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Effects of Leucine on Skeletal Muscle During 14 d Bed Rest in Middle-aged Adults

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Effects of Leucine on Skeletal Muscle During 14 d Bed Rest in Middle-aged Adults

by

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Dedication

To my dad, who daily modeled the character, excellence, and work ethic necessary for any great achievement, exceeded the highest standard of a father, and whom I daily try to emulate

To my mom: “As iron sharpens iron…”

To Charity, who selflessly supported this endeavor with very real daily effort; you were not only integral to this achievement, it would be hollow without you to share it with…I love you

To Isabella: Пойдем кататься на велосипедах

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Effects of Leucine on Skeletal Muscle During 14 d Bed Rest in Middle-aged Adults

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Supervisor: Douglas Paddon-Jones

Aging is associated with a slow, progressive loss of muscle mass and strength. Mechanical unloading, such as that commonly experienced during hospitalization or spaceflight, results in a rapid loss of muscle mass and strength, particularly in older adults. Exercise, a potent countermeasure to such losses, is often impractical in acutely ill patients. The essential amino acid leucine has been shown to acutely stimulate muscle protein synthesis (MPS), a decrease in which mechanistically drives inactivity-induced losses in muscle. This study evaluated the effects of leucine supplementation (0.06 g · kg · meal^{-1}; LEU) with each of three daily meals in middle-aged adults, a largely unstudied age group, during 14 d bed rest (BR) and subsequent 7 d rehabilitation. Primary findings were: 1) leucine attenuated the loss of whole body lean mass during the first 7 d of BR compared to control subjects (LEU: -0.6±0.2 kg vs. CON: -1.1±0.2 kg, $p<0.05$) and reduced or prevented decrements in knee extensor strength (LEU: -8±3% vs. CON: -15±3%, $p<0.05$), ankle extensor strength (LEU: -13±5% vs. CON: -20±5%, $p<0.05$), and knee extensor endurance (LEU: -2±4% vs. CON: -14±3%, $p<0.05$) during 14 d BR; 2) LEU maintained both post-absorptive and post-prandial MPS during BR; in contrast, BR decreased post-absorptive MPS (pre-BR: 0.061% · h^{-1} vs. post-BR: 0.043% · h^{-1}, $p<0.05$); 3) insulin area under the curve during an oral glucose tolerance test was unchanged in LEU after BR (21±8%) but elevated in CON (52±23%, $p<0.05$) and whole body insulin sensitivity in LEU was significantly increased above pre-BR values after 7 d rehabilitation (17±10% vs. CON: -9±9%, $p<0.05$). Leucine is an inexpensive, low volume supplement that can be easily incorporated into the daily meals of middle-aged adults to maintain muscle protein synthesis and protect muscle mass, strength, and insulin sensitivity during periods of physical inactivity characteristic of hospitalized acute illness and spaceflight.

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List of Abbreviations

1-g	Earth's gravitational acceleration; 9.81 $m \cdot s^{-2}$
4E-BP1	eukaryotic initiation factor 4E binding protein 1
ADL	activities of daily living
AF	activity factor
Akt	protein kinase B
ARED	Advanced Resistive Exercise Device
AUC	area under the curve
BCAA	branched chain amino acid
BF	body fat
BMI	body mass index
BR	bed rest
CEVIS	Cycle Ergometer with Vibration Isolation System
CHO	carbohydrate
CON	control (alanine-supplemented) group
CSA	cross-sectional area
DEXA	dual energy x-ray absorptiometry
DM2	Type II diabetes mellitus
EAA	essential amino acid
eIF4E	elongation initiation factor 4E
eIF4G	elongation initiation factor 4G
ESA	European Space Agency

FSR	fractional synthetic rate
FWED	flywheel ergometer exercise device
GCMS	gas chromatograph mass spectrometer
GLUT4	glucose transporter type 4
iRED	interim Resistive Exercise Device
ISS	International Space Station
IRS-1	insulin receptor substrate 1
ITS-CRC	Institute for Translational Sciences-Clinical Research Center
kcal	kilocalorie
LEU	leucine-supplemented group
LLM	leg lean mass
MHC	myosin heavy chain
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRI	magnetic resonance spectroscopy
mTOR	mammalian target of rapamycin
NASA	National Aeronautics and Space Administration
OGTT	oral glucose tolerance test
PI3K	phosphatidylinositol 3-kinase
RDA	recommended daily allowance
RER	respiratory exchange ratio
S6K1	70-kDa ribosomal protein S6 kinase 1
SAID	Specific Adaptation to Imposed Demands

SD	standard deviation
SE/SEM	standard error of the mean
SMI	skeletal muscle mass index
STS	Space Transportation System
T2	Treadmill 2
TVIS	Treadmill with Vibration Isolation System
UTMB	University of Texas Medical Branch
WBFM	whole body fat mass
WBISI	whole body insulin sensitivity index
WBLM	whole body lean mass

Chapter 1: Introduction[1]

"Think long. Write short." –George Lois

AGING

Sarcopenia is an age-related, multi-factorial process that is phenotypically characterized by the loss of lean tissue mass. To draw attention to this serious consequence of aging, Rosenberg coined the term in 1989 which comes from the Latin words 'sarco' and 'penia,' literally meaning, "poverty of the flesh" (Rosenberg 1989). The onset of sarcopenia is insidious, but its progression may be greatly accelerated by physical inactivity and poor nutrition. The basic descriptors of sarcopenia are well known. After the age of 30, adults lose 3-8% of their muscle mass per decade. Over time, the loss of lean tissue contributes to a decrease in muscle strength and power which are important predictors of balance (Lord et al. 1994, Hess et al. 2006), the occurrence of falls (Wolfson et al. 1995) and mortality (Manini et al. 2007). Sarcopenia is highly prevalent in American older adults; approximately 20% of community-dwelling elders < 70 years old and more than 50% of those over 80 years of age can be characterized as sarcopenic (> 2 SD below the appendicular skeletal muscle mass (kg)/height (m^2) of a young reference population) (Baumgartner et al. 1998).

