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A MECHANISTIC EXAMINATION OF THE METABOLIC REGULATION OF INSULIN RESISTANCE, SARCOPENIA, AND OSTEOPOROSIS

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A MECHANISTIC EXAMINATION OF THE METABOLIC REGULATION OF INSULIN RESISTANCE, SARCOPENIA, AND OSTEOPOROSIS

by

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Dissertation

Presented to the Faculty of The University of Texas Graduate School of

Biomedical Sciences at Galveston

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

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December, 2007
Galveston, Texas

Key words: aging, glucose tolerance, muscle atrophy, bone loss

Dedicated to my wife, my family, and my friends.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my mentor, Dr. Melinda Sheffield-Moore, for her continuous support and advice throughout my dissertation work. Her expertise as a research scientist has been invaluable for the development of my skills in the laboratory and as a writer. Additionally, her positive attitude and patience has made my graduate career one of the greatest experiences I could imagine. I sincerely thank Dr. Randall Urban for sharing his knowledge and time. I am deeply grateful to him for letting me be part of his research team. His support has been invaluable by allowing me to gain experience in both a scientifically and clinically relevant environment. I thank Dr. Blake Rasmussen for sharing his expertise on the molecular pathways of muscle protein metabolism. I extend my gratitude to Dr. Concepcion Diaz-Arrastia, and Dr. Kevin Short for providing me with sound advice and assistance during the writing process.

There have been many additional individuals that contributed throughout my graduate career. I thank Tony Angel and Shanon Casperson for their friendship in the lab. Their support and assistance during the various clinical studies is greatly appreciated. Tricia Smith, Becky Soliz, and Ta'Wanda Fuqua have been invaluable for their assistance with administrative tasks. Dr. Mort Janghorbani was instrumental in the development of the noninvasive breath test. Dr. Micah Drummond provided valuable assistance with my western blot techniques. I sincerely thank Dr. Doug Paddon-Jones for sharing his knowledge and guidance during the writing of the manuscripts. I extent my thanks to the entire GCRC staff for the help and support they have provided during the various clinical studies. I would like to thank my colleagues at Enterprise Advisory Services, Inc. and NASA/JSC for their support as I started this endeavor. I extend my deepest gratitude to Dr. Golda Kevetter-Leonard for supporting me from the first day I set foot on campus.

I would not have been able to get here without the support of my family and friends. And most of all, I would like to thank my wife and best friend, Alyssa, for her patience, encouragement, and support.

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Publication No._____

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The University of Texas Graduate School of Biomedical Sciences at Galveston, 2007

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Aging is associated with a number of physiological changes, including increased risk of insulin resistance and type 2 diabetes mellitus (T2DM), decreased muscle mass and strength, and decreased bone mass and bone mineral density (BMD). The aim of the studies presented were to investigate 1) the kinetics of glucose-derived breath CO_2 between drug naïve impaired glucose tolerant (IGT) and normal glucose tolerant (NGT) individuals following an oral glucose load, 2) the effects of chronic oral supplementation of essential amino acids (EAA) on the acute muscle protein synthetic response to an oral bolus of EAA in older men and women, and 3) the effects of continuous and monthly cycled administration of testosterone on markers of bone metabolism in older men with low normal endogenous testosterone production.

Study 1) During a 10-h oral glucose tolerance test, blood and breath samples were collected following ingestion of 75 g of glucose isotopically labeled with 150 mg of U-$^{13}C_6$-Glucose. Glucose-derived breath $^{13}CO_2$ was lower in IGT as compared to NGT from 1 to 3.5 h post-glucose ($P \leq 0.05$). Glucose-derived breath CO_2 kinetics measured during the immediate post-glucose ingestion period may assist in recognition of undiagnosed IGT in at-risk individuals during the pre-diabetes stage of T2DM.

Study 2) Older men and women received daily between-meal supplements of either 7.5 g EAA (LO), 15 g EAA (HI), or placebo (PL) for three months. Chronic EAA supplementation increased net phenylalanine uptake in the leg of LO and HI ($P<0.05$) without altering the magnitude of the acute FSR or total and phosphorylated Akt, mTOR, S6K1 or 4E-BP1 response. The magnitude of the anabolic response to an oral EAA ingestion in healthy older men and women remains intact after 3 months of EAA supplementation.

Study 3) Older men received continuous weekly testosterone injections for 5 months (TE), monthly cycled testosterone treatment (MO) or weekly placebo (PL). Continuous weekly testosterone administration resulted in decreased serum n-telopeptide (NTX) and serum osteocalcin (OC) in TE. Continuous testosterone treatment results in a decrease in both bone resorption and bone formation in older men.

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CHAPTER 1

INTRODUCTION

Aging is associated with a gradual decline in the function of a number of physiological systems. While many of these changes are commonly accepted as naturally occurring adaptations associated with the aging process, fundamental questions remain regarding the underlying mechanisms involved. Additionally, many of the physiological alterations that take place share similarities with conditions also observed in pathophysiologies that are independent of the normal aging process. Some of these changes include increased risk of insulin resistance and risk of type 2 diabetes mellitus (T2DM), decreased muscle mass and strength, and decreased bone mass and bone mineral density (BMD). Knowledge gained from exploring these areas of interest will have broad applications.

STUDY 1: INSULIN RESISTANCE AND PRE-DIABETES

Assessment of Insulin Resistance is an Important Public Health Objective

Insulin resistance, impaired biological response to circulating insulin (52), is the hallmark of pathogenesis of a number of chronic disorders that have become a major public health concern (108). The pathophysiologies linked to insulin resistance include obesity, dyslipidemia, and hypertension (52, 108). These disorders form a predictive risk profile for T2DM and cardiovascular disease (49, 76, 82) and are collectively characterized as the Metabolic Syndrome. In the United States, 21-24% of the population suffers from conditions associated with the Metabolic Syndrome, with further increasing

incidence with advancing age (39). In 2005, an estimated 20.8 million people (7% of population) in the U.S. had diabetes, 80% of whom were overweight (93). Recently, the risk of acquiring the associated disorders of the Metabolic Syndrome have significantly increased in the pediatric population (138). Large-scale testing for insulin resistance is needed in both adults and pediatric populations to detect both T2DM and its predecessor, pre-diabetes.

Current Methods of Determining Insulin Sensitivity

The most sensitive indices for assessing those at risk for developing diabetes take advantage of the fact that both plasma glucose and insulin increase during the initial stages of insulin resistance. This occurs prior to pre-diabetes when individuals will have worsening insulin resistance (83, 84, 122, 142). Thus, the use of plasma glucose and its corresponding insulin concentration provides a higher sensitivity than plasma glucose concentration alone, regardless of whether a fasting or oral glucose tolerance test (OGTT) protocol is employed. Three commonly used indices of insulin resistance based on this interaction are: the homeostatic model assessment-insulin resistance (HOMA-IR), the insulin sensitivity index (ISI), and the whole-body insulin sensitivity index (WBISI). These indices have recently been evaluated against the "Gold Standard" of insulin resistance, i.e. the glucose-clamp (33, 143). The first, HOMA-IR, is calculated as the product of fasting glucose and insulin concentrations divided by a constant (84). It is inversely related to the M-value (rate of peripheral glucose uptake obtained from the glucose-clamp), but it primarily reflects impairment in the suppressive action of insulin on hepatic glucose production (HGP) which is the main disturbance leading to hyperglycemia in the fasting state (143). The latter two indices, ISI and WBISI, combine

the glucose-insulin concentration parameters for both fasting and OGTT, and each provide a strong direct correlation with the M-value of the glucose-clamp (143). For example, WBISI is calculated as follows:

$$WBISI = \frac{10{,}000}{\sqrt{(\text{fasting glucose X fasting insulin})\,\text{X}\,(\text{mean OGTT glucose X mean OGTT insulin})}} \quad (83)$$

Yeckel, et al (143) have evaluated these three indices against the M-value of the glucose-clamp in obese children and adolescents (aged 8-18 years) with either normal glucose tolerance (NGT), or impaired glucose tolerance (IGT); defining IGT as a 2-h blood glucose concentration of 140-200 mg/dL, according to the American Diabetes Association guidelines. Against the M-value, ISI (r = 0.74) and WBISI (r = 0.78) yielded a higher level of agreement than HOMA-IR (143). However, the overlap in WBISI values between the IGT and NGT groups of these obese children were such that one third of the NGT cohort had similar levels of poor insulin sensitivity based on WBISI values (143). Thus, despite the numerous indices of insulin resistance currently available, there remains a need for improved methods of identifying individuals with insulin resistance regardless of age.

Insulin and Lipid Oxidation vs. Glucose Oxidation

The traditional concepts on which the development of indices of insulin resistance is based rely on changes in plasma concentrations of glucose and/or insulin resulting from impairment of glucose homeostasis and/or intracellular impairment in pancreatic insulin secretory capacity. McGarry has recently reported an over-reliance on this

"glucocentric" approach and has suggested that a more "lipocentric" approach may be warranted (85). The "lipocentric" concept is based on the established notion that insulin resistance is tightly associated with alterations in lipid homeostasis, even if cause/effect issues are yet unresolved, and that this may occur earlier than measurable impairments in glucose homeostasis (85). Because the consequences of insulin resistance involve a much wider spectrum of homeostatic impairments in cellular fuel metabolism than disturbed glucose metabolism alone (40, 104), an alternative approach to either the "glucocentric", or the "lipocentric" concepts may be to focus on an appropriate end-point combining these derangements. Such a "fuelcentric" approach recognizes the long-established concepts that cellular respiratory fuels, primarily free fatty acids (FFAs) and glucose, compete with each other (the glucose fatty acid cycle of Randle) (40, 104). The utilization of glucose and FFAs as cellular energy sources in response to insulin's action (or lack thereof) are inversely related and regardless of the current uncertainties about some mechanistic aspects, disturbances in metabolism of both lipids and carbohydrates occur early and are integral hallmarks of insulin resistance (85).

Insulin resistance dramatically alters the ratio of substrates used for cellular energy production (40, 85, 104). Under normal physiologic conditions of fasting (8-10 hours after a meal), postprandial (during several hours following a mixed meal), or OGTT (75-100 g glucose), cellular fuel consists primarily of free fatty acids (FFAs) and glucose, with protein metabolism making a minor contribution (24, 36, 40, 104). In the fasting (and resting) state, > 60% of cellular fuel for insulin-dependent peripheral tissues (muscle) is derived from FFAs (16, 36, 41, 51, 53, 60). The portion of plasma-derived and intramyocellular-derived FFAs depends on factors such as the intracellular concentration of triglycerides (TGs); with possible other factors affecting this

contribution (45, 85). When a carbohydrate-containing meal is consumed, or an oral dose of glucose (75 g) is administered, contribution of FFAs to whole-body fuel oxidation is reduced dramatically in insulin-sensitive subjects. (36). This switch is brought about by: (a) suppression of hepatic glucose production (HGP) thereby reducing its contribution to plasma glucose input, (b) drastic suppression of adipose tissue lipolysis leading to a marked decrease in concentration of plasma FFAs and their flux into the muscle cells, (c) up-regulation of glucose uptake of peripheral tissues, primarily muscle, through insulin receptor-mediated translocation of glucose receptors (GLUT 4) to myocellular plasma membrane, (d) insulin-mediated enzymatic regulation of the complex carbohydrate-lipid pathways, such as glycogen synthesis, and (e) enzymatic and non-enzymatic product-feedback regulation of various steps in glycolysis and cellular fatty acid metabolism, such as feedback regulation of pyruvate dehydrogenase complex (PDH) by mitochondrial acetyl-coA (AcCoA), or citrate regulation of phosphofructokinase (40, 69, 104). These complex processes contribute to a dramatic alteration in the contribution of FFAs and glucose to mitochondrial AcCoA, the entry point to Kreb's cycle. This results in a large shift in the source of CO_2 produced by cellular respiration from FFAs to glucose.

Development of insulin resistance profoundly alters this physiologic picture (40, 104) by: (a) impairment of glucose-stimulated suppression of HGP, leading to two strong inputs for plasma glucose (oral and hepatic), (b) impaired suppression of lipolysis in adipose tissue (probably more so than intramyocellular suppression (45)), leading to a disproportionate flux of FFAs into muscle mitochondria relative to pyruvate (from glycolysis), (c) decreased effectiveness of insulin-mediated muscle glucose uptake, (d) impairment of glycolytic pathways via AcCoA-inhibition of pyruvate dehydrogenase, citrate inhibition of phosphofructokinase and product inhibition of hexokinase; all leading

to impaired glucose contribution to AcCoA as the substrate for the Kreb's cycle. The lower carbohydrate contribution to mitochondrial AcCoA is compensated for by higher input from FFA β-oxidation to maintain energy demands (33).

The clinical capability to accurately and non-invasively discern how the metabolic switch from FFA to glucose oxidation is functioning in an individual would be an invaluable tool to aid in the early detection of insulin resistance and development of pre-diabetes.

STUDY 2: SARCOPENIA AND AMINO ACIDS

Aging and the Regulation of Protein Synthesis

Several stimuli such as amino acids (96) or exercise (42) can result in acute increases in muscle protein synthesis in healthy young and elderly. Short term effects of oral amino acids administered to elderly subjects have been studied extensively and induce a stimulatory response in muscle protein anabolism similar to those observed in young (96). Mixed muscle fractional synthetic rates and phenylalanine net balance across the leg increased in both young and elderly subjects following a bolus ingestion of 15 g essential amino acids (96). Yet, there are several striking differences between the responses observed in muscles from young and elderly subjects. The reported increase in phenylalanine net balance in elderly subjects responded slower and was sustained over a longer period compared to young subjects (96). How the differences in time course relate to underlying mechanism of protein synthesis is currently not well understood but may be related to factors such as increased insulin resistance and decreased muscle perfusion (21). Elucidating the age-related differences in the mechanisms of muscle protein synthesis is of great clinical importance. Halting or slowing the gradual loss of lean body

mass that occurs during sarcopenia likely involves long term measures aimed at improving both prandial and postprandial rates of muscle protein synthesis in elderly.

Essential Amino Acids and Mechanisms of Protein Synthesis

Amino acids are known to stimulate muscle protein synthesis (22, 80, 96, 137). The effects of amino acids on protein synthesis are beyond that which can be explained by the simple increase in their presence as substrate alone (109). Prior research indicates a regulatory role of amino acids on gene expression at the level of initiation of mRNA translation leading to increased synthesis of ribosomal proteins (28, 72, 86, 102, 127). This stimulatory effect is detected only when EAAs are present in the treatment and is in particular attributed to the action of the branched chain amino acids (BCAAs) leucine, isoleucine and valine (73). Among these three BCAAs, leucine is the most potent in activating protein synthetic processes *in vitro* (15). The stimulatory effect of the strictly ketogenic amino acid leucine on muscle protein synthesis is also evident *in vivo* in the presence of either insulin or carbohydrates (3). Single oral loads of leucine, in amounts up to 100% of the daily requirement of this amino acid, have been shown to increase skeletal muscle protein synthesis in a concentration dependent manner in food deprived rats (22). Increasing leucine intakes were positively associated with hyperphosphorylation of eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1), dissociation of 4E-BP1-eIF4E, phosphorylation of eIF4G Ser^{1108}, association of eIF4G-eIF4E, and phosphorylation of p70 S6 kinase 1 (S6K1) Thr^{389}. In addition to stimulating protein synthesis, leucine also raises serum insulin levels (4) and suppresses skeletal muscle protein degradation (92) in rats. While insulin is now widely regarded as an anabolic hormone, it has been proposed that additional signals (i.e.

essential amino acids) are necessary for full activation of protein synthesis (3, 8, 19, 48). Stimulation of protein synthesis by leucine can be abolished in perfused rat muscle by co-administration of somatostatin (3). Somatostatin is an endogenous inhibitor of pancreatic insulin release and is produced and released by the pancreas in response to elevated levels of circulating insulin. The aforementioned experiments point to the presence of multiple signaling pathways necessary for proteins synthetic processes to take place. The function of insulin may be to help control protein synthesis when other signals are present. This permissive action of insulin could be a major obstacle to overcome in individuals where insulin sensitivity has been lowered, such as individuals with T2DM and the elderly. As aging is associated with both increased insulin resistance and sarcopenia, the proposed link between insulin resistance and the reduced response in muscle protein synthesis is likely an important factor (106).

Insulin and Amino Acid Signaling

Insulin activates PI3K through the Insulin Receptor Substrate (IRS). PI3K directly activates Akt (PKB) through its phosphorylation. Activation of Akt results in activation of a number of pathways that include stimulation of protein synthesis as well as inhibition of protein degradation. Phosphorylation of the forkhead protein FOXO through Akt/PKB prevents FOXO from translocating to the nucleus where it is involved in upregulation of expression of several E3 ubiquitin ligases such as MURF1 and MAFbx/Atrogen (31, 63). MURF1 and MAFbx/Atrogen are involved in protein degradation through the ubiquitin proteosomal pathway. Akt/PKB activation also results in downregulation of the caspase-9/ caspase-3 protein degradation pathway, thereby promoting cell survival.

A simplified schematic of the pathway through which EAAs and insulin are thought to regulate protein synthesis is depicted in Figure 1.1. Stimulation of pathways of protein synthesis is believed to involve inhibition of TSC2 (73). TSC2 (tuberin), when complexed with TSC1 (harmatin), functions as a GTPase activating protein (GAP). Its regulation through multiple phosphorylation sites is complex and TSC2 activation results in reduction of Rheb bound GTP to GDP. Phosphorylation of TSC2 on separate Ser residues through Akt/PKB, ERK, or $p90^{rsk}$ all result in deactivation of its GAP activity. Inversely, phosphorylation by AMPK on a separate site activates GAP activity of TSC2. The GTPase Rheb (Ras homologue enhanced in brain) is an activator of mTOR (114). Rheb activity is enhanced when bound to GTP and decreases when its bound GTP is hydrolyzed to GDP by the TSC2/TSC1 complex. Recharging of Rheb-GDP to Rheb-GTP requires the replacement of GDP by GTP through a guanine exchange factor (GEF). GTP loading of Rheb has been shown to be blocked by amino acid depletion *in vitro* (81, 114). Rheb is required for activation of mTOR, resulting in signal transduction to downstream targets such as 4E-BP1 and S6K1.

Amino acid availability has been shown to increase S6K1 activity *in vitro* (136). Activation of 4E-BP1 and S6K1 by mTOR require protein interaction through raptor (73). Raptor binds and forms a stable but inactive complex with mTOR until activated by Rheb. Conformational changes are thought to take place resulting in a less stable but active complex, allowing raptor to bind and phosphorylate 4E-BP-1 and S6K1. In addition to raptor, other proteins such as GβL and rictor regulate mTOR activity (73). Activation of 4E-BP1 results in its disassociation from eukaryotic initiation factor (eIF)4E which can then combine with activated eIF4G and eIF4A to form the eIF4F complex at the 5' end of messenger (m)RNA. Phosphorylation of eIF4G and its binding

to eIF4E is upregulated in perfused rat hindlimb by increased leucine concentrations (8, 92). The eIF4F complex is a necessary step in the initiation of translation from mRNA to proteins. Activation of S6K1 by mTOR results in both the activation of the S6 subunit of the ribosomal 40S protein as well as the eIF3 complex (55). In addition to activating translation initiation, leucine and insulin also activate elongation through activation (dephosphorylation) of eEF2 through an mTOR mediated pathway (14, 102, 103). It is not clear how leucine and other amino acids relay the signal to the mTOR pathway. Several suggestions have been made including increasing GEF activity and GTP-bound Rheb, promotion of Rheb binding to mTOR, stabilization of the activated mTOR-raptor complex, or mTOR/raptor activation through an alternative PI3 kinase parallel to that controlled though insulin (73, 81, 94). It is possible that leucine regulates several initiation factors through separate molecular pathways. Whether the leucine induced signal pathways described above include leucine itself, leucine metabolites or additional intermediates is not clear. While some acute effect of administration of EAAs on muscle protein synthesis in elderly subjects have been demonstrated, the effects of long-term daily administration of amino acids on the pathways involved in protein synthesis are not known.

