any defects in the replication, transcription, or translation of either genome could theoretically have attributed to a blunted exercise response in the obese phenotype. Considering that the vast majority of mitochondrial matrix and membrane proteins are encoded by nuclear DNA (only 13 out of ~1500 proteins are encoded by the organelle's own genome), in addition to the fact that replication, transcription, and translation of mtDNA are ultimately controlled by nuclear-encoded proteins (41), we believe that insulin resistance may be associated with multiple

dysfunctions in the manufacture and import of nuclear proteins, leading to a blunted anabolic response (i.e. mitochondrial FSR) following acute contractile activity.

To our knowledge, the molecular mechanisms causing differential mitochondrial adaptations in obese and insulin-resistant individuals following exercise remain largely unknown. Since we were unable to measure signal transduction immediately following RE (~0-3 h post) due to the necessity of a 24-h ^2H_2O enrichment period, the intracellular events responsible for an attenuated increase in mitochondrial FSR will be elucidated in future studies. Mitochondrial biogenesis is thought to be highly dependent upon the activation of AMPK-regulated transcriptional coactivator PPARγ coactivator-1 (PGC1α) and its interaction with sequence-specific DNA binding proteins, mainly nuclear respiratory factors 1 and 2 (NRF-1/NRF-2). Previous research has indicated that acute muscle contractions do not stimulate AMPKα1 activity in obese Zucker rats, whereas the same contractile stimulus causes ~2-fold increase in AMPKα1 activity in lean rats (7). Although it is generally accepted that PGC1α is regulated by AMPK, PGC1α activation following acute exercise is not dependent upon either α1 or α2 isoforms of this enzyme (76). In further support of those findings, acute aerobic exercise amplifies PGC1α and NRF-1 mRNA expression in obese and type 2 diabetic subjects to the same extent as in lean individuals, despite a robust down-regulation of AMPK-signaling in the former (117). That same research group reported that obese Zucker rats are able to augment PGC1α protein expression equally well compared to their lean counterparts with chronic endurance exercise, although the obese phenotype exhibits a reduced expression of LKB1 and attenuated activation of AMPK (118). The underlying molecular mechanisms

are currently unknown, but LKB1 is a 'master kinase' that regulates the activity of 13 AMPK-related kinases and CaMK (in conjunction with calmodulin and intracellular Ca^{2+}), all of which may compensate for the lack of contractile-induced stimulation of AMPK. MAPK signaling has also been shown to be necessary to release PGC1α from its competing repressor protein ($p160^{MBP}$), but compared to lean humans and rodents the time-course and magnitude of ERK1/ERK2, p90RSK, and p38 phosphorylation following a single bout of contractile exercise is preserved or greater in their obese counterparts (27, 78, 88). Although cytosolic to nuclear abundances of PGC1α and SIRT-1 (or SIRT1-mediated deacetylation of the former) have not been assessed in exercise intervention studies, it appears that activation of PGC1α subsequent to physical activity is normal in the insulin-resistant state and may therefore not be responsible for altered mitochondrial adaptations. Numerous other transcription factors, corepressors, and coactivators have been implicated in mediating the anabolic response of mitochondria in concert with PGC1α, including yin-yang 1, peroxisome proliferator-activated receptors (PPARα, PPARβ/δ, and PPARγ), RIP140, and PRC, but few comparative studies have been conducted to date.

Calcium signaling through calcineurin, PKC, and CaMK are postulated to play significant roles in mitochondrial biogenesis since calcium-induced muscle contractions are ultimately dependent upon a steady energy supply from the organelle. The obese Zucker rat exhibits elevated intracellular Ca^{2+} levels and reduced Ca^{2+} ATP activity (89, 90, 142), in addition to increased levels of intramuscular diacylglycerol and ceramide (5, 127), which is associated with chronic activation/membrane translocation of

conventional [α and β] and novel PKCs [δ, ε, and θ] (5). Chronic activation of PKC (θ) is strongly linked to skeletal muscle's insulin resistance to glucose disposal (Lowell 2005), but whether or not a constant basal activation of these enzymes dampens the ability to sense contractile-induced stimuli is unknown. A recent study reported a blunted augmentation in soleus muscle mass and fiber cross-sectional area following synergistic ablation in the obese Zucker rat, which was mirrored by an attenuated activation of calcineurin (59). Although the role of calcineurin in mitochondrial biogenesis have been questioned (55), these findings lend support to the notion that calcium-regulated enzymatic activity is attenuated in obese and insulin-resistant states. Lastly, emerging evidence for the role of the evolutionary conserved TOR pathway in the regulation of mitochondrial biogenesis (10, 26, 111) indicates that Raptor-mediated activation of mTOR is imperative to maintain mitochondrial morphology and function. Previous research has shown that the obese Zucker rat exhibits a blunted activation of the Akt-mTOR-$p70^{s6k}$ pathway 1-3 h following high-force electrical stimulation, suggesting that global synthesis rates, including mitochondrial FSR, are attenuated compared to the lean phenotype (77). Considering that translation of the vast majority of mitochondrial proteins ultimately is under the control of TOR signaling, it makes intuitive sense that an alteration in this pathway could have a fundamental impact on mitochondrial FSR following exercise.