This natural, age-associated loss of muscle mass is problematic for several reasons. Most obviously, as a contractile tissue, only skeletal muscle is capable of voluntary force production—a capability that is essential for mobility, the performance of activities of daily living (ADL), and indeed, independent living. Loss of muscle mass is

[1] Excerpts from English KL and Paddon-Jones D. Protecting muscle mass and function in older adults during bed rest. *Curr Opin Clin Nutr Metab Care* 2010, 13(10): 34-39. Used by permission. Promotional and commercial use of this material in print, digital, or mobile device format is prohibited without permission from the publisher Lippincott Williams & Wilkins.

associated with falls (de Rekeneire et al. 2003, Lord et al. 1994, Moreland et al. 2004, Rubenstein 2006), frailty, functional decline (Bendall et al. 1989, Fiatarone and Evans 1990, Rantanen et al. 2001, Vellas et al. 1992), and death (Landi et al. 2012). Falls have been specifically linked to a loss of muscle power—the ability to produce force over a given unit of time (Hess et al. 2006). Thus, the inability to produce force quickly (i.e., to produce adequate power) has been shown to be a cause of falls in the elderly. Skeletal muscle is also a major contributor to whole body metabolism; it is the largest source of amino acids which are essential to the synthesis of new proteins, a primary target of insulin, it stores glucose in the form of glycogen, and oxidizes both glucose and free fatty acids from the systemic circulation. Substantial decreases in muscle mass can have deleterious consequences on systemic homeostasis and lead to the onset of metabolic disorders such as diabetes mellitus (Clarke 2004).

In 2011, the American population was 311 million people of which 41 million were > 65 years old (13% of total population) (U.S. Census Bureau). By the year 2025, the number of senior citizens will increase to 65 million people (19% of total population), and by 2050 they will have more than doubled (84 million, 21% of total population) their current number (U.S. Census Bureau); indeed, every day 4,500 more Americans reach the age of 65 (U.S. Census Bureau). This is anticipated to put an unprecedented strain on our healthcare system (Caspersen et al. 2012, Janssen et al. 2004, Smith et al. 2013). Older age is associated with increased hospitalization which, in turn, is highly associated with decreased mobility, functional capacity (Visser 2011), ability to perform ADLs (Corona et al. 2013), and ultimately, with a loss of independent living (Han et al. 2013). During inactivity, older adults lose muscle mass much more rapidly than their younger counterparts (English and Paddon-Jones 2010, Kortebein et al. 2007, Paddon-Jones et al. 2004). This, coupled with incomplete recovery following hospital discharge results in greater muscle loss over time, a phenomenon dubbed the ‘Catabolic Crisis Model’

(English and Paddon-Jones 2010). Thus, it is imperative that interventions are developed and implemented that will prevent these acute, episodic losses of muscle mass.

Aging *per se* also appears to facilitate inactivity-mediated muscle loss (Kortebein et al. 2007). The consequences of accelerated muscle loss in bed-ridden elders may be further complicated by the fact that approximately 71% of male and 42% of female older Americans (≥ 65 y) can already be characterized as moderately sarcopenic based on skeletal muscle index [SMI = muscle mass (kg)/height (m^2); males: 8.51-10.75; females: 5.76-6.75] (Janssen 2006). Further, 17% and 11% of older men and women, respectively, are severely sarcopenic (males: SMI < 8.51; females SMI < 5.76). Severe sarcopenia is also associated with a 79% greater likelihood of disability (Janssen 2006). Thus, with advancing age, it becomes increasingly likely that even a brief, clinically mandated period of bed rest could initiate a serious decline in muscle strength and functional capacity, i.e., a "tipping point" from which some may not fully recover (Covinsky et al. 2003, Hirsch et al. 1990, Visser et al. 2000). It is essential to note the clear conceptual distinction between the traditional, insidious sarcopenic process and the accelerated episodic loss of muscle and functional capacity during a "catabolic crisis;" this distinction is highlighted in Figure 1.

The fundamental mechanisms of sarcopenia are not well understood. Protein breakdown does not appear to be elevated in healthy older adults (Volpi et al. 2001) and investigators have been largely unable to identify differences in basal (post-absorptive) muscle protein synthesis (MPS) between young and elderly subjects (Cuthbertson et al. 2004, Volpi et al. 2001). However, a number of studies have documented a diminished anabolic response in elderly subjects to feeding (Cuthbertson et al. 2004, Katsanos et al. 2005, Katsanos et al. 2006, Volpi et al. 2000) and exercise (Kosek et al. 2006), both potent anabolic stimuli. The diminished response to feeding has been termed "anabolic resistance" (Phillips et al. 2009, Rennie et al. 2010); this is possibly the mechanism underlying the sarcopenia of aging.