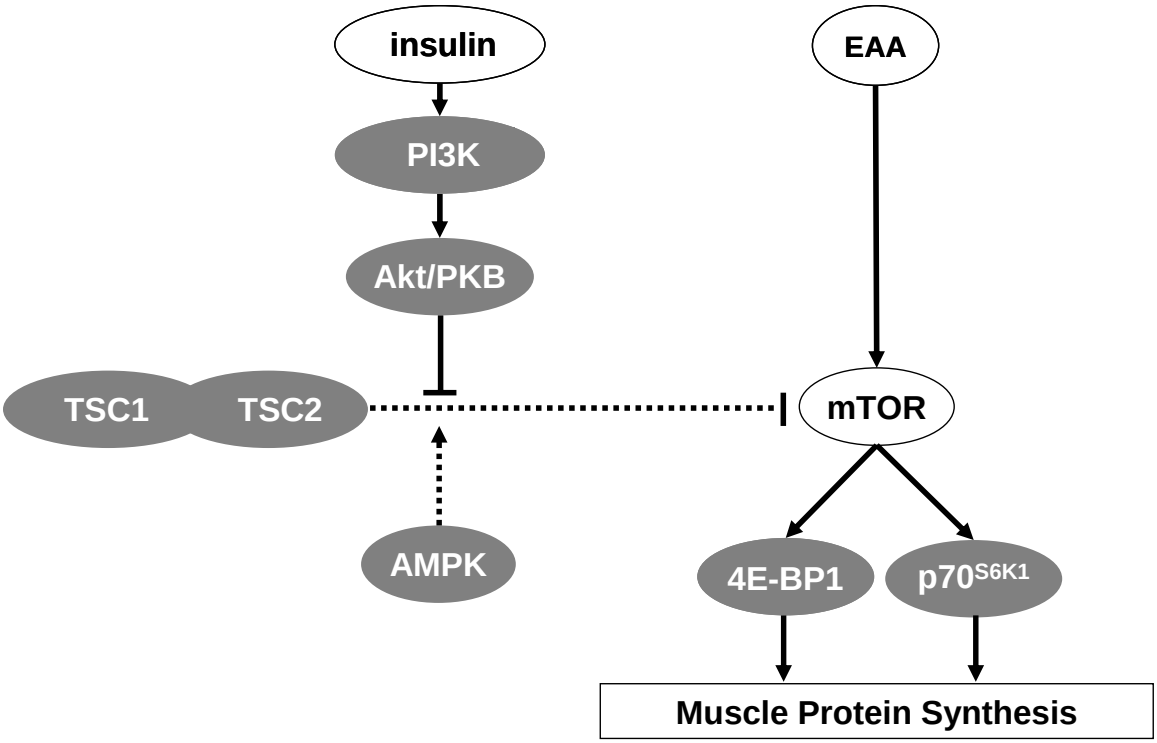


Figure 1.1: mTOR Pathway.

Simplified pathway of protein synthesis through mTOR. Essential amino acids (EAA) stimulate activation of mTOR. Activation of mTOR results in downstream activation of 4E-BP1, S6K1, eEF1, and subsequent upregulation of protein synthesis. The action of mTOR is inhibited by the TSC complex. Insulin likely plays a permissive role in EAA induced protein synthesis through inhibition of the TSC complex through the PI3K/Akt pathway.

STUDY 3: OSTEOPOROSIS AND ANDROGENS

Decreased Muscle Mass and its relation to Bone Mass

Loss of lean body mass in the elderly population poses a significant health concern affecting both males and females. These losses can be attributable to reductions in muscle mass and strength (sarcopenia) as well as bone mineral density (BMD). Although no direct correlation between sarcopenia and diminished BMD has been shown, the incidence of sarcopenia is 33.9% higher in postmenopausal women with osteoporosis compared to those with normal BMD (134). A cross-sectional study using data from NHANES III revealed that the estimated direct healthcare cost in 2000 due to sarcopenia related disabilities was $18.5 billion in the U.S. alone (59). This amount exceeds a 1995 estimate of $13.5 billion attributable to osteoporotic fractures (107). Reduced muscle strength among elderly is believed to be an important risk factor for functional limitations, disability, and mortality (105). The risk of disabilities among elderly may be between 1.4 to 4.7 times higher in those with low muscle mass (less than 10.76 kg/m^2 for men, 6.76 kg/m^2 for women) compared to elderly with normal muscle mass (equal or more than 10.76 kg/m^2 for men, 6.76 kg/m^2 for women) (58). However, comorbidities such as obesity may be important factors in this estimate as sarcopenia alone was only found to be a moderate predictor on the development of physical disabilities among elderly women (57). The reduction in muscle mass during aging is believed to be largely related to reduced production of sex hormones, inactivity, and reduced quality of nutrition, leading to decreased muscle protein synthesis (29, 91). While many factors are involved in the age related loss of muscle and bone mass, adequate physical activity and

nutrition are believed to be important preventative measures aiding to increased quality of life among the elderly.

Aging is Associated with Decreased Bone Mineral Density (BMD)

Bone mineral density (BMD) is classically considered an independent predictor of fracture risk in postmenopausal women. While limited data exists on men, increasing evidence suggests that BMD can be applied as a predictor for fracture risk in aging males as well as females (7, 12, 23, 61, 113). Mechanistically, the age-related loss of bone mass and density seems to coincide with a gradual increase in bone turnover after middle age, with increases in bone resorptive processes exceeding those of bone formation (75, 119, 125).

Reducing the incidence of osteoporosis and related bone disorders constitutes a major goal in current healthcare. This is evidenced by the increased promotion of anti-osteoporotic drugs such as bisphosphonates (i.e. Fosamax®, Actonel®, Evista®), parathyroid hormone (i.e. Forteo®), or calcitonin (i.e. Calcimar®). As serum testosterone levels are generally inversely associated with markers of bone resorption (71, 77), and the efficacy of testosterone therapy in reducing age related sarcopenia looks promising, its simultaneous use as an anti-osteoporotic agent should be explored.

Sex Hormones and Bone Metabolism

Both androgens and estrogens influence bone metabolism in aging adults, possibly by preventing the rates of bone resorption and bone formation from increasing and contributing to high turnover (type I) osteoporosis. (75, 119, 125). Osteoblasts and osteocytes express androgen receptors (AR) as well as estrogen receptors (ER). While

human osteoclasts also express ER, the expression of AR has not been detected (130). The classic view of the sex hormone receptors is that as members of the nuclear receptor family, directly interacting with DNA where it can influence the expression of downstream effectors. However, it is now known that the AR and ER also work through second messenger systems by mediating the Src/Shc/ERK pathway and downregulating the c-Jun/c-Fos pathway (56, 74, 78, 130). Although both androgens and estrogens positively influence bone mass, the direct mechanisms of their actions are not identical. Most studies suggest that the direct effects of estrogen on bone outweigh those of androgens and that some of the observed effects of androgens are through their aromatization into estrogen (77). Additionally, opposite effects of androgens and estrogens on bone metabolism have also been observed (70). While estrogen seems to upregulate osteoprotegrin (OPG) production, testosterone reduces OPG levels (54, 70). The androgen 5α-dihydrotestosterone (DHT) as well as estradiol (E2) were found to inhibit receptor activator of nuclear factor kappa B ligand (RANKL) induced osteoclast formation *in vitro* through downregulation of the JNK1 pathway as evidenced by decreased c-Jun expression, AP-1/DNA binding, and osteoclast numbers (56). Despite subtle differences in direct effects of androgens and estrogens, bone resorption is decreased by both agents. Estrogen seems to have a more profound effect on bone metabolism than androgens over the short term. However, as males generally reach a higher peak bone mass than females, it is possible that exposure to male sex hormones such as testosterone over a long time period induces a stronger bone forming response than estrogen.

Aging is associated with a decline in sex hormones in both genders. In males, the decline in circulating testosterone induces an increase in circulating sex hormone binding

globulin (SHBG), further diminishing the bioavailability of the hormone (112). A similar decrease in estrogen levels, leading to a drop in growth hormone (GH) secretion and IGF-I production, adds to this increase in SHBG production. Low IGF-I levels were found to be associated with decreased BMD at the total hip, femoral neck, and femoral trochanter among elderly men (115). However, other investigators have failed to find similar correlations between IGF-I and either BMD or excretion of bone turnover markers (47). Finally, the indirect effects of increased muscle mass on bone formation due to increased mechanical loading should not be ignored. Musculoskeletal loading is considered one of the most important factors affecting bone. Therefore it is possible that the main positive effect of androgens on bone mass is indirectly through their stimulatory effects on muscle protein synthesis and muscle mass.

Bone Resorption

Serum testosterone levels are generally inversely associated with markers of bone resorption. Negative correlations between bioavailable testosterone and n-telopeptide (NTX) levels in both urine and serum were found in elderly men aged 60-90 years (71). Serum NTX levels were also negatively associated with total testosterone in this group. Administration of continuous biweekly intramuscular (IM) testosterone injection (200 mg IM) in elderly men resulted in a significant decrease in urinary NTX excretion after 6 months. Induction of hypogonadism by administration of a GnRH analog to healthy 22-40 year old men resulted in increases in urinary NTX, urinary deoxypyridinoline (DPD), as well as serum NTX levels in as early as 4 weeks compared to baseline values (77). This increase was blunted by coadministration of testosterone.

N-telopeptide (NTX) is a type-I collagen crosslink and is widely used as a marker for bone resorption either by its detection in serum or urine. While the serum measurements are considered more reliable, both serum levels and urinary levels are positively correlated with bone collagen breakdown. Deoxypyrrodinoline (DPD) and Pyrrodinoline (PYD) are additional bone collagen crosslinks widely used as markers of bone resorption. The measurements of these fragments in urine can provide supporting information on the extent of bone resorption in addition to NTX data. Urinary collagen crosslink levels in 24 h urine pools must be normalized to either total daily urine volume or urine creatinine concentration to account for diurnal changes in bone collagen crosslink output. Although both methods are widely used, normalization by daily urine volume is the more reliable method (120). Parathyroid hormone (PTH) is important for normal blood calcium homeostasis. Small decreases in circulating Ca^{2+} levels result in the upregulation of PTH secretion (20). PTH immediately increase bone mineral mobilization from interstitial fluid by osteocytes and acts on osteoblasts to increase the number of osteoclast, resulting in increased bone resorption. This hormone also stimulates the conversion of 25(OH) vitamin D to its active form 1,25(OH)2 Vitamin D resulting in increased Ca^{2+} absorption from the small intestinal lumen. In addition, loss of Ca^{2+} through urinary excretion is reduced by stimulating reabsorption of Ca^{2+} from the glomerular filtrate. Measurement of intact PTH (iPTH) or n-terminal fragment is preferred over that of the C-terminal fragment as interference due to inactive PTH fragments is reduced.

Bone Formation

The effects of sex hormones on markers of bone formation in adults are more complex than the effects on bone resorption. While stimulating bone development and growth in young, sex hormones have a regulatory effect on the process of bone formation in elderly. Osteocalcin, a protein produced by osteoblasts, is widely used as an indicator for bone formation (50). Osteocalcin has been shown to be specific to osteoblasts and a fraction is directly released into circulation after synthesis. Serum total testosterone levels correlated positively to levels of osteocalcin in young (aged 22-39 yr) and middle aged (aged 40-59 yr) subjects, but not in elderly (aged 60-90 yr) (71). Additionally, elderly prostate cancer patients receiving a hypogonadic GnRH analog treatment experienced increases in osteocalcin and bone specific alkaline phosphatase (BSAP) levels after 6 months of treatment (88). BSAP is a glycoprotein found on the surface of osteoblasts where it is believed to be involved in bone mineralization (34, 100, 101). Administration of continuous biweekly testosterone injections (200 mg IM) in elderly men significantly decreased BSAP after 6 months (1). Osteoprotegrin (OPG), a recently discovered cytokine and inhibitory ligand of RANKL, is expressed in several tissues including bone and liver (118). Binding of OPG to RANKL prevents the latter from binding and activating RANK on the osteoclast cell surface and thus acts to inhibit bone resorption. While not widely used as a clinical marker, circulating levels of osteoprotegrin (OPG) are positively associated with age (124) and this decoy receptor likely provides a physiological mechanism to counteract the increase in bone resorption that occurs during the same period. Interestingly, although both sex hormones ultimately counteract bone resorption, testosterone and estrogen seem to have opposite direct effects on serum OPG levels. Testosterone administration to GnRH induced hypogonadal elderly men resulted

in reduced OPG levels; whereas estrogen administration markedly increased OPG levels (70). The negative effects of androgens on OPG production by osteoblast have also been shown *in vitro* (54).

Dual Energy X-ray Absorptiometry (DEXA)

DEXA offers the clinical capability to non-invasively quantify lean body and bone mass at different body regions. Bone can be quantified in terms of total mass (bone mineral content, BMC) or mass per area (bone mineral density, BMD). BMD is the more widely reported parameter and is often used as an independent predictor of fracture risk in postmenopausal women, and more recently, also in men (61). Total hip or lumbar spine BMD is widely used as a predictor for fracture risk in women and men, snd wrist BMD has recently been proposed as an additional site of interest for the prediction of fracture risk in men (87). However, changes in total bone mass (i.e. BMC) can occur without changes in bone density (BMD). It is therefore prudent to monitor both parameters during interventions that are expected to affect bone metabolism.

Administration of continuous biweekly testosterone injections (200 mg IM) in elderly men has shown to significantly increase BMD in the lumbar spine and trochanteric region after 6 months (1). Conversely, negative associations between serum free testosterone and spinal BMD have been reported (30, 89). The effects of cycled administration of testosterone on BMD in elderly subjects are unknown.

SUMMARY

Despite the continuous progress in the field of aging research, many of the mechanisms behind the physiological declines associated with older age remain obscure.

While the physiological changes associated with aging, such as decreased insulin sensitivity, sarcopenia, and osteoporosis, are commonly addressed in their own right, there are undeniably shared mechanisms behind the onset of these conditions. By systematically addressing each of the systems involved, the commonalities between them can be uncovered. The studies described herein are intended to explore three distinct areas of interest concerning the physiology of aging.

CHAPTER 2

GLUCOSE DERIVED BREATH CO_2 KINETICS IN IGT AND NGT ADULTS FOLLOWING AN ORAL GLUCOSE LOAD

ABSTRACT

Impaired glucose tolerance (IGT) is a risk-factor for future development of T2DM. Diagnosis of IGT in the pre-diabetes phase of T2DM primarily occurs by means of an oral glucose tolerance test (OGTT). The aim of this study was to characterize the kinetics of glucose-derived breath CO_2 between drug naïve IGT and NGT (normal glucose tolerance) individuals following an oral glucose load. Blood and breath samples were collected at baseline and every 30 minutes for a 10 h period following ingestion of 75 g of glucose isotopically labeled with 150 mg of U-$^{13}C_6$-Glucose. Age (56 ± 5 vs. 47 ± 3 y) and BMI (31 ± 2 vs. 32 ± 2) were similar between IGT and NGT, respectively. Blood glucose concentrations were significantly higher in IGT compared to NGT from baseline to 4.5 h post-glucose ingestion ($P \leq 0.05$). Plasma insulin was higher in IGT at 3, 4-7, 9.5, and 10 hours post-glucose load as compared to NGT. Glucose-derived breath $^{13}CO_2$ was significantly lower in IGT as compared to NGT from 1 to 3.5 h post-glucose ($P \leq 0.05$). Peak breath $^{13}CO_2$ abundance occurred at 4.5 and 3.5 h in IGT and NGT, respectively (36.87 ± 3.15 vs. 41.36 ± 1.56 ‰ delta over baseline). These results suggest that glucose-derived breath CO_2 kinetics measured during the immediate post-glucose ingestion period (1 to 3.5 h) may assist in recognition of undiagnosed IGT in at-risk individuals during the pre-diabetes stage of T2DM.

INTRODUCTION

Insulin resistance, impaired biological response to circulating insulin (52), is the hallmark of pathogenesis of a number of chronic disorders that have become a major public health concern (108). These disorders include obesity, dyslipidemia, hypertension, and diabetes, all of which have substantial underpinnings in insulin resistance (52, 108). The features in these disorders form a predictive risk profile for T2DM and cardiovascular disease (49, 76, 82) and have been coined the Metabolic Syndrome. In the United States, 21-24% of the population suffers from conditions associated with the Metabolic Syndrome, with a higher incidence as age advances (39). In 2005, an estimated 20.8 million people (7% of population) in the U.S. had diabetes, 80% of whom were overweight (93). Recently, the risk of acquiring the associated disorders of the Metabolic Syndrome have significantly increased in the pediatric population (138). Large-scale testing for insulin resistance is needed in both adults and pediatric populations.

Currently, the most sensitive indices for assessing those at risk for developing diabetes take advantage of the fact that both plasma glucose and insulin increase during the initial stages of insulin resistance. This occurs prior to prediabetes when individuals will have worsening insulin resistance (83, 84, 122, 142). The traditional concepts on which development of indices of insulin resistance is based rely on changes in plasma concentrations of glucose and/or insulin resulting from impairment of glucose homeostasis and/or impairment in pancreatic insulin secretory capacity; complex events that take place intracellularly.

Insulin resistance dramatically alters the mix of fuels used for cellular energy production (40, 85, 104). Under normal physiologic conditions of fasting (8-10 hours after a meal), postprandial (during several hours following a mixed meal), or oral glucose

tolerance test (OGTT) (75-100 g glucose), cellular fuel consists primarily of free fatty acids (FFAs) and glucose, with protein metabolism making a minor contribution (24, 36, 40, 104). In the fasting (and resting) state, > 60% of cellular fuel for insulin-dependent peripheral tissues (muscle) is derived from FFAs (16, 36, 41, 51, 53, 60). The portion of plasma-derived and intramyocellular-derived FFAs depends in part on intracellular concentrations of triglycerides (45, 85). When a carbohydrate-containing meal is consumed, or an oral dose of glucose [75-100 g] is administered, contribution of FFAs to whole-body fuel oxidation is reduced dramatically in insulin-sensitive subjects. (36). This switch is brought about by: (a) suppression of hepatic glucose production, reducing its contribution to plasma glucose input, (b) suppression of adipose tissue lipolysis leading to a marked decrease in concentration of plasma FFAs and their flux into the muscle cells, (c) up-regulation of glucose uptake of peripheral tissues, primarily muscle, through insulin receptor-mediated translocation of glucose receptors (GLUT 4) to myocellular plasma membrane, (d) insulin-mediated enzymatic regulation of the complex carbohydrate-lipid pathways, such as glycogen synthesis, and (e) feedback regulation of various steps in glycolysis and cellular fatty acid metabolism, such as feedback regulation of pyruvate dehydrogenase complex (PDH) by mitochondrial acetyl-coA (AcCoA), or citrate regulation of phosphofructokinase (40, 69, 99, 104). The result of these complex processes is a dramatic shift in contribution of FFAs and glucose to mitochondrial AcCoA, the input to Kreb's cycle. This results in a large shift in the source of CO_2 produced by cellular respiration from FFAs to glucose. Development of insulin resistance profoundly alters this physiologic picture (40, 104) leading to impaired glucose contribution to AcCoA as the substrate for the Kreb's cycle. In individuals with impaired glucose tolerance the reduction in carbohydrate contribution to mitochondrial AcCoA is

compensated for by a higher input from FFA β-oxidation because energy expenditure at any given level of activity (e.g. resting metabolic rate) remains constant (33).

The aim of the present study was to test whether an isotopically labeled oral glucose tolerance test (OGTT) can be used to assess insulin sensitivity from breath CO_2. Lewanckzuk et al. recently compared single measurements of ^{13}C-glucose derived CO_2 in breath to results obtained from a hyperinsulinemic-euglycemic clamp performed on a separate occasion (79). However, to the best of our knowledge no reports have correlated glucose derived CO_2 in breath to blood glucose and plasma insulin measurements collected simultaneously over an extended period of time. We hypothesized that impaired glucose utilization for cellular respiration during a standard OGTT results in delayed glucose-derived CO_2 appearance in breath of impaired glucose tolerant (IGT) individuals compared to normal glucose tolerant (NGT) individuals. We compared the breath CO_2 kinetics from IGT individuals to NGT individuals for 10 hours following an OGTT (75 g glucose load) isotopically labeled with U-$^{13}C_6$-glucose**.** Additionally, these data were correlated to indices of insulin resistance from simultaneously collected blood glucose and plasma insulin measurements.

MATERIAL AND METHODS

Subjects

This research was approved by the University of Texas Medical Branch (UTMB) Institutional Review Board. All subjects provided written informed consent before participating in the study. Screening of all subjects included a blood cell count, thyroid function test, lipid panel, fasting blood glucose, urinalysis, and a urine pregnancy test for pre-menopausal women. Individuals previously diagnosed with T2DM or taking any

medications known to affect glucose or lipid metabolism were excluded from participation. Based upon their 2-h OGTT blood glucose levels, subjects were grouped as either normal glucose tolerant (NGT, 2-h OGTT blood glucose < 7.8 mmol/L, n=10) or impaired glucose tolerant (IGT, 2-h OGTT blood glucose ≥ 7.8 mmol/L, n=7). Subject characteristics are shown in Table 2.1.

Table 2.1: Subject Characteristics.

Subject characteristics of the NGT and IGT subjects. * Significantly different from NGT (P<0.001).

	NGT	IGT
2-h OGTT Blood Glucose (mmol/L)	6.2 ± 0.17	11 ± 0.94*
Gender	5Female/5Male	4Female/3Male
Age (yr)	47 ± 3	56 ± 5
Weight (kg)	89 ± 6	92 ± 6
Height (cm)	166 ± 2	172 ± 3
BMI (kg/m2)	31 ± 2	31 ± 2
Fasting Blood Glucose (mmol/L)	5.1 ± 0.11	6.8 ± 0.56*
Fasting Plasma Insulin (μIU/mL)	7.0 ± 1.7	10.39 ± 2.77
WBISI	8.00 ± 1.22	5.48 ± 1.47
HOMA-IR	1.63 ± 0.43	3.23 ± 0.94
QUICKI	0.37 ± 0.01	0.34 ± 0.01

Methods

The study protocol consisted of 4 days of study activities (Table 2.1). On days 1 – 3 subjects were placed on a standardized, low carbohydrate diet containing 15% carbohydrates, 25% protein, and 60% fat. Individual dietary requirements were determined by a registered dietitian and calculated using the Harris Benedict Equation. During this diet stabilization period all subjects were provided breakfast, lunch, and dinner from the UTMB General Clinical Research Center (GCRC) metabolic kitchen. All meals were picked up on a daily basis for consumption off site. Subjects were required to completely consume these meals and nothing else except water. Height and weight were recorded daily during the visits to the GCRC for pick up of the meals. A DEXA scan was completed for determination of body composition on day 3. On the morning of the fourth day, a 10 h OGTT was performed in the GCRC. After an overnight fast (water allowed), subjects were placed in a bed at the GCRC and an antecubital venous IV was placed for the collection of blood samples. Baseline blood samples were collected twice over a fifteen minute period. During the second baseline blood collection, simultaneous breath samples were collected by having the subjects breathe into breath collection bags fitted with one way valves. Following the baseline sample collection, a drink containing 75 g glucose and 150 mg U-$^{13}C_6$-glucose was administered and consumed within 1 minute. From this point (t=0 minutes) blood and breath samples were collected every 30 minutes for 10 hours. For the collection of breath samples, the subjects were instructed to breath normally, hold their breath for 3 seconds, and exhale completely into the provided collection bags. Subjects remained at rest throughout the 10 h OGTT and were only allowed to move around the room to use the restroom. Water was provided *ad libitum*

throughout the 10 h OGTT. After collection of the final samples at t=10 hours and removal of the IV, the subjects were fed and discharged.