Many of the metabolic aberrations associated with type 2 diabetes and obesity would be expected to cause abnormal protein turnover, but few studies have looked at the effect of obesity-induced insulin resistance on intracellular mechanisms governing

skeletal muscle growth (e.g., manufacture and degradation of proteins). Previous studies in type 2 diabetics have mainly focused on proximal muscle groups (e.g., quadriceps) despite documented peripheral vascular disease and neural dysfunction in distal limb regions of this population (110). Furthermore, assessments of protein turnover have typically been done on the mixed protein fraction, which is particularly problematic in metabolic disease conditions where subtle changes in synthesis/degradation of specific myocellular compartments may be masked in the total protein pool. Previous research in the obese Zucker rat has indicated that an increased rate of degradation (4) coupled with a reduced rate of protein synthesis (37) and suppressed satellite cell proliferation (102) collectively confer the atrophic phenotype in this rat strain. Nevertheless, most of the current knowledge pertaining to protein metabolism in this metabolic rodent model is limited to the mixed protein fraction and much less is known about the individual contribution of specific myocellular sub-compartments to the overall condition. Thus, an important goal of our study was to elucidate if insulin resistance is associated with uniform perturbations in FSR in regards to proximodistal location of muscle groups and biological function of various myocellular compartments (substrate metabolism [cytosol/mitochondria] vs. contractile activity [myofibrils]). As previously mentioned, we utilized a novel stable isotopic method that offers several methodological advantages over the traditional precursor-product techniques (flooding dose, *in vitro* incubation, and continuous infusion protocols). The most important distinction is that the $^{2}H_{2}O$ method allows for long-term assessments of biosynthesis rates in free living conditions, meaning that measurements can be done over long periods of time without perturbing normal

activity patterns (feeding and sleep) or introducing the muscle into an artificial environment (*in vitro*). Herein we provide evidence that basal protein synthesis rates in the insulin-resistant state are differentially affected depending upon proximodistal location of the muscle group. We demonstrate for the first time that 24-h fractional synthesis rates of mixed proteins in quadriceps muscle are significantly elevated in developmentally mature obese Zucker rats, but mildly suppressed in soleus and gastrocnemius. The latter results should be interpreted with caution since phenotypical differences in distal muscle groups were partially attributed to modest augmentations of mixed FSR following RE in the lean cohort. To our knowledge, there are no previously published reports on protein metabolism in isolated quadriceps muscle in this rat strain. Previous research has demonstrated that protein turnover of thigh muscles, which presumably included posterior flexor muscles, is lower in obese vs. lean rats and that this suppression becomes less marked with advancing age (106). Thus, although the obese phenotype may exhibit a significant repression of mixed protein synthesis rates in earlier developmental stages (106), the current study indicates that these perturbations disappear or even reverse when the animal reaches musculoskeletal maturity. A recent report involving human subjects supports the notion that basal protein synthesis and degradation rates are elevated in quadriceps muscle of obese subjects with poorly controlled type 2 diabetes (9), but more research is clearly needed to confirm these findings and elucidate underlying mechanisms. Considering that much less is known about the effects of human insulin resistance on protein metabolism in distal muscle groups such as the plantar flexors, most of our current knowledge have been inferred

from research completed in metabolic rodent models. The fact that the obese Zucker rat exhibits similar vascular and neural dysfunctions that are present in human obesity/type 2 diabetic conditions makes it a particularly appealing candidate for studying the effects of insulin resistance on protein metabolism in distal muscle groups (99). Although our results generally corroborate earlier work that has assessed protein turnover in distal muscle groups in this rat strain (37, 47, 106), we extend previous observations by demonstrating that the suppression is less or non-existent in comparison to what has been previously reported, and when a suppression is observed, it is found in sub-fractions involved in contractile function rather than substrate metabolism. In light of the observation that 24-h protein synthesis rates are significantly lower in the myofibrillar sub-fraction of the obese phenotype [gastrocnemius], and largely unaffected in mitochondrial and cytosolic compartments [gastrocnemius and quadriceps], we surmise that the metabolic syndrome is associated with a preferential down-regulation of contractile protein synthesis rates whereas manufacture of metabolic enzymes are maintained [or slightly elevated] to cope with nutrient excess.