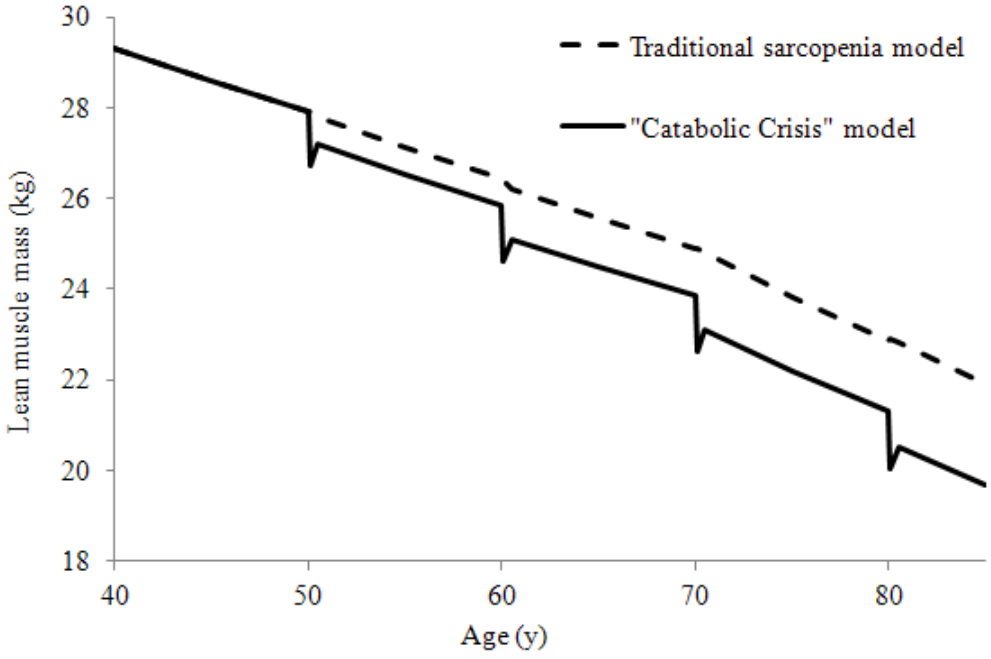


Figure 1. Proposed model of age-related muscle loss punctuated by episodes of acute inactivity (e.g., hospitalization) and characterized by accelerated muscle loss and incomplete recovery. Used by permission. (English and Paddon-Jones 2010)

Muscle loss during bed rest (BR) appears to be driven primarily by a reduction in muscle protein synthesis (Kortebein et al. 2007, Paddon-Jones et al. 2006, Symons et al. 2009). However, while it appears that muscle protein breakdown is largely unaltered by BR in young adults (Ferrando et al. 1996, Symons et al. 2009), we are lacking corresponding data in older populations. Although an increase in muscle protein breakdown would be consistent with the accelerated loss of muscle mass in older adults, anabolic resistance (Phillips et al. 2009, Rennie et al. 2010) or a blunted protein synthetic response to mixed nutrient meals is likely to play a much greater role (Volpi et al. 2000). Although not studied during BR, Volpi et al. examined muscle protein synthesis, breakdown, and amino acid transport in young and older adults following administration of a simulated protein/carbohydrate mixed meal (40 g amino acids, 40 g glucose) (Volpi

et al. 2000). Consistent with the *anabolic resistance* hypothesis, muscle protein breakdown did not change in either age group, however, muscle protein synthesis increased only in the young. Supporting data have also been reported by researchers using a 14 day unilateral knee immobilization protocol (Glover et al. 2008). In a cohort of young healthy subjects, post-absorptive, basal muscle protein synthesis in the vastus lateralis of the immobilized leg fell by 27%. Further, after 14 days the non-immobilized leg had up to a 68% greater increase in muscle protein synthesis than the immobilized leg in response to increased amino acid availability. The authors conclude that the decrease in post-absorptive muscle protein synthesis coupled with an anabolic resistance to feeding causes much of the muscle loss that occurs during immobilization (Glover et al. 2008).

SPACEFLIGHT

While aging is a universal phenomenon that effects muscle loss, the microgravity exposure of spaceflight represents a unique unloaded environment that elicits acute decreases in muscle mass and muscle strength. Human space flight began on April 12, 1961 with Soviet Union cosmonaut Yuri Gagarin's solo orbital flight; less than a month later on May 5, 1961, Alan Shepard became the first American in space. The remainder of the decade was filled with humans traveling to space, climaxing on July 20, 1969 with the Apollo 11 Moon landing. However, all of the 1960s space missions were less than two weeks in duration.

In 1971, the Soviet Union launched Salyut 1, its first orbiting space station which was later manned for 23 days. Over the next decades, the duration of missions grew increasingly longer as America and the Soviet Union each launched orbiting space stations: the American Skylab 4 mission lasted 84 days in 1973-74 and the Soviet *Salyut* 6 hosted a 185 day mission in 1980. This pattern of manned missions lasting ~6 months

continued through the 1990s into present day utilization of the International Space Station (ISS) although Soviet cosmonauts often stayed longer on Mir; Valery Polyakov holds the record for longest mission ever at 437 days (1994-95).