Subjects were considered either normal glucose tolerant (NGT) or impaired glucose tolerant (IGT) based on the 2-hour OGTT blood glucose cutoff value of 140 mg/dl (7.8 mmol/L) suggested by the American Diabetes Association (www.diabetes.org).

Measurements of blood glucose

Blood glucose concentrations were determined immediately after collection of the samples using a 2300 STAT Plus Glucose analyzer.

Measurements of plasma insulin

Plasma insulin concentrations were determined using an Immulite 2000 chemiluminescence immunoassay system (Diagnostic Products Corporation, Los Angeles, CA).

Measurements of breath CO_2

The ratios of $^{13}CO_2$ to $^{12}CO_2$ in breath samples were measured using a UBiT-IR300 infrared spectrophotometer (Otsuka Electronics Co., Ltd, Hirakata, Osaka). All results are calculated as per mille (‰) change of $^{13}CO_2$ abundance from the baseline breath sample and expressed as ‰ delta over baseline (‰DOB).

Calculations of indices of insulin resistance

Whole body index of insulin sensitivity (WBISI) (83), homeostatic model assessment-insulin resistance (HOMA-IR) (84), and quantitative insulin sensitivity check index (QUICKI) (67) were calculated from blood glucose (mg/dL) and plasma insulin (μIU/mL) measurements using the following equations:

$$\text{WBISI} = \frac{10{,}000}{\sqrt{(\text{Fasting Glucose} \bullet \text{Fasting Insulin}) \bullet (\text{Mean OGTT Glucose} \bullet \text{Mean OGTT Insulin})}}$$

$$\text{HOMA} = \frac{\text{Fasting Glucose} \bullet \text{Fasting Insulin}}{405}$$

$$\text{QUICKI} = \frac{1}{\log(\text{Fasting Glucose}) + \log(\text{Fasting Insulin})}$$

Statistical Methods

Statistical analyses were performed using SPSS 13.0 (SPSS Inc.), Prism 4 for Windows (GraphPad Software, Inc.), and Microsoft Excel (Microsoft Corporation). Student t-tests were used to calculate differences between NGT and IGT. $P \leq 0.05$ was chosen to indicate statistical significance.

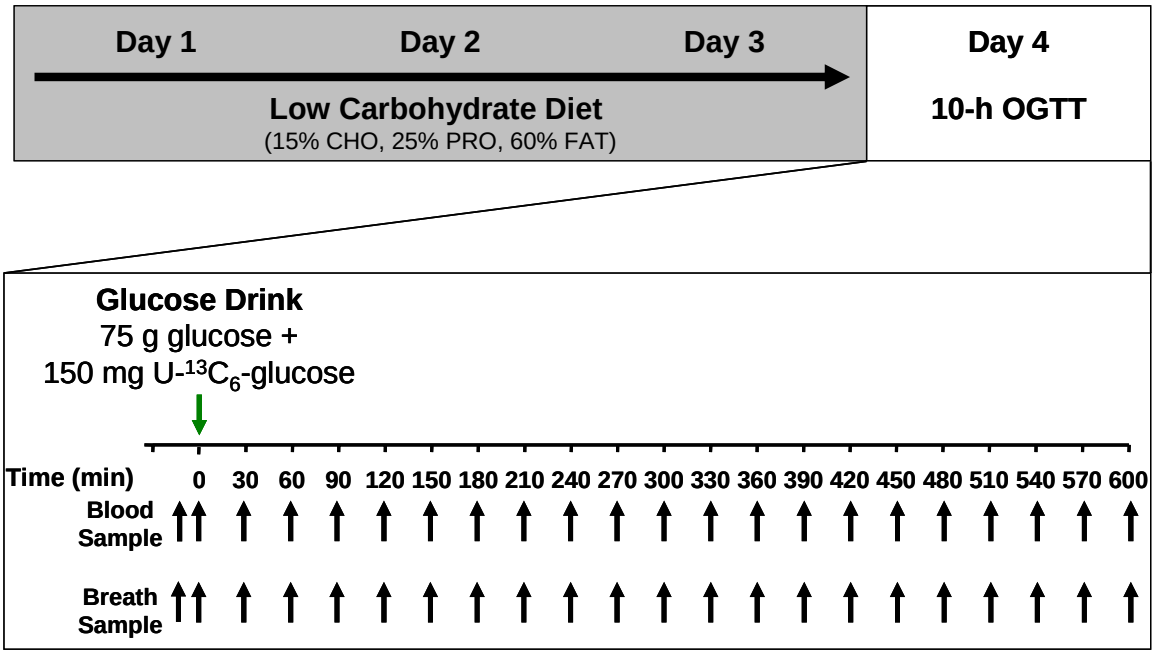


Figure 2.1: Study Timeline.

Subjects followed a three day standardized low carbohydrate diet composed of 15% carbohydrates, 25% protein, and 60% fat. Individual dietary requirements were determined by a registered dietitian and calculated using the Harris Benedict Equation. On the fourth day, after an overnight fast, a 10 h stable-isotopically labeled OGTT was performed and blood and breath samples were collected every 30 minutes.

RESULTS

Subject characteristics

There were no differences in age, height, weight, BMI, or gender between the NGT and IGT groups (Table 2.1). Fasting plasma insulin, WBISI, HOMA-IR, QUICKI were not different between the groups.

Blood glucose concentrations

By design, 2-h OGTT blood glucose concentrations were higher in the IGT (11 ± 0.95 mmol/L) compared to NGT (6.2 ± 0.19 mmol/L) ($P<0.00001$). Fasting blood glucose concentrations were higher in IGT (6.8 ± 0.56 mmol/L) compared to NGT (5.1 ± 0.13 mmol/L) ($P<0.01$). Blood glucose levels were elevated for 3.5 hours post-glucose load as compared to 2.5 hours in NGT (Figure 2.2). There were no differences in blood glucose concentrations from 4 through 10 hours post-glucose load between the groups.

Plasma insulin concentrations

Fasting and early OGTT plasma insulin concentrations showed high within-group variations and were not different between NGT and IGT. Plasma insulin concentrations were significantly elevated in IGT at 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 9.5 and 10 hours post-glucose load as compared to NGT (Figure 2.3).

Breath $^{13}CO_2$ kinetics

Breath $^{13}CO_2$ abundances were significantly lower in IGT compared to NGT between 1 and 3 hours post-glucose load ($P \leq 0.05$) (Figure 2.4). Peak breath $^{13}CO_2$ abundance occurred at 3.5 hours in NGT (41.36 ± 1.56 ‰DOB) and 4.5 hours in IGT (36.87 ± 3.15 ‰DOB). At 9.5 hours post-glucose load, breath $^{13}CO_2$ abundance was lower in NGT compared to IGT (15.11 ± 1.08 vs. 18.63 ±0.89 ‰DOB NGT vs. IGT respectively, $P \leq 0.05$). Areas under the curve (AUC) for breath $^{13}CO_2$ abundance were calculated for each time point and are shown in Figure 2.5. Breath $^{13}CO_2$ abundance AUC were lower in IGT compared to NGT between 1.5 and 4.5 hours post glucose load ($P < 0.05$). This difference disappeared thereafter and remained similar between the groups for the remainder of the study.

Correlation of Breath CO_2 to indices of insulin sensitivity

Glucose derived $^{13}CO_2$ in breath correlated with WBISI, QUICKI and inversely with fasting glucose, 2-hour glucose, HOMA-IR, weight, and height (Table 2.2). Additionally, significant correlations ($P \leq 0.002$) were found between all three measured indices of insulin resistance. WBISI, HOMA-IR and QUICKI correlated to fasting plasma insulin concentrations. HOMA-IR and QUICKI, but not WBISI, correlated to fasting and 2-hour OGTT glucose concentrations. WBISI correlated to 2-hour plasma insulin concentrations. BMI correlated to weight and QUICKI. No correlations were found between age and any of the measured parameters.

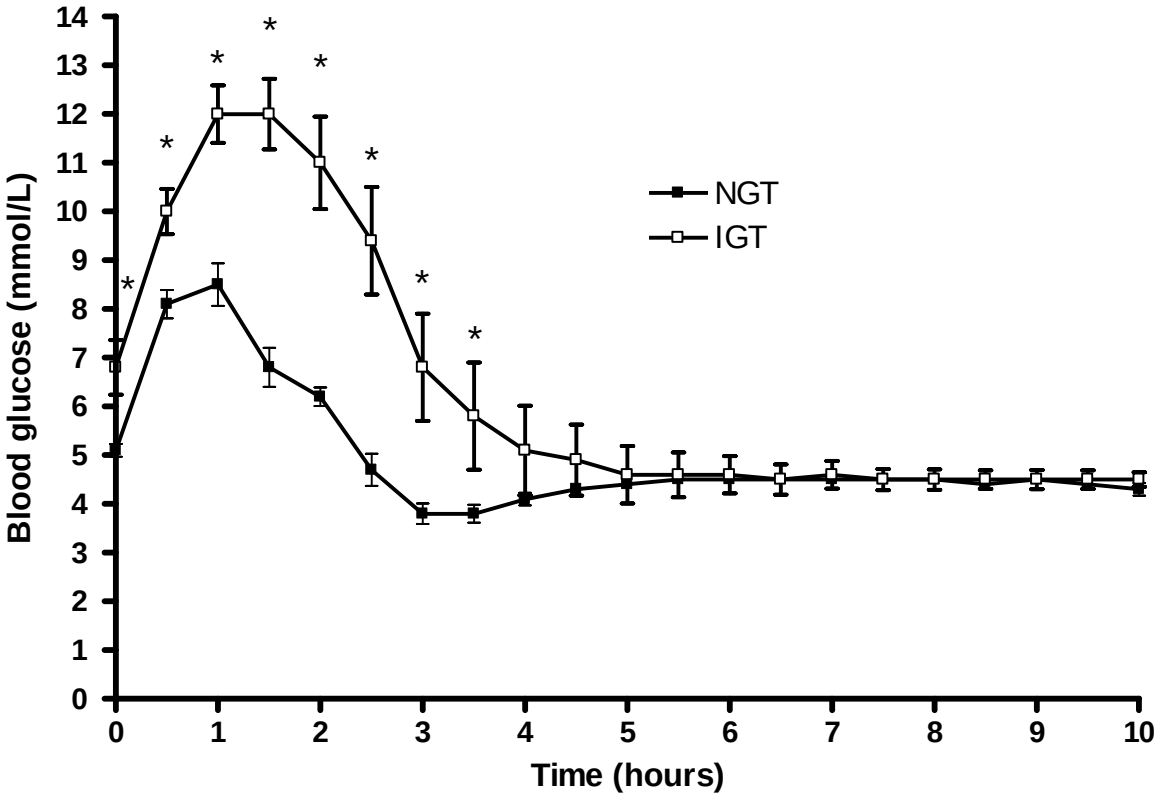


Figure 2.2: Blood glucose concentrations in NGT and IGT during the 10-h OGTT.

*Significant difference between NGT and IGT ($P<0.05$).

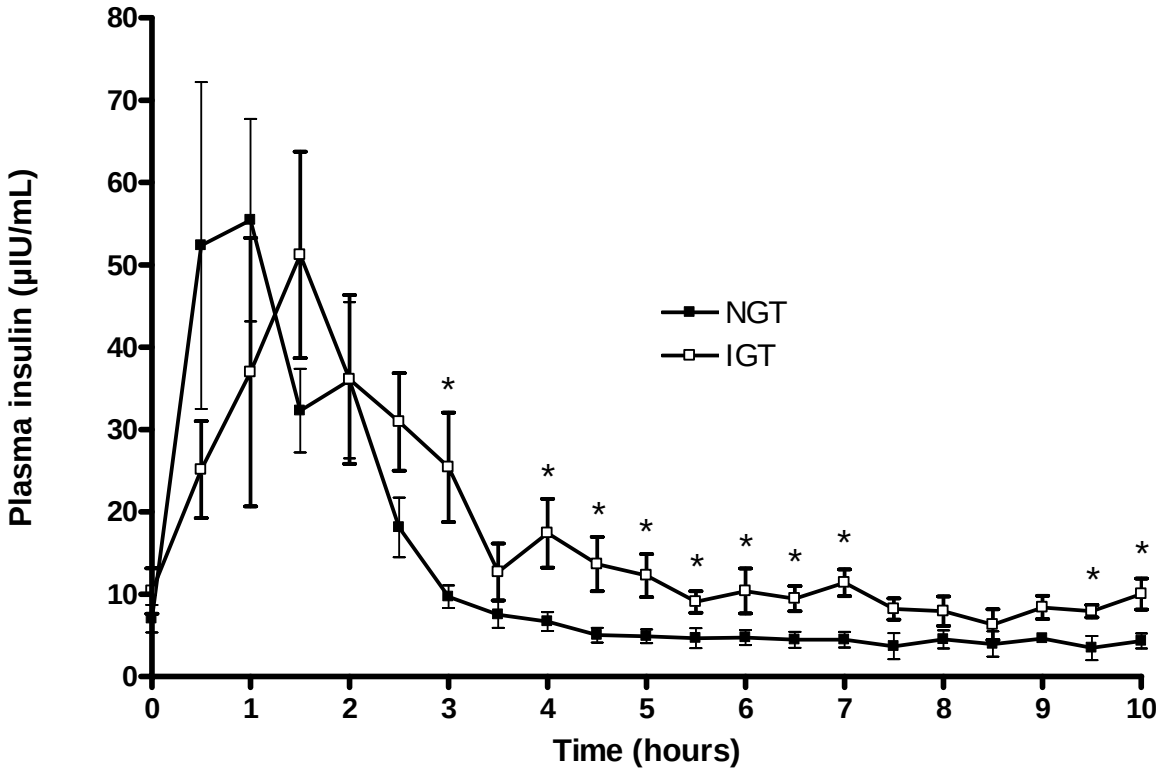


Figure 2.3: Plasma Insulin concentrations in NGT and IGT during the 10-h OGTT.

*Significant difference between NGT and IGT ($P<0.05$).

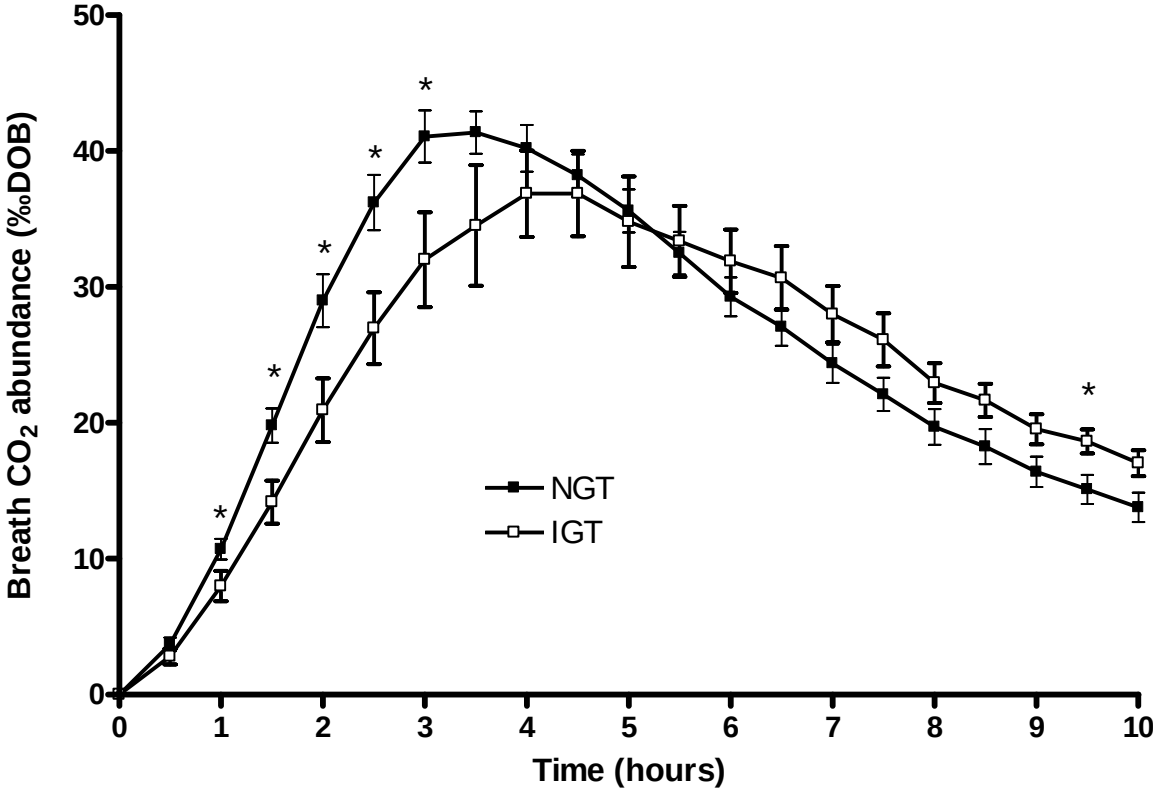


Figure 2.4: Breath $^{13}CO_2$ abundance from NGT and IGT during the 10-h OGTT.

*Significant difference between NGT and IGT ($P<0.05$).

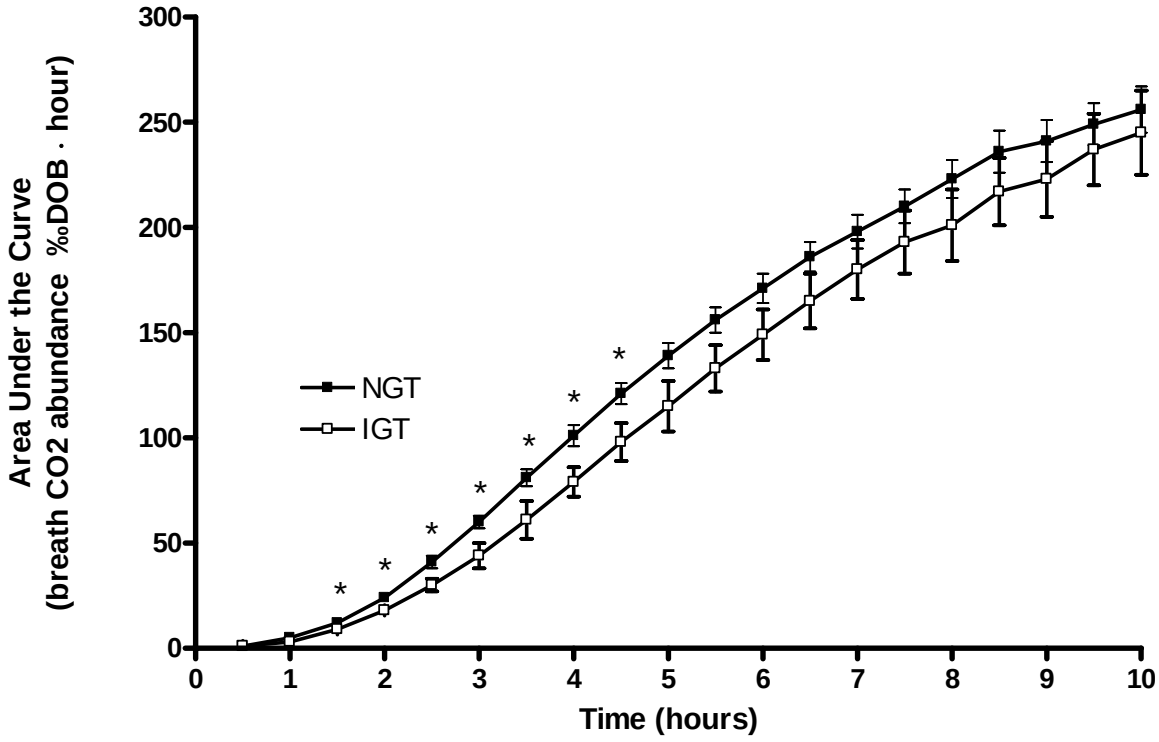


Figure 2.5: Cumulative areas under the curve (AUC) for breath $^{13}CO_2$ abundances.

Cumulative areas under the curve (AUC) for breath $^{13}CO_2$ abundances from NGT and IGT calculated at each time point during the 10-h OGTT. *Significant difference between NGT and IGT ($P<0.05$).

Table 2.2: Correlation coefficients.