Perhaps the most interesting finding of this study is that basal mitochondrial FSR is not reduced in the gastrocnemius muscle (or quadriceps) of the obese phenotype, despite being unable to significantly augment rates of protein synthesis in this sub-fraction in response to contractile activity. These results are supported by the fact that we did not find a phenotypic difference in mitochondrial protein content on a whole muscle level, which was attributed to a 31% increase in mitochondrial density in the obese cohort (Fig. 5*BC*). When adjusting protein synthesis rates for sub-fractional

concentrations, mitochondrial FSR was indeed elevated ~20% in the obese phenotype (Fig. 8*F, insert*). Our finding that the obese Zucker rat exhibited a robust augmentation in mitochondrial density in gastrocnemius muscle corroborates recent work by Holloway et al. (69), who reported a significant upregulation of COX-IV and mtDNA (+30% and +47%, respectively) in the same muscle group and rat strain. Although these values were reported as protein contents, protein expression obtained by Western blotting is technically a measure of protein concentration (expression/ug total protein loaded). The authors also found that mitochondrial oxidation rates, albeit significantly increased compared to the lean phenotype, are attenuated compared to rates of fatty acid transport, causing an increased esterification of fatty acids and intramuscular lipid accumulation (69). Bach and colleagues demonstrated that the obese Zucker rat exhibits smaller and misshapen mitochondria due to a dysregulation in mitochondrial fusion events (mitofusin-2), but total mitochondrial volume is normal since mitochondrial density is elevated compared to its lean counterpart (6). Taken altogether, these results lend support to the notion that mitochondrial dysfunctions *per se*, more specifically reductions in density, total content/volume, oxidation rates, and translational capacity (mtDNA), do not have to be present to induce intramuscular lipid accumulation and skeletal muscle insulin resistance. We extend these findings by showing that mitochondrial synthesis rates, which ultimately regulate biogenesis of the organelle, are not impaired in chronically obese states, but may be desensitized to contractile-induced stimuli.

To further elucidate differences in basal protein synthesis rates between phenotypes we performed studies focused on the expression of proteins known to regulate nuclear mRNA translation initiation and polypeptide elongation in gastrocnemius muscle. We found that the obese Zucker rat exhibits a reduced expression of Akt, mTOR, and Raptor, all of which are important upstream mediators of mRNA translation and polypeptide elongation. Considering that the Akt/mTOR pathway is up-regulated during hypertrophy and down-regulated during muscle atrophy (11), it is not surprising that knockout models suffer from reduced growth and severe loss of muscle mass/function (10, 23, 98, 109). Although a suppressed Akt-mTOR-Raptor expression theoretically may attenuate rates of protein synthesis in the obese Zucker rat, our observation that phosphorylation states of downstream mTOR targets eIF4EBP1 (eukaryotic initiation factor 4 binding protein) and $p70^{s6k}$ (p70 ribosomal protein S6 kinase) are elevated does not support this notion. It is tempting to speculate that, although mTOR expression is suppressed in the obese phenotype, the activation of the enzyme may be constitutively increased compared to its lean counterpart. Although complexation/immunoprecipitation studies need to be completed to confirm this notion, several observations in literature support this concept. The current prevailing view is that mTOR regulates many fundamental biological processes by integrating both intracellular and extracellular signals, including growth factors (insulin) and nutrients (branched-chain amino acids; BCAAs). A previous study demonstrated that the availability and concentrations of BCAAs (primarily leucine) are significantly elevated in hindlimb muscles of the obese Zucker rat (64). BCAAs are known to activate Rag GTPases, the

Ste-20 related kinase MAP4K3, and the class III PI3-K mVps 34, all of which mediate amino acid signaling to mTORC1 and promote $p70^{s6k}$ activation (58, 104). Chronic stimulation of $p70^{s6k}$ induces insulin resistance through a negative feedback loop upon IRS-1 [pSer 307, pSer 636/639] and knockout models have further corroborated $p70^{s6k}$ role in the development of metabolic disease by showing that its deletion protects against diet-induced obesity and improves insulin sensitivity (128). Thus, it is possible that nutrient overload in the obese phenotype facilitates a constitutively higher level of Akt-independent mTOR activation, leading to a more pronounced phosphorylation of downstream targets of mTOR and insulin resistance. Although it may appear perplexing that an augmented phosphorylation of eIF4EBP1 and $p70^{s6k}$ is not mirrored by an elevation in global protein synthesis rates in the obese phenotype, we found that sedentary obese rats exhibited a more potent deactivation of peptide chain elongation compared to lean rats. In support of our findings, recent research has shown that ob/ob mice exhibit a greater repression in global rates of muscle mRNA translation compared to wild type mice, despite increased activation of $p70^{s6k}$/rpS6/eIF4EBP1 and reduced raptor-associated mTOR (138). Another plausible explanation is that up- or down-regulation of nuclear translation/elongation steps may preferentially target specific compartments of the muscle cell, although an increased/decreased translational efficiency of specific mRNA pools is yet to be proven. This would be a particularly appealing adaptive mechanism whereby the obese Zucker rat is able to maintain normal cytosolic and mitochondrial protein synthesis rates in response to an elevated intramuscular lipid load, but at the expense of myofibrillar FSR.