From 1981-2011, the American Space Shuttle made regular flights lasting 2-17 days, durations comparable to the Apollo flights. Short-duration space flight also elicits decreases in muscle mass (LeBlanc et al. 2000, Stein et al. 1999) although some have prevented these losses with exercise (Trappe et al. 2001). Long-duration space flight with its concomitant exposure to microgravity is associated with a host of physiologic maladaptations which include decreased muscle mass (LeBlanc et al. 2000, LeBlanc et al. 2000) and strength (Trappe et al. 2009). These morphologic and performance changes result from disruptions in protein metabolism, primarily a reduction in MPS that causes a net decrease in muscle tissue (Stein et al. 1999, Stein and Schluter 1997).

In the absence of gravitational loading, skeletal muscle enters a de facto state of disuse. Long-duration ISS crew members have employed a wide variety of countermeasures to spaceflight-induced unloading, ranging from bungee-loaded treadmill running to weightlifting with elastic or vacuum-based resistance (Amonette et al. 2004, Everett et al. 2009, Schneider et al. 2003, Smith et al. 2012, Trappe et al. 2009). Although the preservation of muscle strength during long-duration space flight has improved over the first ten to twelve years of the ISS, small to moderate strength losses are still typical for ISS crew members (Gopalakrishnan et al. 2010, Smith et al. 2012).

Regardless of the underlying mechanisms, skeletal muscle mass loss is a primary feature of long-duration space flight. Muscle strength loss is potentially problematic both from an individual and an operational standpoint. Upon return to Earth's gravity, astronauts may be at increased risk for falls and/or experience difficulty with activities of daily living. Operationally, high strength levels are needed both during space flight (e.g., to free jammed hardware, or to move large mass/high inertia objects) and during emergency egress from a vehicle which would likely involve high force activities like

opening a hatch, raising oneself and other crew members out of the vehicle, and running from the vehicle, all performed while wearing a space suit that weighs 121 kg (Norcross et al. 2010). Spaceflight and the unique demands that it places on skeletal muscle are discussed further in Chapter 6.

BED REST

Bed rest (BR) is synonymous with physical inactivity and is typically employed during either hospitalization (clinical purpose or necessity) or research (experimental purpose). BR, or acute inactivity associated with hospitalization or disease state, poses a potent threat to muscle tissue and functional capacity. In the nineteenth and early twentieth centuries, BR was commonly used as a treatment for disease and the resultant losses in bone mineral density, muscle mass, etc. were attributed to the primary pathology (Pavy-Le Traon et al. 2007). During the polio outbreaks of the 1940's, a group of researchers conducted a clinical BR trial on healthy medical students to determine whether the bone loss associated with polio was an inherent outcome of the disease or rather a result of the prolonged BR inactivity (Dietrick et al. 1948); their findings provided some of the earliest scientific evidence of the deleterious effects of BR. Despite this knowledge, in older adults, physical inactivity during hospitalization is a relatively accepted part of the inpatient experience although it clearly contributes to a host of negative outcomes, including a reduction in the ability to perform activities of daily living, increased incidence of readmission, and institutionalization (Covinsky et al. 2003). While reduced or limited physical inactivity may be indicated in many patient populations, the practice of subjecting patients to continuous BR without a clear medical indication is a regrettable default position (Brown et al. 2004).

Experimentally, BR was developed as a ground-based analog to more efficiently investigate spaceflight-induced physiologic (and psychologic) adaptations. This model

originated with Soviet scientists who are reported to have raised the foot of the beds of cosmonauts who had recently returned from spaceflight in order to eliminate the cosmonauts' sensation of slipping off of the foot of the bed (Pavy-Le Traon et al. 2007). Further work by the Soviets found that a 6 degree head down tilt elicited the optimal compromise between comfort and physiologic adaptations; thus, 6 degree head down tilt is the BR model most commonly employed in space flight research (Atkov and Bednenko 1992). The cephalid fluid shift induced by this slight head down tilt initiates a systemic diuresis, reducing blood volume ~10-15% in 1-2 days; this directly mimics what occurs in space as body fluids migrate headward in the absence of Earth's gravity (Pavy-Le Traon et al. 2007).

EXERCISE COUNTERMEASURES

In light of the loss of muscle mass and strength associated with BR and spaceflight, much work has focused on methods to counteract these deleterious changes. Perhaps most intuitively, exercise has been employed to combat the disuse inactivity inherent in these environments. While early space missions of the 1960s incorporated no in-flight exercise, the next four decades of space flight saw a significant emphasis placed on in-flight exercise countermeasures. Exercise hardware evolved from astronauts running in their socks on a slick plate (the Skylab 4 "treadmill") to advanced equipment such as a fully motorized treadmill with vibration isolation and a strength training device (the Advanced Resistive Exercise Device) that provides constant forces up to 273 kg with inline flywheels that simulate the inertial properties of weightlifting in 1-g (English et al. 2008, Loehr et al. 2011). These advances reflect, among other things, the complexities of attempting to replicate the physiologic effects of continual exposure to Earth's gravity which acts even on sedentary (but ambulatory) individuals.