Correlation coefficients between parameters of glucose tolerance and indices of insulin sensitivity. * Pearson correlation is significant at P<0.05. ** Pearson correlation is significant at P<0.001.

	Breath CO_2	Fasting Glucose	Fasting Insulin	2-h Glucose	2-h Insulin	WBISI	HOMA-IR	QUICKI	Weight	Height	BMI	Age
Fasting Glucose	-0.631**	1										
P	0.007											
Fasting Insulin	-0.439	0.360	1									
P	0.078	0.155										
2-h Glucose	-0.689**	0.899**	0.337	1								
P	0.002	<0.0001	0.187									
2-h Insulin	-0.191	-0.095	0.427	-0.069	1							
P	0.462	0.716	0.088	0.793								
WBISI	0.502*	-0.336	-0.732**	-0.412	-0.638**	1						
P	0.040	0.187	0.001	0.100	0.006							
HOMA-IR	-0.555*	0.612**	0.949**	0.546*	0.289	-0.703**	1					
P	0.021	0.009	<0.001	0.024	0.260	0.002						
QUICKI	0.629**	-0.554*	-0.873**	-0.506*	-0.400	0.807**	-0.881**	1				
P	0.007	0.021	<0.001	0.038	0.112	<0.001	<0.001					
Weight	-0.546*	0.344	0.311	0.263	0.364	-0.363	0.372	-0.362	1			
P	0.023	0.177	0.224	0.307	0.151	0.152	0.142	0.154				
Height	-0.507*	0.314	0.132	0.515*	-0.030	-0.254	0.201	-0.190	0.307	1		
P	0.038	0.219	0.613	0.034	0.909	0.326	0.440	0.465	0.231			
BMI	-0.469	0.286	0.369	0.113	0.466	-0.383	0.387	-0.482*	0.900**	-0.032	1	
P	0.057	0.266	0.145	0.667	0.060	0.129	0.125	0.050	<0.001	0.904		
Age	-0.223	0.012	0.382	0.140	0.253	-0.311	0.319	-0.272	-0.088	0.311	-0.151	1
P	0.390	0.964	0.130	0.592	0.326	0.225	0.212	0.291	0.737	0.224	0.563	
Gender	-0.247	0.225	0.050	0.159	-0.400	0.145	0.146	-0.055	-0.002	0.578*	-0.169	0.024
P	0.339	0.386	0.850	0.542	0.111	0.579	0.577	0.834	0.994	0.015	0.516	0.927

DISCUSSION

The results of the present study show marked differences in glucose-derived breath CO_2 kinetics between glucose tolerant and glucose intolerant individuals using a novel breath test within 30 min of a 10 h OGTT. The initial rate of glucose derived CO_2 appearance in breath was much slower in IGT compared to NGT. This finding is consistent with the blood glucose measurements, indicating an impaired clearance of exogenous (oral) glucose from circulation. Impaired glucose uptake due to diminished pancreatic insulin secretion or impaired insulin action on the target tissue (i.e. skeletal muscle) results in blunted glucose uptake and subsequent oxidation in IGT individuals. The $^{13}CO_2$ curves in both NGT and IGT corresponded well with the respective blood glucose profiles. In both groups the time of peak $^{13}CO_2$ appearance was identical to the lowest blood glucose measurement, corresponding to the expected decrease in insulin mediated glucose uptake by skeletal muscle and increase in endogenous glucose production (either through gluconeogenesis or glycolysis) to restore circulatory concentrations of glucose to homeostatic levels.

Results from this study establish at which time points breath $^{13}CO_2$ samples collected from IGT subjects differ from NGT subjects, making it possible to further develop an OGTT protocol utilizing this noninvasive breath procedure. The appearance of glucose derived CO_2 in breath returned to baseline slower in IGT compared to NGT. Whether this was due to a metabolic difference between the two groups or because of a dilution effect remains to be determined. Hypothetically, but unlikely because of the small amounts of stable isotope administered, it is possible that since U-$^{13}C_6$-glucose was cleared slower in IGT compared to NGT this resulted in relatively higher U-$^{13}C_6$-glucose blood enrichment in IGT towards the end of the study. However it is more likely that this

observed difference in glucose derived CO_2 reflects a higher rate of non-insulin dependent glucose uptake in the postabsorptive state of IGT when compared to NGT (40, 139).

Our results support the concept that individuals with IGT are slower to switch between lipid fuel and glucose as a source of metabolic fuel when compared to NGT individuals. It has been suggested that impaired glucose tolerance can be described as "metabolic inflexibility" of the target tissue to respond to a switch from lipid oxidation to glucose oxidation (40). In the postabsorptive state of healthy individuals, free fatty acids constitute the major source of fuel for skeletal muscle. After a meal, under the control of insulin, glucose becomes the predominant metabolic fuel source. Among the glucose transporters, GLUT-4 is the predominant form in skeletal muscle. Glucose uptake through GLUT-4 is mediated by insulin whereas non-insulin dependent glucose uptake occurs though other glucose transporters. In pathologies such as T2DM, this non-insulin dependent glucose uptake may be upregulated to compensate for the lack of insulin dependent GLUT-4 recruitment, leading to a higher than normal glucose oxidation in the fasting state but a lack in insulin mediated glucose oxidation in response to a meal (40, 139).

The breath CO_2 area under the curve suggests that the primary fate of glucose is the same for IGT as it is for NGT. While the area under the curve for breath $^{13}CO_2$ was higher in NGT than IGT from 1.5 through 4.5 hours post-glucose ingestion period, the gap in this difference closed within the 10-h period. This is suggestive of the fact that, while much slower, the primary fate of the oral glucose in IGT was similar to that in NGT and most likely glucose oxidation. If, for instance, the area under the curve had remained significantly lower in IGT this could have indicated a redirection of glucose

toward nonoxidative paths such as glycogenesis. Our data do not support this alternative scenario. Theoretically, the difference in area under the curve for $^{13}CO_2$ appearance between the two groups is expected to reach zero when the following conditions are assumed: a) all glucose is oxidized, b) all subjects were at rest and c) resting energy expenditure (REE) for all subjects were similar. While REE measurements are not available from these experiments, we attempted to control the conditions by keeping all subjects at rest during the 10-h OGTT protocol. Under these conditions the relative $^{13}CO_2$ concentration (i.e. $^{13}CO_2$ to $^{12}CO_2$ ratio) in the breath is expected to follow a similar curve as that of total $^{13}CO_2$ concentration in the breath. Since all subjects ingested equal quantities of U-$^{13}C_6$-Glucose, we argue that given sufficient time, the amount of $^{13}CO_2$ produced would be equal regardless of the state of glucose tolerance. Future studies that include indirect calorimetry measures are needed to confirm how energy expenditure differences between subjects as well as changes in response to glucose ingestion affect the results of the breath test. The time course of appearance of exhaled $^{13}CO_2$ will be different between NGT and IGT. By establishing in which aspect and at which time points breath $^{13}CO_2$ samples collected from IGT subjects differ from NGT subjects, it is possible to develop an OGTT protocol utilizing this noninvasive procedure.

The findings we report are promising for future developments of a breath test to detect the onset of diabetes or prediabetes. The differences in glucose derived CO_2 kinetics between IGT and NGT were most profound during the immediate post-glucose ingestion period corresponding to the same timeframe in which a standard 2-h OGTT is administered. This is promising for the application of this breath test as a noninvasive alternative to blood draws during an OGTT. Our results suggest that both the area under the curve and slope of glucose derive $^{13}CO_2$ can be used to distinguishing NGT from

IGT. While area under the curve is theoretically more accurate as it utilizes multiple data points between t=0 and the final measurement at the peak CO_2 output, the slope may be more practical in a clinical setting as it only requires a baseline sample and one additional sample, near, but before the expected peak CO_2 output.

While low-carbohydrate diets are not typically prescribed before administration of a standard OGTT, the 3-day low-carbohydrate diet stabilization period was intended to remove dietary intake as a variable in the present study. Additionally, by reducing dietary carbohydrate intake we intended to decrease hepatic and muscle glycogen stores, thus minimizing these tissues as fast sources of endogenous glucose. Reducing glycemic load is considered beneficial during the management of glucose tolerance. Reducing glycemic index or total carbohydrate intake for 4 months has been shown to reduce postabsorptive plasma glucose concentrations in IGT subjects (140). Yet others have shown that low carbohydrate intake before administration of an OGTT decreases glucose uptake and disposal through pyruvate dehydrogenase in skeletal muscle (99) and can falsely identify NGT individuals as IGT (135). While not an objective during this study our experience was that some individuals we expected to be IGT based on their fasting blood glucose levels at the time of screening, failed to meet IGT criteria after the three-day low carbohydrate diet. As results from our noninvasive breath test correlated highly to established indices of insulin resistance, we do not feel that the type of diet we used negatively affected our results.

Our results support and expand on results recently reported by Lewanczuk et al. (79). However, there were notable differences between our study designs. First, the amount of glucose Lewanczuk et al administered during the OGTT was of a different composition (25g dextrose solution containing 25 mg ^{13}C-glucose) and was followed by a

single follow-up breath measurement at 90 minutes post-glucose load. Secondly, they correlated breath CO_2 data to results obtained from the "Gold Standard" hyperinsulinemic-euglycemic clamp conducted up to two days later. Lewanczuk et al. reported Pearson correlation coefficients at 1.5 h into the OGTT that were similar to correlation coefficients found in the present study. In our study, the 1.5 h correlation coefficient between breath CO_2 and HOMA-IR was -0.555, compared to -0.531 reported by Lewanczuk et al. Similarly, our correlation coefficient between breath CO_2 and QUICKI of 0.629 corresponded well with 0.593 reported by Lewanczuk et al. (79). While no hyperinsulinemic-euglycemic clamp was utilized, the strength in our present study is that breath CO_2 kinetics were followed over a much longer period (10 hours) and included multiple breath CO_2 measurements in conjunction with simultaneous measurements of both blood glucose and plasma insulin concentrations.

In conclusion, the results of the present study demonstrate that that glucose-derived breath CO_2 kinetics measured during the immediate post-glucose ingestion period (0.5 to 3.5 h) may assist in recognition of undiagnosed IGT in at-risk individuals during the pre-diabetes stage of T2DM. Following a baseline sample, the optimal time for the following breath collection was between 1 and 2.5 hours after the oral glucose load, similar to the timeframe of a standard OGTT. Both the area under the curve, when multiple measurements are made over time, and the slope when only pre- and post measurements are available, were shown to be effective. Furthermore, the results obtained with the noninvasive breath test are highly correlated to several indices of insulin resistance, including the WBISI, HOMA-IR, and QUICKI. Continued development of this application will have tremendous potential applications in the diagnosis of T2DM in children. However, further research is necessary to validate this

method as a viable diagnostic tool that can be used in addition, or in place of, existing methods. Such studies might focus on larger scale clinical studies, validating the breath test against the "Gold Standard" hyperinsulinemic-euglycemic clamp, establishing firm cut-off values between true NGT and IGT individuals, determining within-subject variability and repeatability, elucidating the effects of dietary intake on breath test results, and the exploring the applicability of this method in pediatric care.

ACKNOWLEDGEMENTS

This study was supported by NIH/NIDDK SBIR Grant R43 DK072637-01, awarded to BioChemAnalysis Corporation to (M. Janghorbani). Clinical studies were conducted on the GCRC at UTMB, funded by Grant M01 RR 00073 from the National Center for Research Resources, NIH, USPHS, and subcontract from BioChemAnalysis Corp to M. Sheffield-Moore.

CHAPTER 3

CHRONIC AMINO ACID SUPPLEMENTATION DOES NOT IMPAIR THE ACUTE ANABOLIC RESPONSE TO ORAL AMINO ACIDS IN ELDERLY

ABSTRACT

Essential amino acids (EAA) are acutely anabolic to muscle protein synthesis in healthy elderly. We investigated whether twice daily between meal oral supplementation of EAA for three months impacted the magnitude of the acute muscle protein synthetic response to an oral bolus of 7.5 g EAA.

Older men and women were randomized in double-blinded fashion to receive daily between-meal supplements of either 7.5 g EAA (3.75 g twice daily, LO), 15 g EAA (7.5 g twice daily, HI), or placebo (PL) for three months. At 0 months, muscle biopsies were taken from the *vastus lateralis* before and after a bolus ingestion of 7.5 g EAA for determination of muscle protein kinetics, muscle fractional synthetic rate (FSR) and phosphorylation of Akt, mTOR, S6K1, and 4E-BP1. This protocol was repeated after three months.

Ingestion of 7.5 g EAA acutely stimulated FSR in all three groups at 0 months ($P<0.05$). Net phenylalanine uptake in the leg was significantly higher after three months in LO and HI compared to PL ($P<0.05$). Chronic EAA supplementation did not alter the magnitude of the acute FSR or total and phosphorylated Akt, mTOR, S6K1 or 4E-BP1 response in LO or HI compared to PL.

The magnitude of the anabolic response to an oral EAA ingestion in healthy elderly remained intact after 3 months of EAA supplementation. However, the 7.5 g EAA load was insufficient strong to activate the Akt/mTOR pathway in the elderly.

INTRODUCTION

Loss of lean body mass in the elderly population poses a significant health concern affecting both males and females. Reduced muscle strength among elderly is an important risk factor for functional limitations, disability, and mortality (105). The reduction in muscle mass during aging is believed to be largely related to reduced hormonal levels, inactivity and suboptimal nutrition leading to decreased muscle protein synthesis (29, 91). While many factors are involved in the age-related loss of muscle mass, adequate dietary protein and/or amino acids are among the key measures recommended to the elderly as a functional preventative therapy.

An apparent postprandial defect in the stimulation of muscle protein synthesis has been demonstrated in old rats (90) and elderly humans (6) after ingestion of a normal protein meal. Evidence of this blunting effect is also seen in elderly when small boluses of essential amino acids (EAA; 7 g) are given (65). Conversely, the stimulatory effect of amino acids (6, 96, 133), leucine (110), or protein (123) are capable of normalizing the muscle protein synthetic response if the increase in aminoacidemia is significantly large. Leucine availability appears to be essential for the normalization of muscle protein synthesis (15, 26) via intracellular signaling of translation initiation (2, 5, 44) However, in vitro evidence suggests that protein synthesis in muscle of old rats becomes resistant to the stimulatory effect of leucine in its physiological range (26). This resistance can be overcome when leucine concentrations are greatly increased above its postprandial level

as demonstrated by a normalized protein synthetic response in old rats (27, 111). In elderly humans, plasma leucine excess has greatly improved muscle protein synthesis.

The present experiment investigated whether repeated exposure to excess plasma EAA supplemented between meals to the elderly for 3 months would induce resistance to the normal stimulatory effect of an acute ingestion of essential amino acids. Additionally. we examined whether a chronically higher (7.5 g EAA twice daily) or lower (3.75 g EAA twice daily) amino acid load would alter the acute anabolic response to a 7.5 g EAA load to a similar extent. We hypothesized that the higher chronic dose (7.5 g EAA twice daily) would elicit either a normal acute response or a slightly greater than normal increase in the acute muscle protein synthetic response to 7.5 g of EAA.

MATERIALS AND METHODS

Subjects

Healthy older men and women between the age of 60 and 85 were recruited through the Sealy Center of Aging Volunteers Registry of the University of Texas Medical Branch (UTMB). Informed written consent was obtained on all subjects using consent forms approved by the UTMB Institutional Review Board (IRB). Subject characteristics are shown in Table 3.1. Subjects were randomly assigned to one of three blinded groups. Each group received twice daily between-meal supplementation with capsules containing either 7.5 g EAA (3.75 g in the morning and 3.75 g in the afternoon, LO), 15 g EAA (7.5 g in the morning and 7.5 g in the afternoon, HI), or placebo (PL) for three months.

Methods

Each subject was studied on two occasions, once before start of the supplementation period (0 months) and again after the three month supplementation period (Figure 3.1). The morning after admission, an 18-gauge polyethylene catheter (Insyte-W; Becton Dickinson, Sandy, UT) was inserted in an antecubital vein. Baseline blood samples were drawn for the analysis of background amino acid enrichment and concentration and insulin and glucose concentrations. A second 18-gauge polyethylene catheter was placed in the contralateral wrist for blood sampling for the spectrophotometric (λ = 805 nm) determination of leg plasma flow (62). A primed (2 μmol/kg) continuous (0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) infusion of L-[ring-$^2H_5$]phenylalanine was initiated and maintained throughout the basal and EAA periods.

3-Fr 8-cm polyethylene Cook catheters (Bloomington, IN) were inserted in the femoral artery and vein of one leg under local anesthesia. Arterial and venous blood samples were obtained at 10- to 20-min intervals before and after EAA ingestion for determination of amino acid kinetics and plasma concentrations of glucose and insulin. The femoral artery catheter was also used for indocyanine green (ICG) infusion (infusion rate = 0.2 mg/min). Blood flow was measured on three occasions by infusing ICG into the femoral artery for 20 min. Three 2-ml blood samples were drawn simultaneously from the femoral and wrist vein during the final 10 min of each ICG infusion period. Leg plasma flow was calculated from steady-state ICG concentrations and converted to leg blood flow using hematocrit (11, 62).

Muscle biopsies (~150 mg) were taken from the lateral portion of the vastus lateralis 10-15 cm above the knee with a 5 mm Bergstrom biopsy needle, as previously described (9). The final muscle biopsy was performed 4 h post-EAA ingestion. Muscle

biopsies were snap frozen in liquid nitrogen. All samples were kept at -80°C until further processing and analysis.

The composition of the EAA supplements were identical to that previously described by Paddon-Jones et al. and approximated the distribution of amino acids required to increase the intracellular concentrations of EAA's in proportion to their respective contributions to the synthesis of muscle protein (Table 3.2) (96). The EAA drink contained 0.186 g of L-[ring-$^{2}H_{5}$]phenylalanine to maintain the isotopic enrichment (tracer-to-tracee ratio) in the femoral artery at 0.08 (95). The amino acids were dissolved in 250 ml of a noncaloric, noncaffeinated soft drink and consumed as a bolus.

At the end of the protocol, all peripheral and femoral catheters were removed, and subjects were fed and monitored for a minimum of 2 h before discharge. Before release from the GCRC all subjects were provided with their respective twice daily between-meal supplements. All subjects returned after three months and repeated this protocol. Subjects were encouraged to refrain from making major changes to their regular diet throughout the three month supplementation period. Dietary questionnaires were obtained before reporting to the GCRC and were reviewed by a registered dietician.

Blood

Femoral artery and vein blood samples were immediately mixed and precipitated in preweighed tubes containing a 15% sulfosalicyclic acid solution and an internal standard. The internal standard (100 µl/ml blood) contained 49.3 µmol/l L-[U-^{15}N]phenylalanine. Samples were reweighed and centrifuged, and the supernatant was removed and frozen (-80°C) until analysis. Upon thawing, blood amino acids were extracted from 500 µl of supernatant by cation exchange chromatography (Dowex AG

50W-8X,100-200 mesh H+ form; Bio-Rad Laboratories, Richmond, CA) and dried under vacuum (Savant Instruments, Farmingdale, NY). Phenylalanine enrichments and concentrations were determined on the tert-butyldimethylsilyl derivative using gas-chromatography mass spectrometry (GCMS) (HP model 5989; Hewlett-Packard, Palo Alto, CA) with electron impact ionization. Ions 336, 341, and 346 were monitored (97, 144). Plasma insulin concentrations were determined with an Immulite 2000 chemiluminescence immunoassay analyzer (DPC, Los Angeles, CA). All other blood measures were performed by the UTMB Clinical Laboratory.

Muscle

Muscle biopsy samples from the vastus lateralis were immediately rinsed, blotted, and snap frozen in liquid nitrogen until analysis. Upon thawing, samples were weighed, and the protein was precipitated with 800 μl of 14% perchloroacetic acid. To measure intracellular phenylalanine concentration, an internal standard (2 μl/mg wet wt) containing 3 μmol/l L-[U-^{15}N]phenylalanine was added. Approximately 1.5 ml of supernatant was collected after tissue homogenization and centrifugation and processed in the same manner as the supernatant from blood samples. Intracellular phenylalanine enrichment and concentrations were determined using the tert-butyldimethylsilyl derivative (9, 141). The remaining muscle pellet was washed and dried, and the proteins were hydrolyzed in 6 N HCl at 50°C for 24 h. The protein-bound L-[ring-$^{2}H_5$]phenylalanine enrichment was determined by GCMS (17).

Table 3.1: Subject Characteristics.

Subject characteristics of the PL, LO, and HI groups at month 0.