Our results demonstrate that the anabolic response of insulin-resistant skeletal muscle is blunted following voluntary resistance exercise, and that the effects of insulin resistance on cumulative protein synthesis rates are divergent and dependent upon myocellular sub-fraction and proximodistal location of the muscle group. We acknowledge that physiological adaptations to exercise are intensity-dependent and that obese Zucker rats are inherently inactive. In our hands, the obese phenotype mastered the movement pattern equally well compared to its lean counterpart and did not require an altered absolute exercise intensity or increased negative reinforcement. We believe that we promoted an anabolic response in exercised obese rats since phospho/total eIF4EBP1 was significantly elevated following resistance exercise when compared to sedentary counterparts and even augmented in comparison to the corresponding lean group (Fig. 10). Although the mitochondrial response was blunted in gastrocnemius muscle, a 12% increase in FSR of this organelle further supports a modest anabolic effect in the obese phenotype. The lean rats exhibited a potent augmentation of myofibrillar and cytosolic FSR in quadriceps muscle (+40% and +17%, respectively), in addition to the already discussed outcomes in mixed gastrocnemius muscle. Although unaccustomed exercise may induce muscle damage (91), our progressive RE protocol did not promote significant edema as evidenced by no change in wet mass or total protein concentrations in either phenotype at the end of the study (Fig. 5-6).

CHAPTER III

SUMMARY AND CONCLUSIONS

In summary, we provide evidence that protein synthesis of mixed muscle homogenates is similar in lean and obese rats, even though the obese phenotype exhibits markedly lower muscle mass. While it is tempting to consider that differences in muscle mass must be due to an elevated rate of protein degradation or reduced satellite cell differentiation, this study offers an exciting alternative possibility. While mixed synthesis rates may be similar between phenotypes, the manufacture of specific sub-fractions of the cell may be very different. For example, our data strongly suggests that 24-h protein synthesis rates are near normal (and sometimes augmented) in cellular compartments containing oxidative and glycolytic enzymes [mitochondria and cytosol], but may be relatively repressed in sub-fractions involved in energy utilization and muscle contraction [myofibrils] depending upon proximodistal location of the muscle group. To our knowledge, this study was the first to demonstrate that insulin resistance is not associated with reductions in density or synthesis rates of mitochondrial proteins in gastrocnemius or quadriceps muscles of the obese Zucker rat, perhaps at the expense of the contractile apparatus. Although at present we cannot provide the underlying mechanism(s), it is clear that this fraction-specific phenotypical suppression cannot be explained by a reduced expression/activity of proteins in the Akt/mTOR pathway, since basal phosphorylation of downstream translation initiation factors are augmented in the obese vs. lean phenotype. We surmise that steps involved in polypeptide elongation are more strongly controlled in obese rats, or that translational capacity (total mRNA and/or

ribosome content) and/or translational efficiency (number of transcripts translated for any given mRNA/ribosome content) of specific mRNA pools are altered to cope with nutrient overload. Whether translation of specific mRNA pools are determined exclusively by the availability of transcripts and/or if there is a preferential regulation of what transcripts are targeted for translation remain to be elucidated.

Although exercise is commonly prescribed to reduce whole-body glycemia in type 2 diabetics, the optimal type, frequency, intensity, and duration to achieve therapeutic goals are not known. The current study demonstrates a blunted anabolic response in myofibrillar, cytosolic, and mitochondrial sub-fractions following acute resistance exercise in the insulin-resistant state, suggesting that alternative implementation strategies of traditional RE protocols may be necessary for insulin-resistant individuals to achieve similar gains in muscle growth as healthy subjects. Taken together, these findings underscore the importance of assessing synthesis rates of specific sub-fractions or individual proteins to fully elucidate the subtle differences in protein turnover associated with insulin resistance, and to determine the intracellular mechanisms underlying a differential anabolic response to exercise in insulin-resistant skeletal muscle.

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