Due to the cost and limited opportunities to conduct exercise countermeasures research during actual space flight, BR has been used extensively to evaluate the efficacy of a range of exercise countermeasures. These have included resistance exercise (Alkner and Tesch 2004, Shackelford et al. 2004, Trappe et al. 2007), aerobic exercise with and without lower body negative pressure (Lee et al. 2009, Schneider et al. 2009, Watenpaugh et al. 2000), artificial gravity via a centrifuge (Caiozzo et al. 2009, Stenger et al. 2012), and vibration (Garman et al. 2007, Mulder et al. 2009). These countermeasures have been relatively successful at the prevention of muscular deconditioning in BR, but due to practical issues (e.g., prohibitively large mass), they have not been similarly implemented in spaceflight.

LEUCINE

Leucine is a branched-chain amino acid (BCAA) that stimulates MPS in vitro (Buse and Reid 1975, Hong and Layman 1984), in rats (Crozier et al. 2005), and in humans (McNurlan et al. 1993, Rennie et al. 1982) while the other BCAAs, valine and isoleucine, are, alone, ineffective and inhibitory, respectively (Buse and Reid 1975). Together, all three BCAAs stimulate MPS, but without leucine, the others are ineffective (Vary et al. 1999). Leucine exerts this protein synthetic effect by stimulation of mRNA translation initiation (Anthony et al. 2000, Buse and Reid 1975). Specifically, in rat muscle preparations, leucine-stimulated translation initiation (and protein synthesis) is facilitated by increased binding of the elongation initiation factor 4E (eIF4E) · elongation initiation factor 4G (eIF4G) assembly, possibly secondary to increased eIF4G phosphorylation; these effects were seen independent of activation of the mammalian target of rapamycin (mTOR) pathway as phosphorylation of eukaryotic initiation factor (eiF)4E binding protein-1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase 1 (S6K1) was not increased with supraphysiologic levels of leucine (Bolster et al. 2004). A PI3-

kinase inhibitor in this study also clearly demonstrated leucine's ability to stimulate MPS in the absence of insulin's effects. Nevertheless, insulin still has potent synergistic effects with leucine on mTOR activation, subsequent translation initiation via phosphorylation of S6K1 and 4E-BP1 and assembly of eIF4G and eIF4E, and ultimately, MPS (Bolster et al. 2004, Crozier et al. 2005, Preedy and Garlick 1986, Svanberg et al. 1996).

Leucine has also been shown to stimulate MPS in humans via the mTOR pathway and its downstream targets, S6K1 and 4E-BP1 (Glynn et al. 2010). Young adults given a 10 g essential amino acid (EAA) drink with either 1.8 or 3.5 g leucine similarly increased MPS and net balance over a 3 h period. Although synthetic responses were similar, a slightly greater decrease in muscle protein breakdown (MPB) was seen in the high-leucine group (Glynn et al. 2010). Further, extra leucine was associated with a prolonged increase in insulin as well as enhanced anabolic signaling, particularly of 4E-BP1 (Glynn et al. 2010).

The stimulatory effects of EAA and leucine on MPS are synergistically enhanced in combination with other potent anabolic stimuli such as exercise. Aggregate data from the Rasmussen lab show that, in young men, resistance exercise alone produced a 40% increase in MPS above fasted levels (Drummond and Rasmussen 2008). EAA plus carbohydrate (EAA+CHO) without exercise elicited a 100% increase in MPS over the same fasted baseline while exercise plus the EAA+CHO produced the greatest rise in MPS—145% (Drummond and Rasmussen 2008). Worthy of note is the fact that, by itself, nutrition stimulated MPS to a far greater degree than did exercise alone.

Most of the above studies utilized young subjects. In many circumstances, older adults (> 60 y) exhibit similar responses to nutrition, and EAA in particular, as their younger counterparts; however, in other situations, deficiencies are apparent. Basal muscle protein turnover is not different between young and old adults although the elderly exhibit slightly greater rates of synthesis and breakdown (Volpi et al. 2000, Volpi et al. 1999, Volpi et al. 2001). Also similar to young adults, a bolus ingestion of 15 g

EAA (2.8 g leucine) produced a significant increase in MPS in older adults despite no change in plasma insulin levels (Paddon-Jones et al. 2004). On the other hand, older adults are unable to mount an anabolic response to an EAA/CHO mixture; Volpi et al. found that MPS did not increase in response to 40 g of amino acids (leucine: 3.2 g) with 40 g glucose in the elderly although it did so in young adults; in this case, insulin was increased with nutrient ingestion in both age groups, indicating a possible resistance to the anabolic effects of insulin in the elderly despite normal glucose tolerance (Volpi et al. 2000). Also unlike young adults, older adults do not increase MPS after administration of a small (6.7 g; leucine: 1.7 g) serving of EAA despite similar increases in plasma leucine concentrations (Katsanos et al. 2005). However, when the leucine content of the 6.7 g EAA supplement was increased to 2.8 g, the elderly did mount a synthetic response similar to young controls; incidentally, MPS was not further stimulated in the young in response to the added leucine which suggests a threshold-type response to leucine (albeit with different thresholds for young and old), not a dose-dependent one (Katsanos et al. 2006). Finally, Rieu et al. demonstrated that an orally ingested, semi-liquid mixed meal (~755 kilocalories: 98 g CHO, 27 g fat, 30 g protein (leucine: 2.4 g)) supplemented with leucine (~3.9 g; total leucine = 6.3 g) increased MPS in older adults while the same meal with supplementary alanine failed to do so (Rieu et al. 2006). Insulin levels were increased with feeding and not different between groups; this seems to rule out an impaired anabolic response to insulin in these older adults. Why this mixed meal increased MPS and that of Volpi et al. (discussed above) did not is unclear (Rieu et al. 2006, Volpi et al. 2000). Plasma leucine concentrations were similar between the two studies as was the fold-increase in plasma leucine after the meal (~2.5). The obvious differences were that the meal of Rieu et al. had more kcal (755 vs. 320) and more leucine (6.3 g vs. 3.2 g) than that of Volpi et al. (Rieu et al. 2006, Volpi et al. 2000).