	PL n=10	LO n=9	HI n=7
Gender (Female/Male)	7F/3M	6F/3M	7F/0M
Age (yr)	68 ± 2	65 ± 2	67 ± 1
Height (cm)	166 ± 2	169 ± 1	169 ± 1
Weight (kg)	73 ± 3	81 ± 8	76 ± 5
Lean Body Mass (kg)	46 ± 3	49 ± 5	43 ± 3

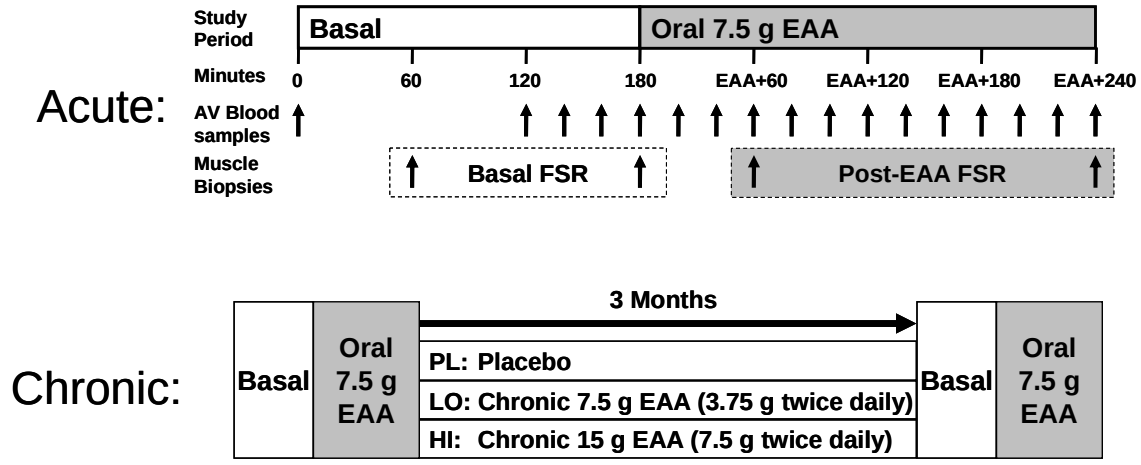


Figure 3.1: Study Timeline.

Study timeline showing the acute and chronic oral EAA experimental protocol.

Table 3.2: Composition of EAA Supplements.

The composition of the essential amino acid mixture was identical to that used by Paddon-Jones, et al. (96). The 7.5 g EAA supplement was used as the acute bolus during the kinetic studies at 0 and 3 months for all groups. The LO group received 3.75 g EAA twice a day to total 7.5 g EAA/day. The HI group received 7.5 g EAA twice a day to total 15 g EAA/day.

Amino Acid	**3.75 g EAA**	**7.5 g EAA**	**Total (%)**
Acute (bolus)		All groups (= 7.5 g)	
Chronic (twice daily)	LO (= 7.5 g)	HI (= 15 g)	
Histidine	0.41	0.82	10.9
Isoleucine	0.39	0.78	10.4
Leucine	0.70	1.39	18.6
Lysine	0.58	1.17	15.5
Methionine	0.12	0.23	3.1
Phenylalanine	0.58	1.17	15.5
Threonine	0.55	1.10	14.7
Valine	0.43	0.86	11.5
Total	**3.76**	**7.52**	**100**

Analytical Methods

Phenylalanine enrichments and concentrations in blood and muscle samples were analyzed by GCMS as previously described (17, 117). Protein-bound phenylalanine enrichment was analyzed by GCMS after protein hydrolysis and amino acid extraction (141), using the external curve approach for low enrichments (17, 98).

Calculations

Phenylalanine was selected to trace muscle protein kinetics because it is neither produced nor metabolized in skeletal muscle. Mixed muscle fractional synthetic rate (FSR) was calculated at steady state by measuring the direct incorporation of L-[ring-$^{2}H_5$]phenylalanine into protein, using the precursor-product model:

$$FSR = [(EP2 - EP1)/(EM \cdot t)] \bullet 60 \bullet 100 \qquad (\%\bullet h^{-1})$$

where EP1 and EP2 are the enrichments of bound L-[ring-$^{2}H_5$]phenylalanine in consecutive muscle biopsies, t is the time interval between biopsies and EM is the mean L-[ring-$^{2}H_5$]phenylalanine enrichment in the muscle intracellular pool (11, 131). Data are expressed as $\%\bullet h^{-1}$.

Protein synthesis (Rd), protein breakdown (Ra) and net phenylalanine balance (NB) were calculated as follows:

$$Rd = (Ca \bullet Ea - Cv \bullet Ev)/(Ea \bullet BF) \qquad (nmol \bullet min^{-1} \bullet 100ml\ leg^{-1})$$

$$Ra = Rd - NB \qquad (nmol \bullet min^{-1} \bullet 100ml\ leg^{-1})$$

$$NB = (Ca - Cv) \bullet BF \qquad (nmol \bullet min^{-1} \bullet 100ml\ leg^{-1})$$

where Ca and Cv represent the phenylalanine concentrations in the femoral artery and vein, respectively (141). BF represents leg blood flow, as determined by the ICG dye dilution method (62). Leg volume was determined anthropometrically (64). Data are expressed as nmol • min^{-1} • 100ml leg^{-1} (11).

Western blot analysis

Protein was isolated from muscle biopsy samples by slicing frozen muscle in very small pieces using a clean razor blade and thawing the tissue in lysis buffer (150 mM NaCl, 10 mM Tris, 1% Triton X-100, 1% Na Deoxycholate, 0.1% SDS, 5 mM EDTA) containing proteinase inhibitors (1 mM PMSF, 1 mM Benamidine, 10 mg/ml Aprotinin, 50 mg/ml Leupeptin, 1 mg/ml Pepstatin A) at a concentration of approximately 3 ml of ice cold lysis buffer per gram of tissue. The tissue was homogenized with a Dounce homogenizer (4°C), centrifuged at 15,000 X g for 20 min, the supernatant removed and centrifuged again to result in total cell lysate. After determination of protein content, using the Bradford protein assay, cell lysates were frozen at -80°C for further analysis. For western blot analyses of Akt, mTOR, S6K1 and 4E-BP1, aliquots containing 80 µg of protein were mixed with equal volumes of SDS-sample buffer, heated to 80°C for 3 min, and electrophoresed on NuPAGE 4-12% Bis-Tris gradient gels using a XCell SureLock Mini-Cell system (Invitrogen Corp., CA). Proteins were transferred to PVDF membranes. Following blocking with 5% non fat dry milk in Tris-buffered saline-Tween 20 (TBST), membranes were incubated overnight (at 4°C) with rabbit primary antibodies to either total or phosphorylated Akt (Ser473), mTOR (Ser2448), S6K1 (Thr389), and

4E-BP1 (Thr37/46) (Cell Signaling Technology, Inc., MA). GAPDH antibody was used to correct the results for protein loading of the gel. Blots were washed with TBST and incubated in horseradish peroxidase conjugated anti-rabbit IgG secondary antibody and developed with enhanced chemiluminescense reagents (Amersham Biosciences, NJ). Developed blots were imaged and analyzed using a ChemiDoc XRS system (Bio-Rad Laboratories, CA). All densitometry results are expressed in arbitrary units.

RESULTS

Physical Characteristics

Subject characteristics are shown in Table 3.1. There were no differences in age, height, weight, or lean body mass between the groups. Lean and total body mass were increased in HI but not in LO or PL after three months.

Phenylalanine Enrichment and Concentration

Arterial phenylalanine enrichment remained stable from basal to EAA in all groups at month 0 and 3 (

Figure 3.2). Basal arterial and venous phenylalanine concentrations were similar between all groups at month 0 (54.6 ± 2.55, 61.52 ± 6.25, and 56.99 ± 4.31 $nmol \cdot mL^{-1}$ for artery in PL, LO, and HI, respectively) and were not changed at month 3. Both arterial and venous phenylalanine concentrations increased immediately after EAA ingestion and remained elevated throughout the 4 hour study period in all groups at months 0 and 3 ($P<0.01$, Figure 3.3). At 3 months, basal concentrations in both the artery and vein were higher in the HI group when compared to PL ($P<0.05$) and tended to stay higher after

EAA ingestion at 135, 150, 165, 180, and 225 minutes ($P<0.05$). There were no differences in arterial or venous concentrations between month 0 and 3 in the PL or LO group at any time.

Mixed Muscle Fractional Synthesis Rate (FSR)

All groups showed similar increases in FSR in response to acute 7.5 g EAA at 0 months (PL = 0.0546 ± 0.00396 vs. 0.104 ± 0.0109, LO = 0.0583 ± 0.00357 vs. 0.0997 ± 0.0105, HI = 0.0634 ± 0.00388 vs. 0.0937 ± 0.00896 $\%\bullet h^{-1}$, $P<0.05$) (Figure 3.4). At month 3, the magnitude of the increase in FSR was not significantly different among PL (0.104 ± 0.0104, $P<0.01$), LO (0.0841 ± 0.00650, $P=0.07$), and HI (0.103 ± 0.0115, $P=0.13$) As previously reported, basal FSR was higher in LO (0.0679 ± 0.00343, $P<0.01$) and HI (0.0804 ± 0.00567, $P<0.05$) after 3 months when compared to 0 months but remained unchanged in PL (0.0617 ± 0.00365) (116). Thus, while the acute change in FSR at 3 months remained in PL, this was no longer significant in the groups receiving chronic EAA.

Net Phenylalanine Balance (NB)

Basal phenylalanine NB was similar between all three groups at 0 months (-17.17 ± 3.33, -17.09 ± 4.28, and -17.79 ± 7.28 $nmol\cdot min^{-1}\cdot 100\ mL\cdot leg^{-1}$ for PL, LO, and HI respectively) and after three months (-17.79 ± 4.26, -21.27 ± 4.25, and -18.05 ± 3.32) (Figure 3.5). 7.5 g EAA resulted in a shift from a net negative to positive NB and peaked at 30 minutes in all three groups both at 0 months (117.26 ± 15.41, 144.53 ± 18.47, and 112.46 ± 53.34) and after three months (201.23 ± 19.33, 182.36 ± 14.05, and 153.01 ± 35.23). NB returned to basal levels within 1 hour for all three groups at 0 months. After

three months, NB in LO (35.82 ± 20.56) and HI (20.50 ± 23.07) were significantly higher at 1 hour as compared to 0 months ($P<0.05$) while PL returned to basal values (-29.67 ± 16.04). The difference in acute net phenylalanine accumulation in the leg (measured as NB area under the curve for the entire 240 post-EAA period) between month 0 and month 3 was elevated in LO and HI when compared to PL (Figure 3.6, $P<0.05$).

Model-derived protein synthesis and breakdown (Phenylalanine Ra and Rd)

Phenylalanine Ra did not change after EAA ingestion at 0 or 3 months and were similar between the groups (not shown). Average basal vs post-EAA Ra values were 39.9 ± 4.9 vs. 38.8 ± 5.3 nmol Phe·min^{-1}·100 ml leg vol^{-1} (0 months PL), 42.9 ± 6.4 vs. 49.7 ± 5.6 (3-month PL), 43.9 ± 7.2 vs. 43.3 ± 6.9 (0 months LO), 51.5 ± 9.6 vs. 52.0 ± 7.2 (3-month LO), 34.1 ± 8.2 vs. 42.1 ± 4.1 (0 months HI), and 44.9 ± 5.7 vs. 51.2 ± 9.0 (3-month HI).

Basal Rd was similar between all groups at 0 months and at 3 months respectively 22.7 ± 2.6 and 25.1 ± 6.0 nmol Phe·min^{-1}·100 ml leg vol^{-1} (PL), 26.8 ± 3.8 and 30.2 ± 9.7 (LO), and 16.3 ± 6.8 and 26.5 ± 5.7 (HI). At 0 months, Rd increased immediately after EAA ingestion in every group and peaked at 30 minutes 167.9 ± 20.1 (PL, $P<0.0001$), 198.9 ± 22.4 (LO, $P<0.0001$), and 175.3 ± 54.9 (HI, $P<0.05$). By 60 minutes post-EAA ingestion Rd had returned to basal levels in all three groups. At three months Rd peaked at 15 minutes in the HI group (233.9 ± 60.3, $P<0.01$) and at 30 minutes in PL and LO (252.2 ± 13.1, 260.1 ± 20.8, and 215.1 ± 47.7 for PL, LO, and HI at 30 minutes respectively, $P<0.01$). Rd returned to basal levels within 60 minutes for PL and HI and returned to basal levels within 75 minutes for LO. Compared to 0 months, at 3 months the

post-EAA Rd was significantly higher at 30 minutes ($P<0.05$) for PL, at 45 ($P<0.05$) and 75 minutes ($P<0.01$) for LO, and at 15 minutes ($P<0.05$) for HI.

Plasma Insulin

All three groups elicited an acute insulin response after oral EAA ingestion before ($P<0.05$) and after three months ($P<0.01$) of supplementation (Figure 3.7). Insulin returned to basal concentrations or below basal concentrations within 1 hour in all groups. There were no differences in insulin concentrations between any of the groups at any time. Net insulin release (calculated from AUC over the entire 240 minute periods) at month 0 vs. month 3 were 6.8 ± 1.2 vs. 6.7 ± 1.4, 7.2 ± 1.0 vs. 6.7 ± 1.2, and 7.6 ± 2.3 vs. 7.1 ± 2.6 $\mu IU \cdot mL^{-1} \cdot min^{-1}$ for PL, LO, and HI respectively.

Leg Blood Flow

Leg blood flow was not affected by acute EAA ingestion. Blood flow values pre vs. post-EAA were 2.8 ± 0.1 vs. 2.6 ± 0.2 ml·min-1·100 ml leg vol^{-1} (0 months PL), 2.9 ± 0.2 vs. 2.9 ± 0.1 (3 month PL), 3.0 ± 0.3 vs. 3.0 ± 0.3 (0 months LO), 3.1 ± 0.2 vs. 3.0 ± 0.3 (3 month LO), 3.0 ± 0.2 vs. 3.1 ± 0.3 (0 months HI), and 2.9 ± 0.4 vs. 2.9 ± 0.4 (3 month HI).

Western immunoblots

No significant changes in total protein or phosphorylation of Akt, mTOR, S6K1, or 4E-BP1 were observed at 1 hour or 4 hours after acute oral EAA in any of the groups at 0 months or after the 3 month supplementation period (Figure 3.8). Pooled data from

all three groups revealed increased phosphorylation of S6K1 within 4 hours following the acute bolus of 7.5 g EAA before and after 3 months ($P<0.05$). Phosphorylation of 4E-BP1 was increased within 1 hour following the acute bolus of 7.5 g EAA in pooled data at 3 months ($P<0.05$).

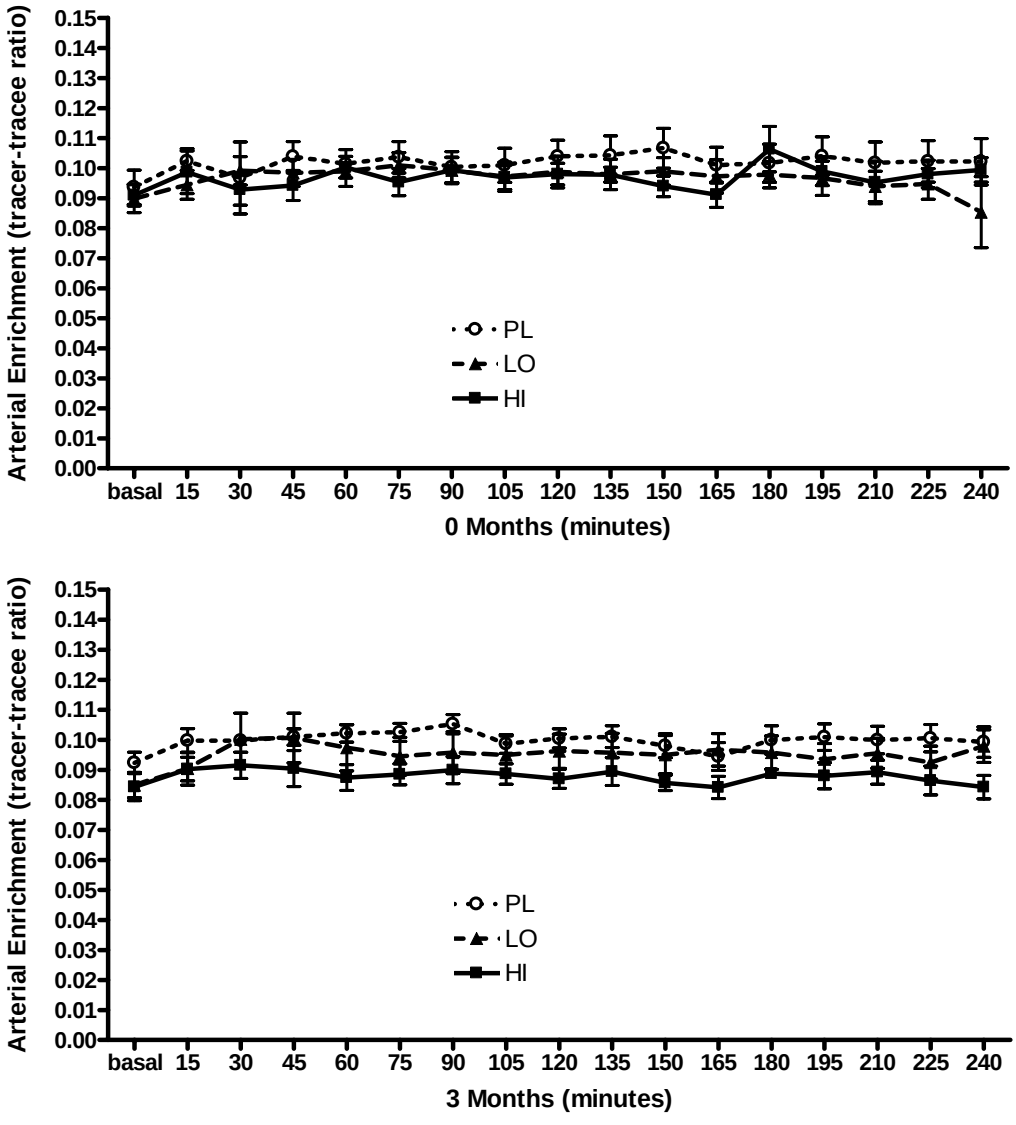


Figure 3.2: Arterial phenylalanine enrichment at 0 months and 3 months.

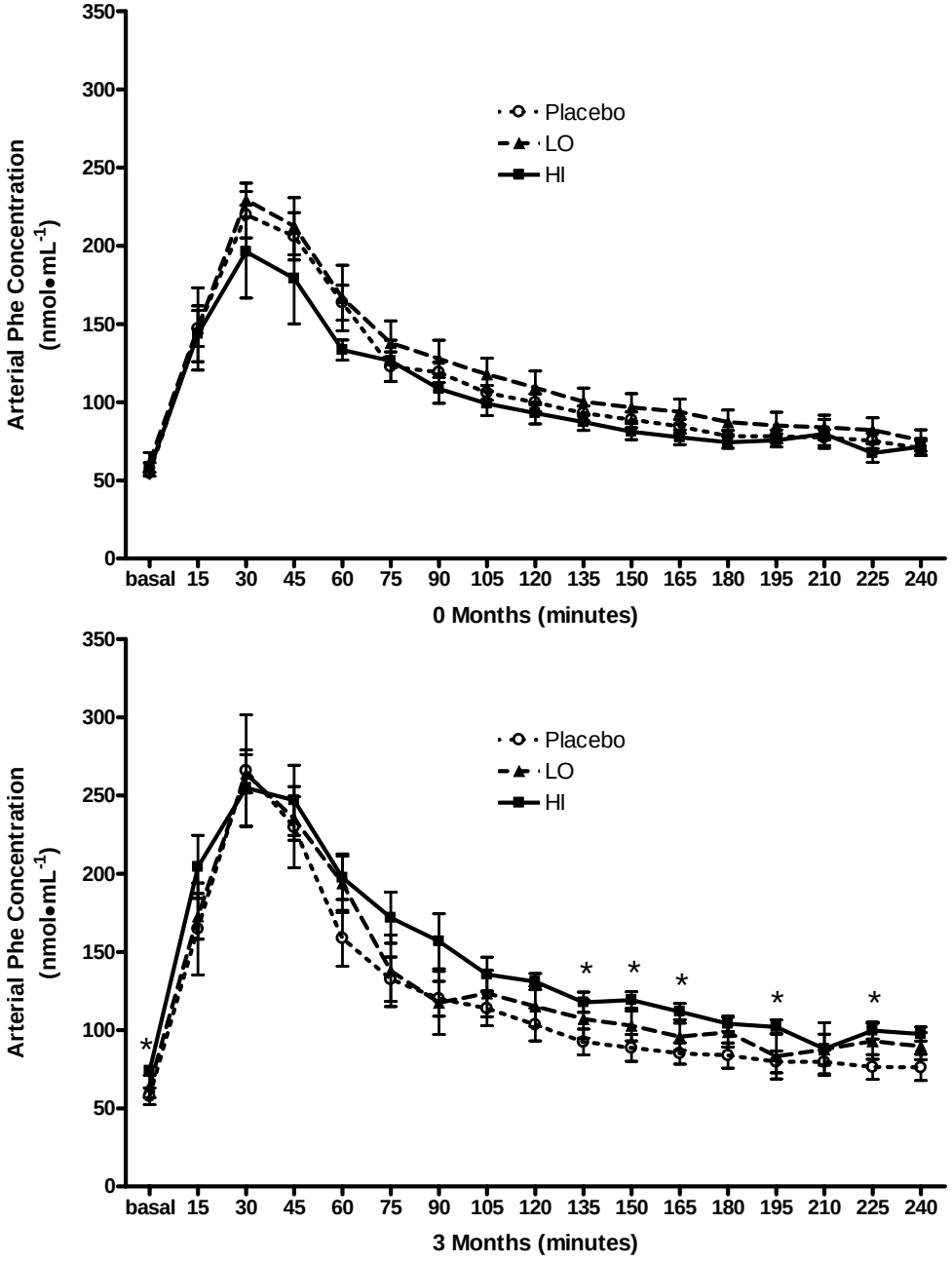


Figure 3.3: Arterial phenylalanine concentrations at 0 months and 3 months.

*Significant difference between PL and HI group P<0.05.

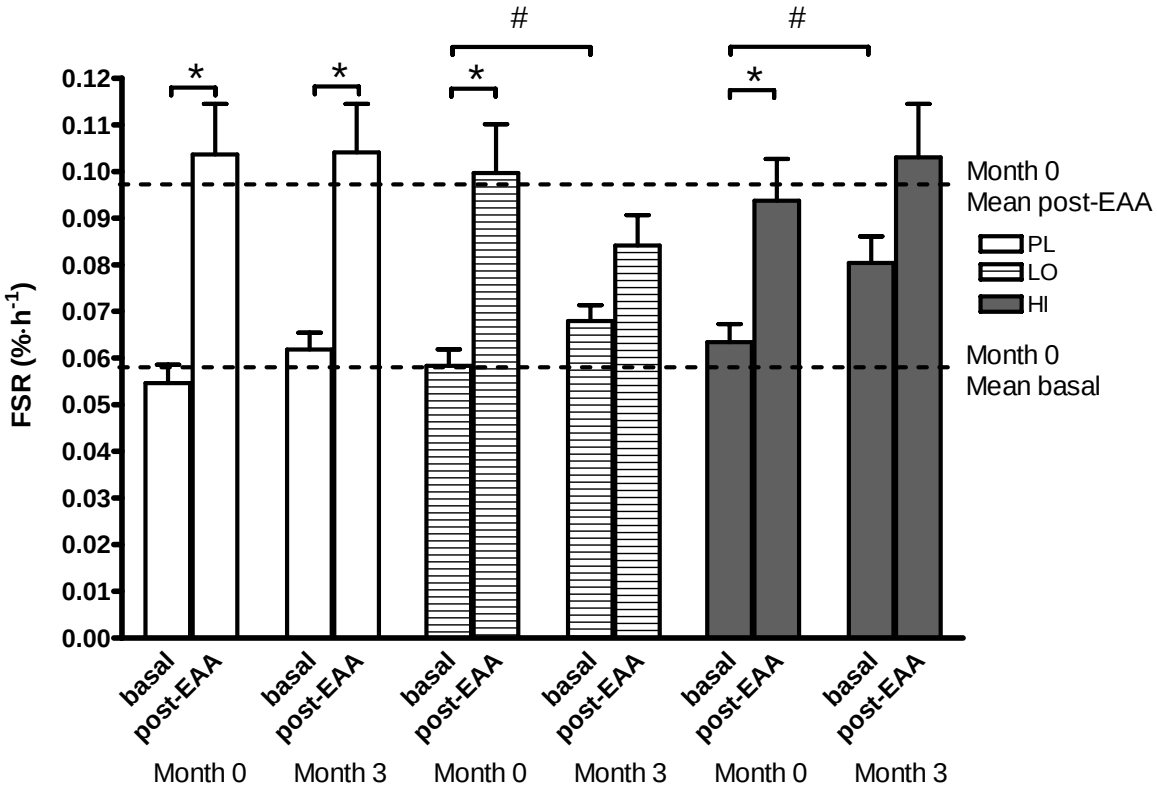


Figure 3.4: Mixed muscle fractional synthetic rate (FSR).

Mixed muscle fractional synthetic rate (FSR) in basal and post-EAA state at 0 months and 3 months. Dotted lines are representative of pooled averages from all three groups at month 0. # Basal at month 3 significantly different vs. basal at month 0 ($P<0.05$). * Post-EAA different vs. basal ($P<0.05$).

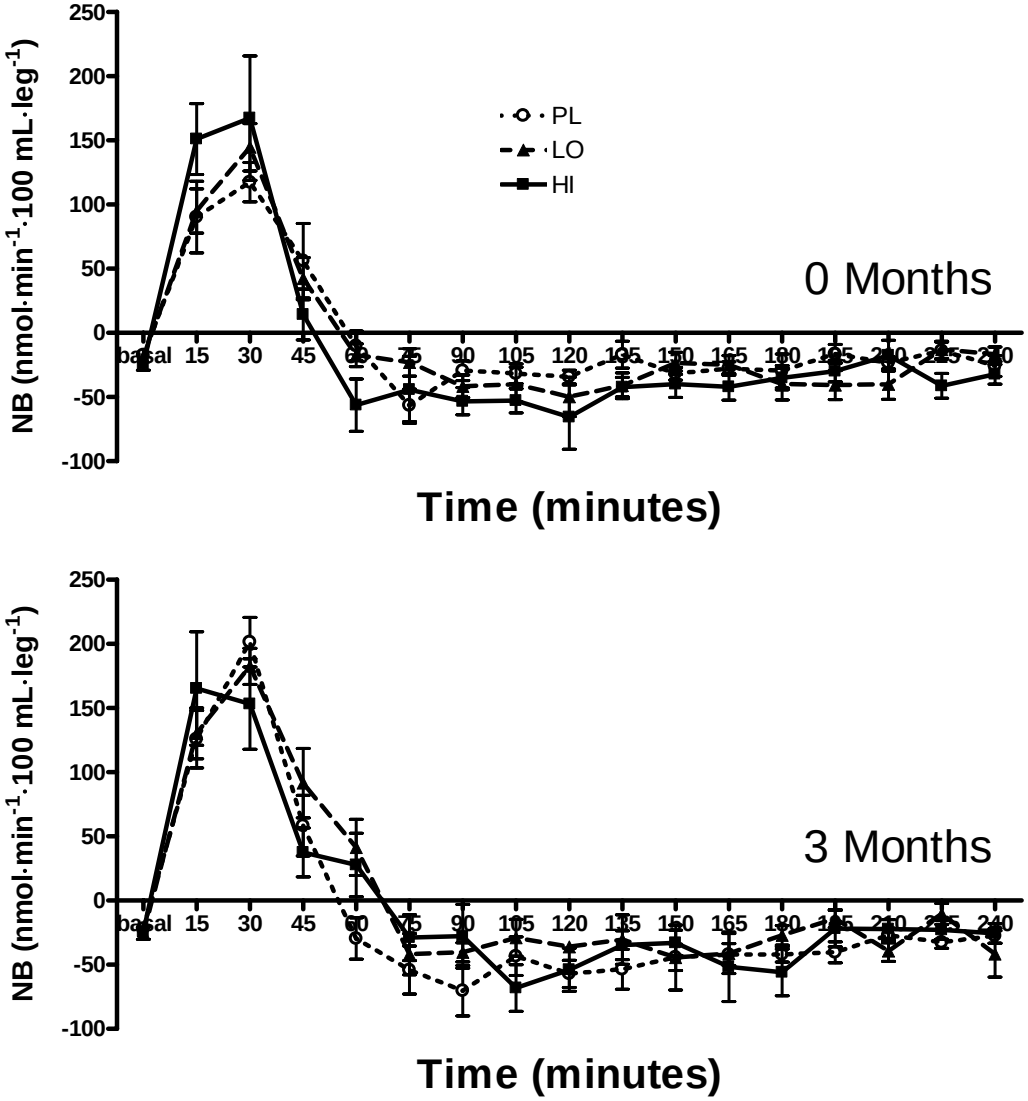


Figure 3.5: Phenylalanine net balance (NB) across the leg at 0 months and 3 months.

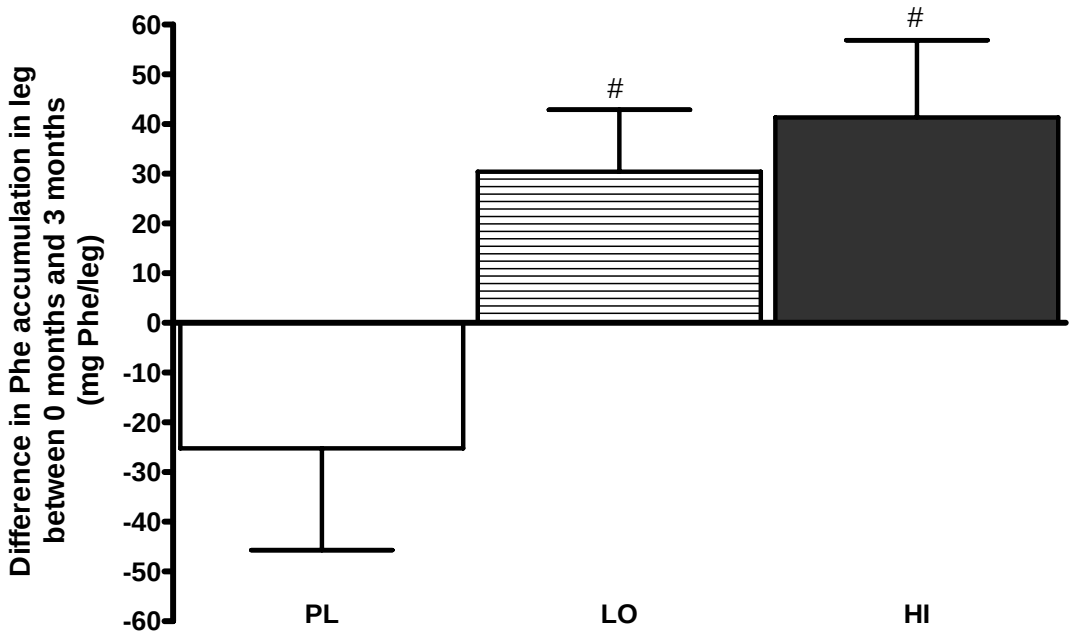


Figure 3.6: Difference in phenylalanine net accumulation in the leg.

Difference in phenylalanine net accumulation in the leg between month 0 and month 3. Phenylalanine net accumulation was calculated from the NB area under the curve (AUC) for the entire 240 min post-EAA period. #Different from PL ($P<0.05$)

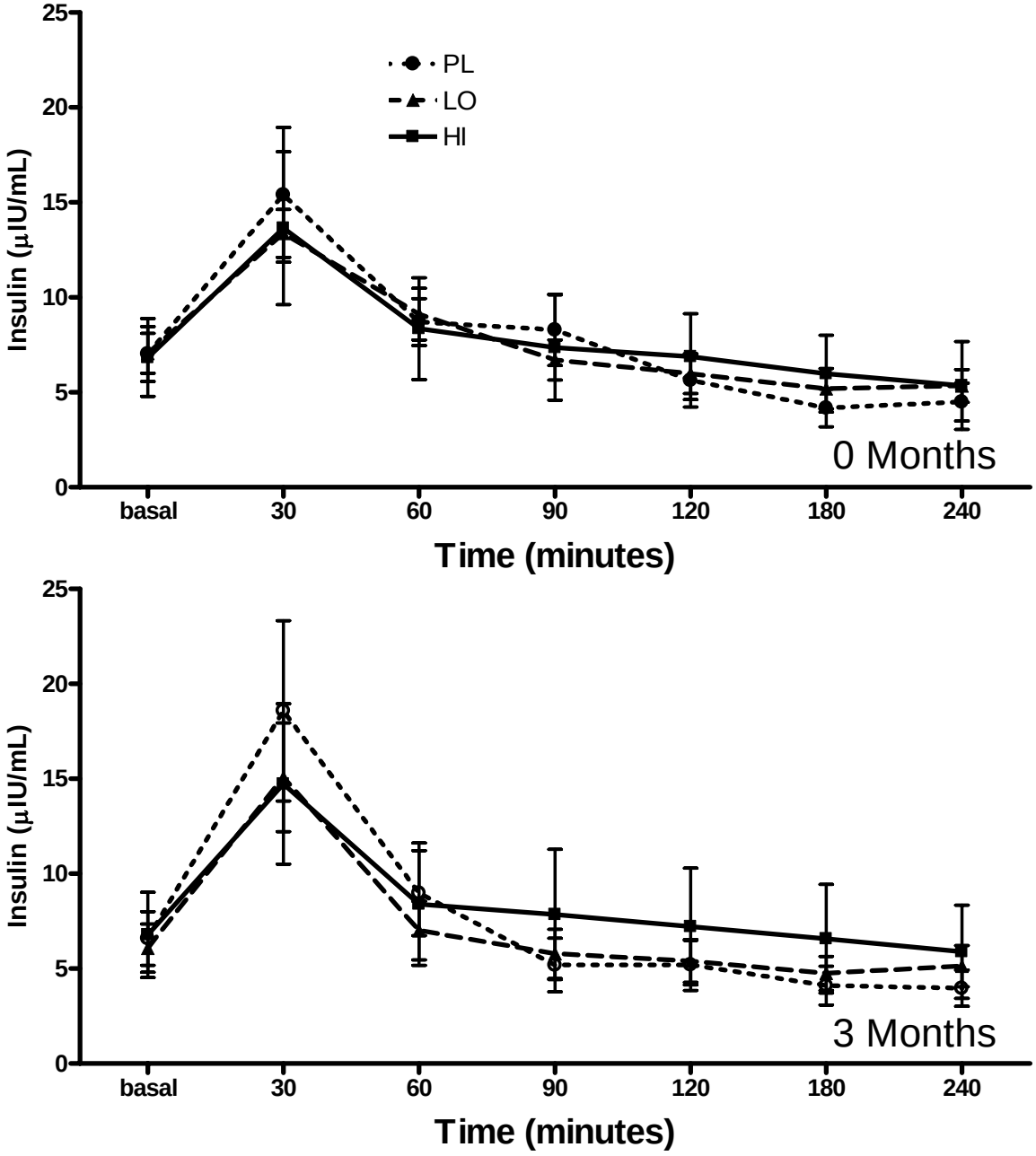


Figure 3.7: Plasma Insulin concentrations at 0 months and 3 months.

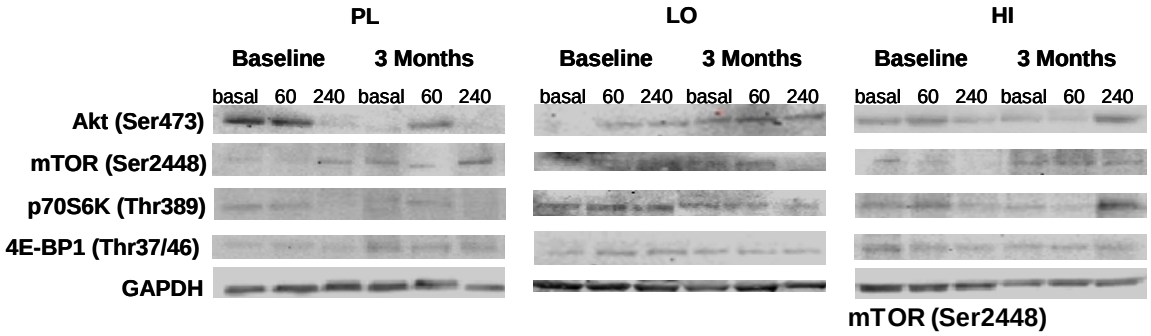
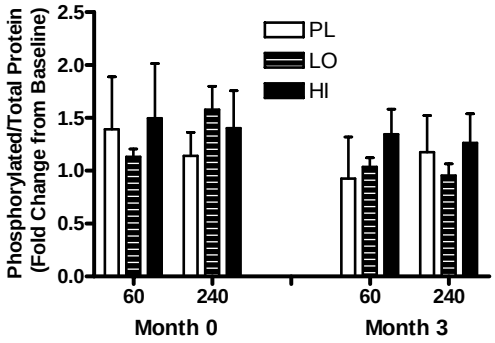
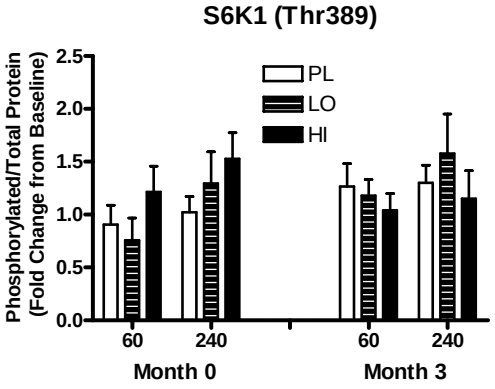
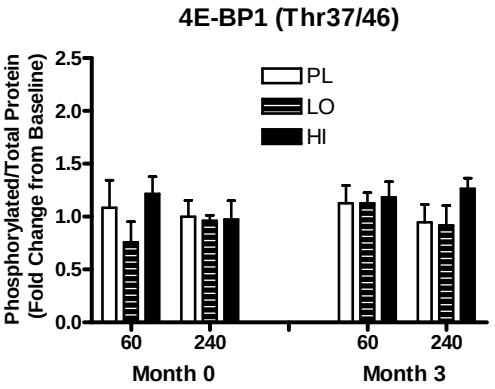


Figure 3.8: Phosphorylation of mTOR, S6K1, and 4E-BP1 in skeletal muscle.

Phosphorylation of mTOR, S6K1, and 4E-BP1 in skeletal muscle at 0 months (basal state) and 3 months (basal, 60 min post-EAA, 240 min post-EAA).







DISCUSSION

Our results demonstrate that daily between meal oral supplementation of 7.5 g and 15 g EAA for three months does not alter the acute anabolic response to oral essential amino acids in elderly. We recently reported that chronic supplementation of EAA increases basal phenylalanine concentration and mixed muscle FSR (116). Here we demonstrate that chronic supplementation does not alter the magnitude of the acute anabolic response to a bolus ingestion of 7.5 g EAA. In fact, while there was no difference in mixed muscle FSR, three months of daily EAA supplementation resulted in a slightly but significantly prolonged period of positive NB after acute oral EAA ingestion. Hence, chronic supplementation may prolong the acute response in FSR after acute ingestion of 7.5 g EAA but does not raise the maximum rate itself. It must be noted that FSR was measured from muscle biopsies collected at 1 and 4 hours after oral EAA ingestion while NB had generally returned to basal levels after 1 hour. Therefore, absorptive FSR measurements were made during a period where NB was negative and may slightly underestimate the absolute rate in protein synthesis immediately after the oral loads. Nevertheless, to our best knowledge, these results show for the first time that chronic supplementation of essential amino acids positively affects the acute anabolic response to ingestion of these amino acids.

Paddon-Jones et al. showed that mixed muscle FSR and phenylalanine NB increased in both young and elderly subjects following a bolus ingestion of 15 g essential amino acids (96). While the relative composition of oral EAA used was identical, the amount of EAA administered acutely was double of that used in the present study. In response to a 15 g EAA load, the initiation of the net balance of phenylalanine in elderly was delayed, blunted, and prolonged compared to young (96). Interestingly, while plasma

insulin levels increased in young subjects following the oral dose of essential amino acids, elderly subjects lacked this response in insulin levels. This is strikingly different from our present results in elderly where insulin concentrations acutely increased following EAA ingestion and NB returned to basal levels more rapidly.

The leucine content in our study was relatively low in comparison to that used in other recently published studies. Indeed, Katsanos et al. (65) found that the administration of 7 g EAA was insufficient to elicit a strong anabolic effect on muscle protein synthesis in a group of elderly. However, when the authors increased the leucine content of the 7 g EAA supplement (from 1.7 to 2.8 g Leu) this was sufficient to induce an anabolic response in elderly to the magnitude found in young (66). Dardavet et al. recently demonstrated *in vitro* that the response in S6K activity to the presence of low concentrations of leucine is less sensitive in muscle of old rats when compared to adult rats (26). However, at high concentrations of leucine S6K activity in muscle of old rats was similar to that in adult rats. In comparison, our LO group received 3.75 g EAA (0.70 g Leu) twice a day to give a daily total of 7.5 g EAA (1.39 g Leu) per day (Table 3.2), which was lower than the amount used in the low-Leucine group published by Katsanos et al. (66). The daily amount of leucine that the LO group received, while raising basal levels of muscle protein synthesis over a 3 month period (116), was likely insufficient to elicit a functional promoting effect on acute muscle protein metabolism. Nevertheless, despite the slight trend for a reduction in the magnitude of the acute FSR response in this group at month 3 when compared to month 0, this was not statistically significant (Figure 3.4). Furthermore, the absence of significant increases in post-EAA FSR in the LO and HI groups at month 3 were due to the increased basal FSR levels in these subjects and not

due to a diminished acute anabolic response as there were no differences in post EAA FSR between the groups.

The major pathways through which EAA are known to induce protein synthesis involve activation of mammalian target of rapamycin (mTOR) and its downstream effectors eukaryotic initiation factor (eIF)4E-binding protein (4E-BP1) and the ribosomal protein S6 kinase (S6K1). This pathway is downstream and sensitive to Akt signaling and insulin is believed to play at least a permissive role in the activation of the mTOR pathway (3). The stimulatory effects of amino acids are detected only when EAAs are present in the treatment and is in particular attributed to the action of the branched chain amino acids (BCAAs) leucine, isoleucine and valine (73). Among these three BCAAs, leucine is the most potent in activating protein synthetic processes *in vitro* (15), and when in the presence of either insulin or carbohydrates, *in vivo* (3). We observed acute increases in insulin concentrations in responses to oral EAA both at 0 months and after the three month supplementation period. However, Akt phosphorylation in skeletal muscle was unchanged following the increase in circulating insulin concentrations suggestive of impaired insulin sensitivity in skeletal muscle. Additionally, phosphorylation of mTOR, and its downstream effectors S6K1 and 4E-BP1, did not increase significantly in any of the groups at 0 months or 3 months of supplementation. Pooled data from all three groups revealed a slight increase in phosphorylation of S6K1 (4 hours post-EAA) at 0 and 3 months and 4E-BP1 (1 hour post-EAA) at 3 months ($P<0.05$, data not shown). While the reports in the literature vary widely, there is an increasing body of evidence suggesting that while essential amino acids are capable of inducing an acute increase in muscle protein synthesis, signaling through the insulin/Akt/mTOR pathway is blunted in elderly when compared to younger individuals

(25, 46, 106, 122, 132). We suggest that with higher oral concentrations of leucine than used in our present study, the anabolic response meditated through the mTOR pathway could be enhanced.