In light of these findings, leucine has been investigated as a potential intervention to prevent the muscle loss associated with aging and unloading (e.g., spaceflight or

clinical bed rest). To this end, Trappe et al. evaluated leucine as a countermeasure during 60 d BR in young women and reported losses in lean mass and strength similar to or worse than controls (Trappe et al. 2007); this study is discussed in greater detail in Chapter 3. Verhoeven et al. supplemented healthy older men with 2.5 g leucine with each of their three daily meals (7.5 g · d^{-1}). After 3 months of supplementation, no changes in lean mass, strength, insulin sensitivity, or lipid profile were observed (Verhoeven et al. 2009). They found similar non-effects in a group of older Type II diabetic men who followed the same supplementation regimen for 6 months (Leenders et al. 2011). The subjects in these studies consumed a reasonably high protein diet (~1.0 g protein · kg^1 · d^{-1}) which may explain their lack of response to supplemental leucine, i.e., leucine may only effect changes at the whole body level in individuals who are in a compromised state (e.g., inactive or consuming a low protein diet). Evidence for this may be found in results from Casperson et al. who reported that 14 d supplementation with 4 g leucine · $meal^{-1}$ (12 g · d^{-1}) in healthy older adults consuming 0.8 g protein · kg^1 · d^{-1} resulted in increased basal and post-prandial MPS as well as greater mTOR, S6K1, and 4E-BP1 expression (Casperson et al. 2012).

HYPOTHESES AND SPECIFIC AIMS

This study will test the following hypotheses:

1) Decrements in lean muscle mass, muscle strength, functional capacity, and insulin sensitivity following 14 days of bed rest in middle-aged individuals (45-60 y) will be associated with a decrease in muscle protein synthesis and facilitated by a down regulation of mTOR intracellular signaling and its downstream targets.

2) Leucine supplementation during 14 days of bed rest will reduce the decrease in muscle protein synthetic capacity and partially preserve lean muscle mass, muscle strength, functional capacity, and insulin sensitivity by stimulating nutrient sensing and insulin-dependent protein synthetic pathways.

3) Leucine supplementation during 7 days of rehabilitation immediately following bed rest will facilitate a faster recovery of muscle mass, muscle strength, functional capacity, and insulin sensitivity by stimulating nutrient sensing and insulin-dependent protein synthetic pathways.

This study will address the following specific aims:

1) To determine the effect of 14 days of bed rest on skeletal muscle protein synthesis, cellular signaling, muscle mass, muscle strength, aerobic capacity, and insulin sensitivity in middle-aged individuals.

2) To determine the effect of leucine-enriched meals on skeletal muscle protein synthesis, cellular signaling, muscle mass, muscle strength, aerobic capacity, and insulin sensitivity in middle-aged individuals during 14 day of bed rest.

3) To determine the effect of leucine-enriched meals on skeletal muscle protein synthesis, cellular signaling, muscle mass, muscle strength, aerobic capacity, and insulin sensitivity in middle-aged individuals during a 7 day rehabilitation period following bed rest.

Chapter 2: Methods

SUBJECTS

Subjects were healthy, community-dwelling 45-60 y old males and females that were recruited via print and internet advertisements. After providing written informed consent, subjects were screened in the University of Texas Medical Branch's Institute for Translational Sciences-Clinical Research Center (ITS-CRC) for a variety of pathological conditions including cardiac abnormalities, kidney disease, coagulation disorders, hypertension, HIV, and hepatitis. Other exclusion critiera included narcotics usage, impaired glucose tolerance, history of cancer, BMI > 30 kg · m^{-2}, current smoking or > 20 pack years smoking history, history of anabolic steroid usage, aggression disorders, previous stroke, or gastrointestinal bleeding. Subjects also performed a treadmill stress test with 12-lead electrocardiogram (Bruce protocol) and completed a Doppler ultrasound examination of the bilateral lower extremity vasculature to rule out cardiovascular disease and increased blood clotting risk, respectively.

STUDY DESIGN

Subjects reported to the ITS-CRC on the morning of Day 1 for a 26 d inpatient stay; the study was divided into three periods: 1) Pre-bed rest (Days 1-3), during which subjects consumed a controlled research diet but were permitted to ambulate freely on the research unit; 2) Bed rest (Days 4-18): 14 d of supine bed rest; 3) Rehabilitation (Days 19-26): 7 d of standard of care physical therapy (Figure 2).