Our findings of blunted signaling responses in muscle of elderly are consistent with reports of others (25, 46). However, we are aware that other factors could have contributed to these results. It may be possible that while an increase in Akt/mTOR signaling indeed took place, the timing of our muscle biopsies were such that the signals (i.e. phosphorylation) were missed in our tissues. The timing of the biopsies coincided with times where insulin concentrations were relatively low and phenylalanine net balance values were negative. Since insulin levels returned to baseline before the first muscle biopsies were collected 1 hour after acute oral EAA, it is possible that downregulation of the Akt pathway had already taken place. Consequently, it would be possible that if any significant activation of mTOR, S6K1 or 4E-BP1 occurred, it returned to basal levels within the first hour, before the first muscle biopsies were collected.

We chose to use standardized 7.5 g EAA (for acute effects), and 7.5 g or 15 g EAA (for chronic effects) supplementations opposed to supplementation based on individual lean body weight. Similarly, we did not actively control for any dietary differences between our subjects throughout the 3-month supplementation period. While we are aware that differences in subjects lean body weight, and dietary habits may have contributed to the variation in our results, the reason for this choice is that we were interested in testing a practical method of supplementation that is more likely to succeed outside a clinical setting.

In conclusion, twice daily between meal supplementation of essential amino acids does not alter the anabolic response after the acute ingestion of an essential amino acid load and extends the duration of positive net balance in healthy older women and men. These data support the rationale for establishing targeted nutritional interventions, such as leucine enriched supplements, in aging and other populations undergoing progressive losses in lean body mass. Additionally, our results emphasize the need for further research aimed at age-associated insulin resistance and elucidation of the pathways through which amino acids mediate protein metabolism in elderly.

ACKNOWLEDGEMENTS

This project was funded by NIH/NIA R01 AG21539 to M. Sheffield-Moore and P60 AG17231 to J.S. Goodwin and R.J. Urban. Studies were conducted on the GCRC at UTMB and funded by grant M01 RR 00073 from the National Center for Research Resources, NIH, USPHS

CHAPTER 4

WEEKLY TESTOSTERONE ADMINISTRATION FOR 5 MONTHS RESULTS IN REDUCED BONE TURNOVER IN OLDER MEN

ABSTRACT

Men and women undergo a progressive reduction in lean muscle mass and bone mineral density (BMD) with advancing age. Maintenance of circulating testosterone concentrations is beneficial to skeletal muscle mass and BMD in older men, but the potential benefit of testosterone therapy on bone metabolism in older men with testosterone concentration in the normal range remains unclear. We investigated whether continuous and monthly cycled administration of testosterone in older men with low normal endogenous testosterone concentrations would lead to improvements in markers of bone metabolism.

Older men (68.92 ± 1.59 yrs, n=13) with endogenous testosterone levels below 500 ng/dL at the time of enrollment completed a 5-month randomized blinded placebo controlled protocol. Subjects received either continuous weekly testosterone (100 mg testosterone-enanthate, IM injection) for the entire 5-month period (TE, n=4), monthly testosterone treatment (one month testosterone/one month placebo, MO, n=4), or weekly placebo for the entire period (PL, n=5). Whole-body and regional DEXA scans were obtained at baseline and after 5 months for measurements of lean mass and BMD. Two-way ANOVA with repeated measures followed by a Dunnett's post hoc comparison to PL were used to indicate statistical significance at $P \leq 0.05$. All data are presented as mean ± SEM.

Serum n-telopeptide (NTX) decreased in TE and showed a trend for decrease in MO when compared to PL. Urinary deoxypyrrodinoline (DPD) correlated significantly to serum NTX but did not change in any of the groups. Serum osteocalcin (OC) decreased significantly in TE but not in MO when compared to PL. There were no changes in bone-specific alkaline phosphatase (BSAP), intact-parathyroid hormone (iPTH), or calcitonin (CAL) in any of the groups. Bone mineral content (BMC) and density (BMD) did not change significantly in any of the groups.

Continuous but not monthly cycled testosterone treatment for 5 months resulted in a decrease in markers of bone resorption and bone formation. These data show beneficial effects of weekly testosterone therapy on markers of bone turnover in older men with low-normal endogenous testosterone concentrations.

INTRODUCTION

The risk for osteoporosis increases with age for both females and males. While the underlying factors leading to osteoporosis such as menopause vs. andropause differ between the genders, sex hormones play a major role in bone development and metabolism of both women and men. Both estrogen and testosterone have been shown to positively affect bone mass in elderly. Regardless of gender, aging is associated with a decrease in circulating levels of sex hormones, decreased bone mass, and high bone turnover as indicated by increased levels of biochemical markers of both bone resorption and bone formation (75, 119, 125). Hypogonadism, characterized by low serum testosterone levels, has been associated with diminished BMD in elderly males (115). The use of testosterone replacement is the current standard of care for improving muscle mass and strength among hypogonadal men (10, 13, 37, 38, 128). However, the long term

side effects of continuous testosterone administration are not fully known and can be associated with increased hematocrit levels and increased risks of prostate cancer (18). Therefore, alternate dosing regimens such as cycling the periods of testosterone treatment (i.e. one month on, one month off) could be favorable by both reducing the risks associated with continuous treatments as well as maximizing the initial stimulating properties of testosterone on muscle protein synthesis. Monthly cycled regimens would allow for normalization of the hypothalamic-pituitary-gonadal axis between the cycles of testosterone treatment. As serum testosterone levels are generally inversely associated with markers of bone resorption (71, 77), the simultaneous use of this androgen as an anti-osteoporotic agent would be an additional benefit to its therapeutic use.

Continuous testosterone replacement has shown positive outcomes on BMD in elderly hypogonadal men (1). The effects of testosterone administration on bone metabolism of elderly with low-normal endogenous testosterone concentrations are unclear but are expected to have at least a protective effect on existing bone mass. The potential beneficial effects of testosterone therapy on bone metabolism of elderly men could have clinical applications for both men and women in the prevention or treatment of age related osteoporosis. We investigated whether continuous and monthly cycled administration of testosterone would lead to improvements in markers of bone metabolism in older men with low-normal endogenous testosterone concentrations.

MATERIALS AND METHODS

Subjects

Males between age 60 and 85 were recruited through the Sealy Center of Aging Volunteers Registry of the University of Texas Medical Branch (UTMB). Informed

written consent was obtained from all subjects using consent forms approved by the UTMB Institutional Review Board (IRB). Subject characteristics are shown in Table 4.1.

Additional enrollment criteria included general good health as determined by a blood panel and heart stress test, and endogenous testosterone levels between 200-500 ng/dL at the time of enrollment. Qualified subjects were randomized into one of three double-blinded groups. These groups were to receive either continuous weekly intramuscular (IM) injections of testosterone (100 mg testosterone-enanthate) for the entire 5-month period (TE, n=4), monthly testosterone (one month testosterone/one month placebo, MO, n=4), or weekly placebo for the entire period (PL, n=5).

Experimental Procedures

The 5-month experimental protocol is outlined in (Figure 4.1). At month 0 of the 5-month protocol, subjects arrived at the GCRC for a baseline whole-body Dual Energy X-ray Absorptiometry (DEXA) scan. Collection of a 24 h urine pool was started at noon that same day. The following day, fasting blood samples were collected for the determination of serum markers of bone metabolism. Before discharge, the first injection dose (100 mg testosterone-enanthate or placebo) was administered. For the next 5 months, subjects returned weekly for administration of scheduled treatment injections and monthly for blood and urine sample collection as described above. Each month, subjects completed questionnaires for monitoring changes in mood, level of activity, and dietary preferences. Three-day dietary records were obtained at month 0, month 3, and month 5. Baseline blood samples were collected each month for the analysis of markers of bone metabolism. All samples were frozen (-80°C) until analysis. Serum n-telopeptide (NTX, Wampole Laboratories, NJ), bone specific alkaline phosphatase (BSAP, Quidel

Corporation, CA), and osteocalcin (Quidel Corporation, CA) were determined using commercially available ELISA kits. Serum calcitonin, intact parathyroid hormone (iPTH), and total testosterone were measured using an Immulite 2000 chemiluminescence immunoassay analyzer (DPC, Los Angeles, CA).

Urine

Urine was collected as a 24 h pool. Briefly, after discarding the first void and noting the time, all subsequent voids were collected in a single container. After 24 hours, a final void was collected to complete the 24 h pool. After thorough mixing, aliquots of this 24 h pool were frozen (-80°C) until analysis. Urinary deoxypyridinoline (DPD) was measured using a Pyrilinks®-D kit for the Immulite 2000 chemiluminescence immunoassay analyzer (DPC, Los Angeles, CA).

Dual Energy X-ray Absorptiometry (DEXA)

Whole body DEXA scans were obtained at month 0 and month 5 for the determination of lean body mass, fat mass, bone mineral content (BMC), and bone mineral density (BMD). BMC and BMD of the pelvis, lumbar spine (L1-L4) and forearm were also determined.

Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were evaluated by 2-way ANOVA with repeated measures followed by a post-hoc Dunnett's test, 2-sided. Correlations between selected measurements were determined using the

nonparametric Spearman's rho Correlation Coefficient (ρ). $P \leq 0.05$ was considered statistically significant.

Table 4.1: Subject Characteristics.

Subject Characteristics at Month 0. Subject characteristics of all subjects at the beginning of the study protocol. There were no differences between any of the groups at month 0.

	PL (n=5)	MO (n=4)	TE (n=4)
Age (yrs)	66.1 ± 1.6	68.1 ± 3.1	73.2 ± 4.3
Weight (kg)	97.0 ± 10.5	86.2 ± 6.5	89.3 ± 1.5
BMI (kg/m^2)	31.7 ± 2.9	28.9 ± 2.1	27.0 ± 0.6
Lean Body Mass (kg)	63.5 ± 5.5	60.5 ± 3.0	60.8 ± 2.6
Bone Mineral Density (BMD) (g/cm^2)	1.125 ± 0.067	1.114 ± 0.093	1.184 ± 0.073
Serum Total Testosterone (ng/dL)	385 ± 34	373 ± 56	321 ± 34
Serum Estradiol (pg/mL)	36 ± 4	28 ± 3	27 ± 2

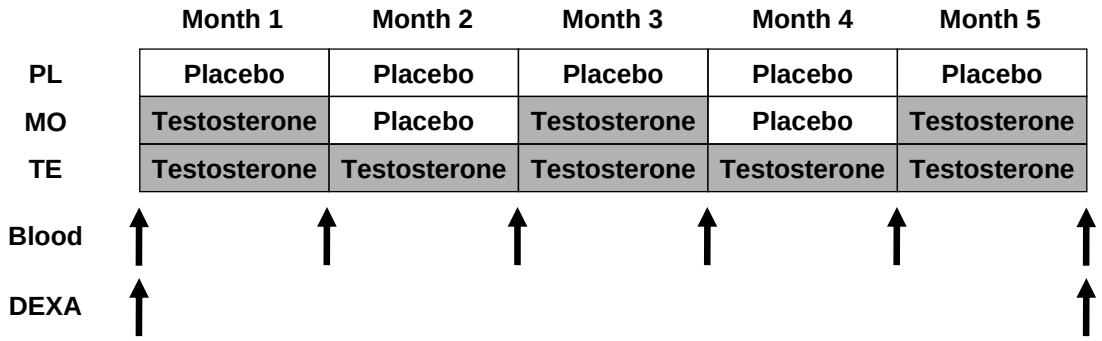


Figure 4.1: Study Timeline.

Older men completed a 5-month randomized blinded placebo controlled protocol. Subjects received either continuous weekly testosterone (100 mg testosterone-enanthate, IM injection) for the entire 5-month period (TE, n=4), monthly testosterone treatment (one month testosterone/one month placebo, MO, n=4), or weekly placebo for the entire period (PL, n=5). Whole-body and regional DEXA scans were obtained at baseline and after 5 months for measurements of lean mass and BMD.

RESULTS

Serum Testosterone and Estradiol

There were no differences in endogenous testosterone concentrations between the groups at month 0 (Table 4.1). Total testosterone was increased at month 1 in the TE group and remained elevated through month 5 (Figure 4.2). In the MO group, total testosterone increased each month following testosterone injections and returned to baseline within one month after receiving placebo.

There were no differences in serum estradiol between any of the groups at month 0 (Table 4.1). Serum estradiol concentrations followed monthly patterns similar to those of total testosterone but showed more month to month variation (Figure 4.3). Estradiol concentrations correlated strongly to total testosterone concentrations when values from all subjects were pooled ($\rho = 0.583$, $P<0.0001$).

Markers of bone resorption

Markers of bone resorption are shown in Table 4.2. Serum NTX decreased over the 5 month treatment period in TE (-23 ± 4%, $P<0.01$) but not significantly in MO (-17 ± 7%, $P=0.07$) when compared to PL (-3 ± 4%) (Figure 4.4). Urine DPD correlated significantly to serum NTX concentrations ($\rho = 0.333$, $P\leq0.01$) but did not change significantly in any of the groups. Serum NTX concentrations tended to correlate inversely to total testosterone concentrations ($\rho = -0.197$, $P=0.086$) when values from all subjects were pooled. Serum iPTH concentrations stayed within normal ranges and did not change in any of the groups.

Markers of bone formation

Markers of bone formation are shown in Table 4.2. Serum osteocalcin decreased in TE (-28 ± 2%, $P<0.05$), but not in MO (6 ± 3%) when compared to PL (-1 ± 5%) (Figure 4.5). Serum osteocalcin correlated negatively to total BMD ($\rho=-0.259$, $P<0.05$) and pelvic BMD ($\rho = -0.258$, $P<0.05$). BSAP did not change in any of the groups but was correlated negatively with total testosterone ($\rho=-0.294$, $P<0.01$). Serum calcitonin stayed within normal physiological ranges and did not change in any of the groups.

Dual Energy X-ray Absorptiometry (DEXA)

Lean body mass increased in TE (8 ± 2%, $P<0.05$) but not in MO (2 ± 5%) when compared to PL (-2 ± 1%). There were no significant changes in BMD between any of the groups at any time. (Table 4.3). Pelvic BMD tended to increase slightly in TE and correlated positively with serum estradiol ($\rho=0.235$, $P<0.05$) when data from all subjects were pooled. While not significant, there was a trend towards decreased total BMC and total BMD in the MO group when compared to PL.

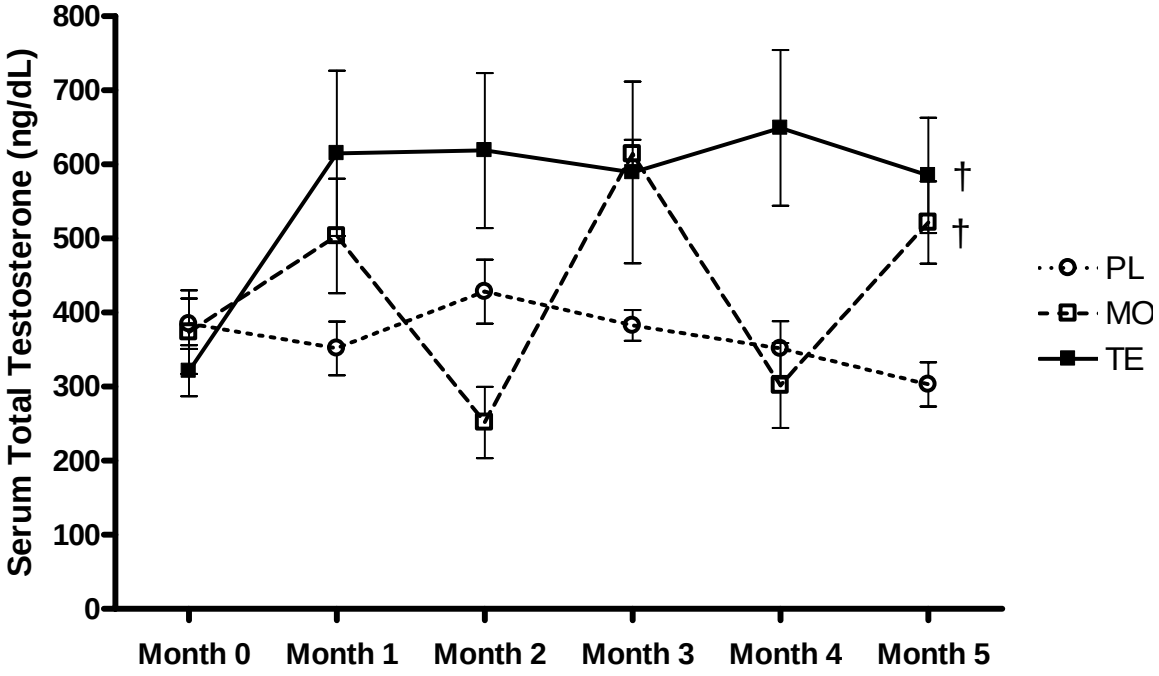


Figure 4.2: Serum Total Testosterone.

Serum Total testosterone concentrations increased at month 1 in the TE group and remained elevated through month 5. In the MO group, total testosterone increased each month following testosterone injections and returned to baseline within one month after receiving placebo. Total testosterone concentrations did not change in PL at any month. † Significantly different from PL, two-way ANOVA with repeated measures, $P<0.05$.

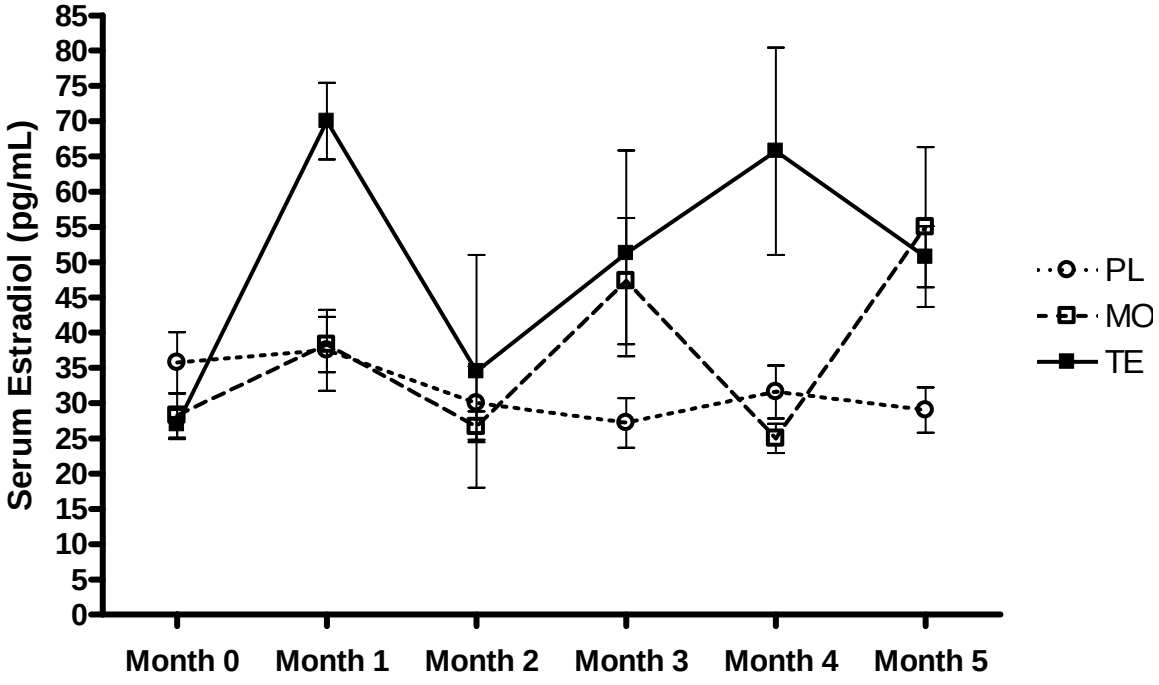


Figure 4.3: Serum Estradiol.

There were trends towards increases in estradiol in the TE and MO groups over time. Serum estradiol concentrations did not change in PL at any month.

Table 4.2: Markers of Bone Metabolism.

Markers of bone resorption (serum NTX, urine DPD, serum iPTH, serum calcitonin) and bone formation (serum BSAP, serum osteocalcin) are shown for each month. All values stayed with normal physiological ranges in all groups.