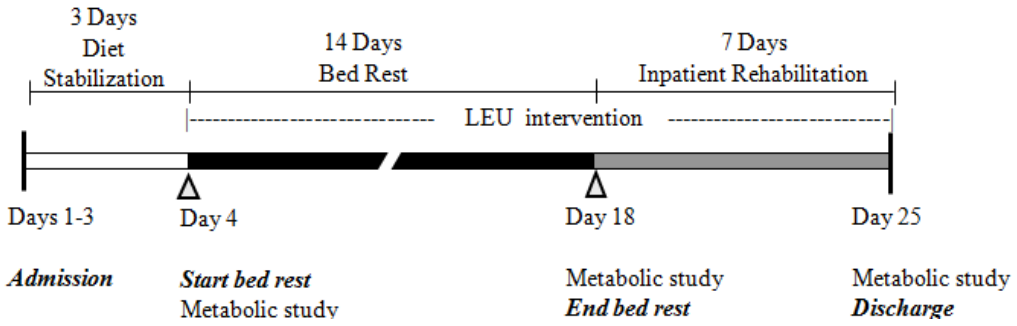


Figure 2. Time line of 26 day inpatient stay.

Subjects were assigned to one of two groups in a randomized, double blind fashion: 1) a supplemented group that received leucine (LEU; 0.06 g · kg^{-1} · $meal^{-1}$) from Day 4-26 with each of their three daily meals and; 2) a control group (CON) that received the non-essential amino acid, alanine (0.06 g · kg^{-1} · $meal^{-1}$), with each of their three daily meals. Metabolic studies using stable isotope tracer techniques were conducted on Days 4, 18 (the actual first and last days of BR), and 25.

BED REST

On the morning of Day 4, subjects were permitted to void and shower upright, and then the BR period began. Beds were set at a 0° angle (horizontal) aside from three, 2-h periods each day that corresponded with the three daily meals when bed backs were raised to 5°; this was done to protect subjects from elevated intraocular pressure and possible vision alterations which have been reported in some head down tilt BR studies (Chiquet et al. 2003, Drozdova and Grishin 1972, Mader et al. 1990). Subjects were allowed two pillows under their head/shoulders. They could turn in bed to alleviate positional discomfort. During the BR period, eating and bathing were performed in bed. Males urinated into a bottle while supine or side lying; bowel movements and female urination was done on a bedside commode that subjects transferred to by using their upper body musculature with no weight bearing.

Subjects had access to a telephone, television, laptop computer with internet access, and any personal material that they brought (e.g., books, playing cards). Visitors were permitted, but were not allowed to bring food or drink, to get in bed with the subject, or to spend the night. Lights were turned out between 2200 and 2300 each night; subjects were awakened and weighed in a pre-weighed hospital gown each morning at ~0600. Napping was permitted between 1400-1600.

Bed rest ended on the afternoon of Day 18 at which time subjects sat upright in a chair in order to gradually reacclimatize to a vertical position. Weight bearing the evening of Day 18 and the morning of Day 19 was kept to an absolute minimum to prevent interference with post-BR strength and functional testing on Day 19.

DIET AND SUPPLEMENTATION

For the entirety of the 26 d inpatient stay, subjects were fed a controlled, isocaloric diet of 55% carbohydrate, 30% fat, and 15% protein. Protein intake was evenly distributed across each of three daily meals. Individual daily caloric intake was determined by the Harris-Benedict equation; activity factors (AF) of 1.6 and 1.3 were used during the ambulatory (pre-BR and rehabilitation) and BR periods, respectively.

Daily energy requirement (kcal) = (66 + (13.7 x kg) + (5 x cm) - (6.8 x y)) x AF

kg = bodyweight; cm = height; y = age; AF = activity factor

Meals were served daily at 0800, 1300, and 1800. Subjects were required to consume all of their food within 30 min; uneaten food items were weighed and recorded. No snacking or eating between meals was permitted. Caffeinated and alcoholic beverages were prohibited, but water was provided *ad libitum*; caloric beverages such as fruit juice and milk were integrated into the diet. Dietary data were analyzed with Nutrition Data System for Research software (v 2006), developed by the Nutrition Coordinating Center

(NCC), University of Minnesota, Minneapolis, MN. All food preparation, food weighing, and dietary preparation was conducted by the ITS-CRC Metabolic Research Kitchen staff.

The nutrition manager of the ITS-CRC held the key to subject group assignment and supervised supplement preparation and distribution. Supplements (leucine or alanine) were dissolved in 200 ml of fruit juice or diet soda; dietary staff verified that subjects consumed their supplement at each meal.

REHABILITATION

Subjects began rehabilitation on Day 20, the second full day after BR. Subjects completed all post-BR strength and functional performance measures on Day 19 and were too fatigued to also perform rehabilitative exercise. Rehabilitation was conducted by a physical therapist or exercise physiologist and incorporated static stretching, bodyweight exercise (e.g., squats, stair climbing), Theraband resistance exercise, and walking. Daily sessions (Days 20-23) lasted 45-60 min. Rehabilitation on Day 24 was limited to 15-30 min of walking to prevent any effects of exercise-induced muscle damage on the Day 25 stable isotope tracer infusion study. The rehabilitation program was designed as a conservative reintroduction to upright activity that would be appropriate for older adults to perform following hospitalization.