		Month 0	**Month 1**	**Month 2**	**Month 3**	**Month 4**	**Month 5**
Serum	PL	13.48± 1.21	13.00± 1.79	11.84± 1.04	12.96± 1.62	12.31± 1.14	12.99± 0.96
NTX	MO	14.25± 1.26	10.90± 1.99	12.96± 1.25	10.06± 1.40	12.27± 2.46	11.59± 0.37
(nM BCE)	TE	14.92± 3.44	11.60± 3.56	13.47± 2.90	12.52± 2.01	10.38± 2.53	11.35± 2.40
Urine	PL	51.17± 7.87	49.26± 5.99	41.29± 10.91	46.91± 10.42	53.99± 6.90	47.45± 10.52
DPD	MO	32.13± 4.73	37.07± 4.25	42.28± 14.21	28.40± 4.53	40.01± 6.53	42.09± 8.97
(nmol/day)	TE	33.38± 4.08	27.76± 3.27	29.21± 4.61	26.10± 2.26	36.35± 7.57	29.22± 6.31
Serum	PL	23.34± 0.64	25.33± 1.71	24.80± 2.89	32.71± 5.58	25.35± 4.10	22.44± 0.40
BSAP	MO	22.02± 4.15	23.16± 2.50	18.08± 2.91	20.13± 2.92	23.77± 6.45	21.31± 3.61
(U/L)	TE	18.49± 1.84	17.75± 1.94	16.90± 1.17	16.89± 1.35	15.50± 1.28	21.48± 5.30
Serum	PL	7.74± 0.15	8.00± 0.81	8.29± 0.52	8.06± 0.50	8.40± 1.04	8.16± 0.50
Osteocalcin	MO	7.59± 0.66	7.47± 1.07	8.32± 1.02	8.56± 0.71	7.50± 0.80	8.09± 0.79
(ng/mL)	TE	8.50± 1.19	8.29± 1.34	8.23± 1.35	6.86± 1.52	7.32± 1.25	6.17± 0.93
Serum	PL	40.06± 3.74	39.46± 4.84	36.16± 3.46	38.90± 5.17	33.46± 4.35	39.54± 6.56
iPTH	MO	32.13± 6.42	37.48± 5.84	26.43± 12.15	30.30± 7.19	37.28± 4.88	26.75± 5.76
(pg/mL)	TE	35.45± 9.12	26.88± 6.36	37.40± 11.70	32.98± 7.00	31.78± 10.05	33.36± 9.24
Serum	PL	3.54± 0.34	2.48± 0.40	2.47± 0.25	2.58± 0.39	2.64± 0.40	2.40± 0.25
Calcitonin	MO	3.90± 1.08	3.12± 0.62	2.92± 1.53	5.63± 2.25	3.10± 0.51	3.51± 0.68
(pg/mL)	TE	2.70± 0.70	3.29± 1.29	2.63± 0.63	3.03± 1.03	3.00± 1.00	2.63± 0.63

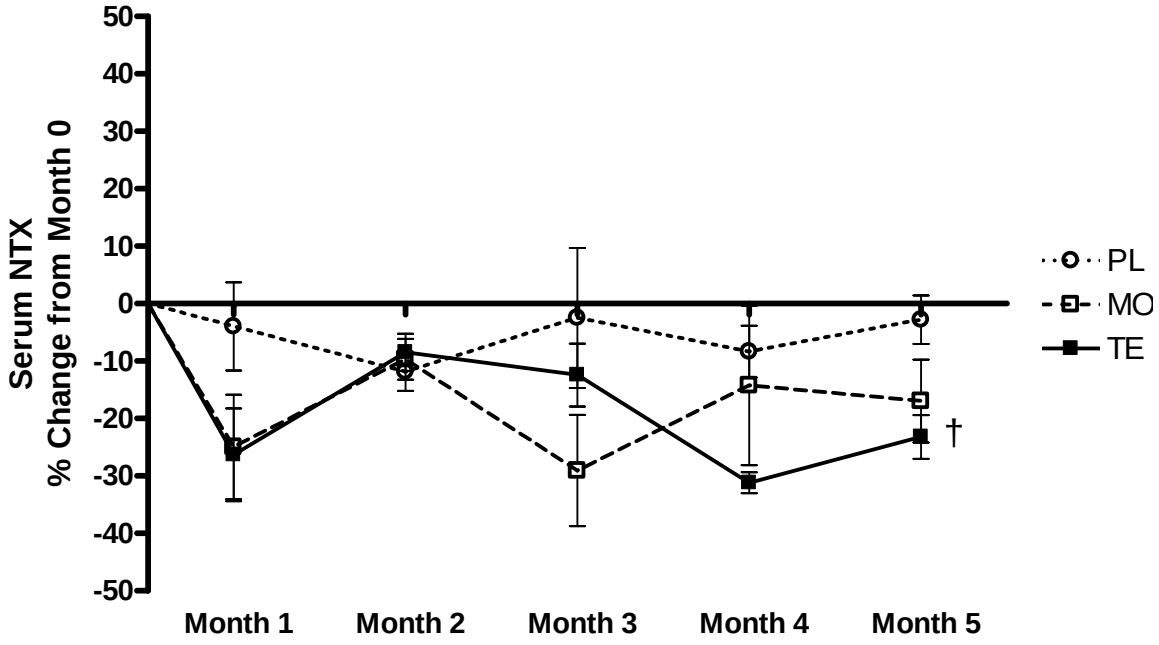


Figure 4.4: Changes in Serum NTX.

Over the 5 month period there was a significant decline in serum NTX in the TE group ($P<0.05$) and a trend for a decrease in the MO group ($P=0.07$). There were no changes in serum NTX in PL ($P=0.54$). † Significantly different from PL, two-way ANOVA with repeated measures, $P<0.05$.

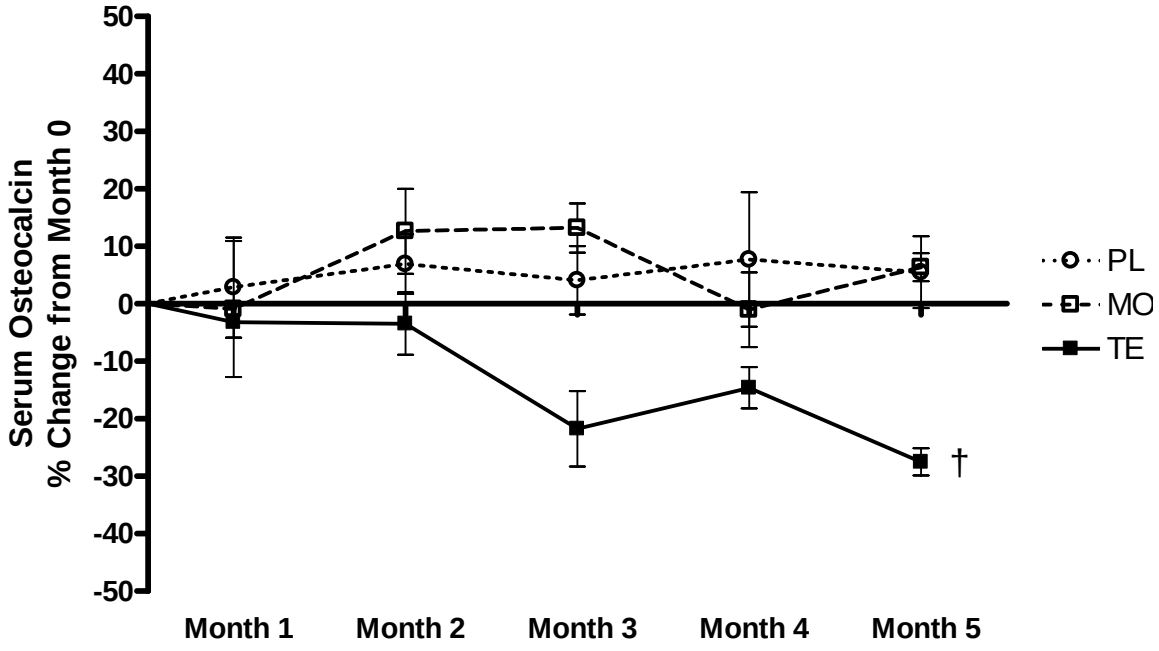


Figure 4.5: Changes in Serum Osteocalcin.

Over the 5 month period there was a decrease in serum osteocalcin in the TE group ($P<0.05$) but not in MO when compared to PL. † Significantly different from PL, two-way ANOVA with repeated measures, $P<0.05$.

Table 4.3: Bone DEXA.

‡ Significantly different from Month 0.

		Month 0	Month 5
Total	PL	1.125± 0.067	1.095± 0.071
BMD	MO	1.114± 0.093	1.032± 0.082‡
(g/cm^2)	TE	1.184± 0.073	1.154± 0.041
Pelvic	PL	1.344± 0.216	1.317± 0.212‡
BMD	MO	1.186± 0.057	1.115± 0.046‡
(g/cm^2)	TE	1.342± 0.047	1.382± 0.055
Forearm	PL	0.580± 0.018	0.587± 0.020
BMD	MO	0.633± 0.039	0.642± 0.041
(g/cm^2)	TE	0.633± 0.020	0.611± 0.016
Lumbar Spine (L1-L4)	PL	1.10± 0.11	1.08± 0.10
BMD	MO	1.01± 0.08	1.03± 0.08
(g/cm^2)	TE	1.17± 0.05	1.17± 0.05
Lumbar Spine (L1-L4)	PL	0.08± 1.04	-0.06± 0.93
T-score	MO	-0.73± 0.77	-0.60± 0.70
	TE	0.73± 0.45	0.73± 0.42

DISCUSSION

Our results indicate that continuous weekly testosterone administration (100 mg IM) in elderly men results in a reduction of biochemical markers of both bone resorption and bone formation. However, monthly cycled administration of testosterone did not result in similar changes. To our best knowledge this is the first report of such differences in responses in biochemical markers of bone metabolism following different testosterone dosing regimens.

There was a trend towards a negative correlation between serum testosterone and serum NTX concentrations among all our subjects and treatments (ρ=-0.197, P=0.086). Serum testosterone levels are generally inversely associated with markers of bone resorption. Negative correlations between bioavailable testosterone and n-telopeptide (NTX) concentrations in both urine and serum have been reported in elderly men aged 60-90 years (71). Administration of continuous biweekly testosterone injection (200 mg IM) in elderly men resulted in a significant decrease in urinary NTX excretion after 6 months. Induction of hypogonadism by administration of a GnRH analog to healthy 22-40 year old men resulted in increases in urinary NTX, urinary deoxypyridinoline (DPD), as well as serum NTX levels in as early as 4 weeks compared to baseline values (77). In that study the increase in serum NTX was blunted by coadministration of testosterone. In our study, serum NTX, but not urinary excretion of DPD, decreased sharply in the group receiving weekly testosterone injections (100 mg) continuously for 5 months.

There was a trend for a decrease in serum NTX in the monthly cycled group which did not reach significance (P=0.07), possibly due to our relatively small number of subjects studied. Urinary DPD excretion correlated directly to serum NTX concentrations (ρ=0.333, P$\leq$0.01) although urinary DPD excretion did not change significantly in any of

the groups. Although the detection of bone collagen crosslinks in urine is the more widely used method in clinical practices, it must be pointed out that serum concentrations are viewed as more reliable and less susceptible to intra-individual variation (32, 35, 126). Nevertheless, the positive correlation between our measurements of serum and urine concentrations of bone collagen crosslinks supports the use of either for clinical purposes.

The effects of sex hormones on markers of bone formation are complex and may be age dependent. Positive associations between serum total testosterone levels and concentrations of osteocalcin were found in young (22-39 yr) and middle aged (40-59 yr) subjects but not among elderly (60-90 yr) (71, 129). Elderly prostate cancer patients receiving a hypogonadic GnRH analog treatment experienced increases in osteocalcin and bone specific alkaline phosphatase (BSAP) levels after 6 months of treatment (88). Additionally, administration of continuous biweekly testosterone injections (200 mg) in elderly men significantly decreased BSAP after 6 months (1).

In the present study, serum osteocalcin decreased in the TE group ($P<0.05$) but not the MO group (Figure 4.5). Additionally, although we found no changes in BSAP in any of the groups, there was a significant negative correlation between serum BSAP and serum testosterone concentrations ($\rho=-0.294$, $P\leq0.01$). While not conclusive, these findings are very consistent with the generally accepted notion that low levels of circulating androgens are associated with increased levels of bone turnover in elderly. However, the reports on the effects of androgen replacement on bone formation vary widely and may be due to numerous factors including subject age (young vs. old), type of steroid used (i.e. testosterone, DHEA, oxandrolone), route of administration (i.e. topical vs. injection), duration of treatment (i.e. weeks to years), and endogenous testosterone

production (i.e. hypogonadal vs. low normal). Testosterone, as well as estrogen, appears to play crucial regulatory role during bone remodeling in elderly by promoting a balance between the processes of bone resorption and formation. The inhibitory effect of sex hormones on bone turnover has been shown by overall increases in processes of both bone resorption and bone formation in older men and women (43, 68, 75, 119, 121, 125). An increase on both sides of the bone-turnover fulcrum generally results in situations where the increase in bone resorption wins out over the associated, but lagging, increases in bone formation. Garnero et al. reported that compared to premenopausal women, the increase in bone resorption is much higher that the increases in bone formation in postmenopausal women (37-52% for markers of bone formation vs. 79-97% for markers of bone resorption) (43). Because the faster process of bone resorption precedes the slow action of bone formation at each bone turnover site by several weeks, an increase in both processes increases the chance of losses in structural integrity, especially in trabecular bone.

Administration of continuous biweekly testosterone injections (200 mg) in elderly men has been reported to significantly increase BMD in the lumbar spine and trochanteric region after 6 months (1). Conversely, a negative association between serum free testosterone and spinal BMD has been reported in other studies (30). While total hip or lumbar spine BMD is widely used as a predictor for fracture risk in women and men, wrist BMD has been proposed as an alternative site of interest among men (87). We did not find changes in wrist or total forearm BMD in any of the groups. We did find subtle but significant decreases in total BMC and BMD in the MO group and in pelvic BMD in both the PL and MO groups. Conversely, there was a slight trend towards increased pelvic BMC and BMD in the TE group. These findings suggest that while continuous

testosterone administration has some protective properties on existing bone, monthly cycled administration may be insufficient in preventing a gradual net loss of BMD over time. However, even continuous weekly testosterone administration did not result in any significant increases in BMC or BMD at any of the sites measured.

The marked difference in the response to testosterone between TE and MO are difficult to interpret. It is possible that a dose-related response was observed, where higher total testosterone administration resulted in a greater anti-resorptive effect. It is important to point out that when viewed over the entire 5 month treatment period the monthly testosterone group (MO) received only half the amount of testosterone when compared to the continuous testosterone group (TE). Alternatively, there may have been a disregulation in bone turnover in the MO group due to the repeated exposure and subsequent removal of increased levels of testosterone. It is possible that bone requires a minimum exposure time to increased concentrations of androgens for a coordinated response in bone metabolism to take place. Further research is required in order to elucidate the mechanisms behind such possible timing related effects of androgens on bone metabolism.

Finally, the indirect effects of increased muscle mass on bone formation due to increased mechanical loading should not be ignored. While we encouraged our subjects to refrain from making any drastic changes in their daily habits we did not actively control for their levels of activity. Musculoskeletal loading is considered one of the important factors affecting bone mass. Indeed, lean body mass increased in the TE group. Therefore it is possible that the some of the positive effects of testosterone on bone turnover was through the stimulatory effects on muscle protein synthesis, muscle mass, and overall physical activity.

In conclusion, continuous weekly administration of testosterone for 5 months resulted in reductions in markers of bone turnover as indicated by a decline in selected markers of both bone resorption and bone formation. Monthly cycled administration of testosterone was insufficient to elicit a similar response within this time frame. While there were no marked changes in bone mineral content or bone mineral density during the 5-month treatment period in any of the groups, long-term testosterone therapy may be beneficial in slowing the progression of age related high-turnover bone loss through a reduction in bone turnover.

ACKNOWLEDGEMENTS

This study was supported by NIH Grant R01 AG022023 awarded to R.J. Urban. Studies were conducted on the GCRC at UTMB, funded by Grant M01 RR 00073 from the National Center for Research Resources, NIH, USPHS.

CHAPTER 5

SUMMARY

The studies presented herein were designed to investigate distinct aspects associated with the aging process. The findings from these studies have potential applications that reach far beyond the field of aging research, such as development of noninvasive diagnostic tools for the early detection and prevention of type-2 diabetes mellitus (T2DM), and the development of nutritional and pharmacological therapies for the prevention of muscle atrophy and bone loss during states of disease, long-term bedrest, and spaceflight.

Noninvasive methods for the early detection of insulin resistance are a welcome alternative to the current methods involving numerous blood draws during a standard OGTT. This study demonstrated that that glucose-derived breath CO_2 kinetics measured during the immediate post-glucose ingestion period (0.5 to 3.5 h) may assist in recognition of undiagnosed IGT in at-risk individuals during the pre-diabetes stage of T2DM. Following a baseline sample, the optimal time for the following breath collection was between 1 and 2.5 hours after the oral glucose load, similar to the timeframe of a standard OGTT. Both the area under the curve, when multiple measurements are made over time, and the slope when only pre- and post measurements are available, were shown to be effective. Furthermore, the results obtained with the noninvasive breath test are highly correlated to several indices of insulin resistance, including the WBISI, HOMA-IR, and QUICKI. Continued development of this application will have tremendous potential applications in the diagnosis of T2DM in children. However, further research is necessary to validate this method as a viable diagnostic tool that can be

used in addition, or in place of, existing methods. Such studies might focus on larger scale clinical studies, validating the breath test against the "Gold Standard" hyperinsulinemic-euglycemic clamp, establishing firm cut-off values between true NGT and IGT individuals, determining within-subject variability and repeatability, elucidating the effects of dietary intake on breath test results, and the exploring the applicability of this method in pediatric care.

Essential amino acids (EAA) are acutely anabolic to muscle protein synthesis in healthy elderly. We investigated whether twice daily between-meal oral supplementation of EAA for three months impacted the magnitude of the acute muscle protein synthetic response to an oral bolus of 7.5 g EAA. In conclusion, twice daily between meal supplementation of essential amino acids does not alter the anabolic response after the acute ingestion of an essential amino acid load in healthy older women and men. These data support the rationale for establishing targeted nutritional interventions, such as leucine enriched supplements, in aging and other populations undergoing progressive losses in lean body mass. Additionally, our results emphasize the need for further research aimed elucidation of the pathways through which amino acids mediate protein metabolism in elderly.

The risk for osteoporosis increases with age for both females and males. While the underlying factors leading to osteoporosis such as menopause vs. andropause differ between the sexes, sex hormones play a major role in bone development and metabolism for both women and men. This study showed that administration of testosterone for 5 months resulted in reduced bone turnover as indicated by a decline in markers of both bone resorption and bone formation. There was a potential dose dependent response to testosterone as continuous therapy induced a greater reduction in bone turnover than

monthly cycled therapy. While there were no marked changes in bone mineral density during the 5-month treatment period in any of the groups, long term testosterone therapy may prove beneficial in slowing the progression of age related high-turnover bone loss. Continued research is needed to determine the long-term effects of different testosterone dosing regimens on the reduction in risk of osteoporosis in both elderly men and women.

Collectively the results presented show tremendous potential for the development of diagnostic tools and treatment therapies in fields such as diabetes, sarcopenia, and osteoporosis. While the focus of the studies presented was largely on aging research, applications based on these methods and therapies can potentially be applied to a wide range of populations. Finally, while each of these studies focused on an isolated aspect within the physiology of aging, it must be understood that the interactions between these systems should not be ignored. Continued research focused on elucidating the interactions between the underlying mechanisms that lead to insulin resistance, muscle atrophy, and bone loss is essential for the development of successful therapies.

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VITA

Edgar Lichar Dillon was born on January 19, 1972 in Longview, TX to Lilian Visser and Charles Raymond Dillon. They moved to Amsterdam, The Netherlands, where he grew up with his two younger brothers, Rayan and Carey, and younger sister Lilian. Lichar graduated from high school at the Scholengemeenschap Reigersbos with his VWO diploma in 1992. He subsequently moved to College Station, TX to attend Texas A&M University where he joined the Corps of Cadets. Lichar graduated in 1997 receiving his B.S. in Nutritional Sciences. Lichar continued his education at Texas A&M University and earned an M.S. degree in Nutrition in 1999.

Following graduation, Lichar worked as a Nutritionist with Women, Infants, and Children (WIC) in Amarillo, TX. In 2000, he obtained a position as nutritional biochemist with Enterprise Advisory Services, Inc at the National Aeronautics and Space Administration (NASA) Johnson Space Center in Houston, TX. In his position at the Nutritional Biochemistry Laboratory he worked on research investigating nutritional status alterations and bone loss in astronauts during longterm spaceflight.

In 2002, while maintaining his position at the NASA Johnson Space Center, Lichar entered the Graduate School of Biomedical Sciences (GSBS), at the University of Texas Medical Branch (UTMB) as a part-time student in Cell Biology. Lichar married Alyssa Ham on July 19, 2003 in Amarillo, TX. Lichar officially joined the laboratory of Dr. Melinda Sheffield-Moore as a full time graduate student in 2006. He was awarded the Virendra B. Mahesh Award for Excellence in Endocrinology from the American Physiological Society in 2007 and Best Student Poster Awards from the UTMB Sealy Center of Aging in 2006 and 2007.

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PUBLICATIONS

Manuscripts

1. Sheffield-Moore M, Paddon-Jones D, Gilkison C, Sanford AP, Casperson SL, Jiang J, **Dillon EL**, Chinkes DL, and Urban RJ. Chronic amino acid supplementation increases lean body mass, basal muscle protein synthesis, and IGF-1 expression in elderly. J Clin Endocrinol Metab (2nd revision, In review), 2007.

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