STATISTICAL ANALYSES

All analyses were performed used Stata 12.1 software (StataCorp LP, College Station, TX). Mixed effects linear regression techniques were employed to analyze all continuously scaled dependant variables. Stata's '*xtmixed'* command was used to generate an initial model for each dependent variable with time and group as fixed effects and subject as a random effect. A Shapiro-Wilk W test for residual normality was performed to insure that the model met the assumptions of normality. Residuals of the

model were then graphed and visually examined for fit. In cases where model residuals were clearly skewed, natural log transformations of the dependent variables were sometimes necessary to meet the normality assumption of these statistical techniques. In some instances it was necessary to exclude overly influential outlying values in order to meet model assumptions. For all dependent variables, this process resulted in the exclusion of zero to six data points from the final model. The output of the final model included simple interaction effects (group x time) for each time point and within-group contrasts between time points. Graphs and tables are expressed using means with SEM in the native scale of each dependent variable.

For each dependent variable, two final models were generated; these models resulted in either three or four simple interaction effects of interest depending on whether or not a mid-BR measure was made for that particular dependent variable. The first model used pre-BR as the reference time point and permitted the examination of differential, between-group changes that occurred: 1) during the first week of BR (if a mid-BR measurement was made; pre-BR to mid-BR), 2) across the entire BR period (pre-BR to post-BR), and 3) across both BR and rehabilitation (pre-BR to post-rehab). The second model, comprised of the same data, used post-BR as the reference time point; this facilitated specific examination of 4) the rehabilitation period (post-BR to post-rehab) and changes that may have occurred during it. The simple interaction effects of both models were of primary interest, as, pursuant to the hypotheses of the study, they evaluated and compared changes between groups, between time points. Individual *a priori* contrasts were also performed to evaluate within-group changes between time points; Bonferroni corrections were made for these contrasts. Between-group/within-time contrasts were not calculated.

Chapter 3: Leucine attenuates the loss of muscle mass and strength and preserves muscle quality during 14 d bed rest in middle-aged adults

INTRODUCTION

Sarcopenia describes the natural loss of skeletal muscle mass that accompanies healthy aging. After the attainment of peak muscle mass between the second and fourth decades of life (Lauretani et al. 2003), lean mass is lost at a rate of 1-2% per year after the age of 50 (Buford et al. 2010, Marcell 2003). With associated costs of $18 billion a year (Janssen et al. 2004) and the burgeoning older American population which will more than double from its 2011 size to 84 million people by 2050, sarcopenia has been called the next important clinical target in translational science (Matthews et al. 2011).

Although sarcopenia is defined by the loss of muscle mass, its practical impact is seen in the concomitant loss of muscle strength, which is typically 2-3 times greater (expressed as percent loss), and causes decrements in functional capacity (Visser et al. 2000), the loss of independent living (Baumgartner et al. 1998, Janssen 2006, Rantanen et al. 2002, Taekema et al. 2010), and an increased mortality risk (Cooper et al. 2010, Ling et al. 2010). Decreased muscle mass is also associated with reduced insulin sensitivity (Kalyani et al. 2012, Schols and Sidossis 2011).

Cross-sectional and modeling studies suggest that the natural (sarcopenic) loss of muscle with healthy aging is linear although no studies have prospectively demonstrated this (Forbes 1999, Frontera et al. 1991, Hughes et al. 2002, Sehl and Yates 2001). In contrast to the gradual and continuous loss of lean tissue attributed to sarcopenia, this author has previously highlighted the rapid loss of muscle mass that accompanies periods of acute physical inactivity (e.g., hospitalization), which when coupled with incomplete recovery, results in sharp net decreases in lean mass (English and Paddon-Jones 2010).

These decrements represent several years' worth of normal, ageing-related muscle loss. Such rapid, inactivity-induced muscle loss has been termed "Catabolic Crisis," in recognition of its episodic and severe nature (English and Paddon-Jones 2010). In addition to inactivity-induced muscle loss, concomitant illness, characterized by elevated cortisol levels, can triple the rate of muscle loss in comparison to inactivity alone (Paddon-Jones et al. 2006). If a lean mass loss greater than 40% of baseline muscle mass is fatal (Roubenoff 2000), it becomes strikingly apparent that swift implementation of targeted and effective therapies in these cases is vital. Fortunately, such catabolic crises are well-suited for the delivery of focused interventions as they not only represent opportunities to mitigate substantial muscle loss, but the inpatient hospital environment provides a controlled setting that is conducive to compliance and monitoring.

Exercise is the optimal intervention during a catabolic crisis (Bamman et al. 1998, Ferrando et al. 1997, Trappe et al. 2001). However, hospital patients do very little voluntary physical activity (Fisher et al. 2011) and, unfortunately, due to weakness, fatigue, patient refusal, and disease complications, even directed physical activity (e.g., inpatient physical therapy) is often not feasible.

Nutritional supplementation is an attractive intervention that can be employed alone or when possible, in conjunction with exercise/physical activity in hospitalized individuals. Increasing protein intake slightly above the Recommended Daily Allowance (~1.0 $g \cdot kg^{-1} \cdot d^{-1}$) does not prevent muscle loss during BR (Ferrando et al. 1996). Protein supplementation also comes with inherent problems such as increased satiety (Pupovac and Anderson 2002) that subvert its intended supplemental role, rendering it a replacement of protein/energy that would otherwise be consumed in the normal diet (Fiatarone Singh et al. 2000). Also, whey protein has been shown to produce an inferior post-prandial muscle protein synthetic response than an isocaloric EAA supplement (Paddon-Jones et al. 2006).

EAA supplementation has been used in BR to successfully prevent lean mass losses and to attenuate muscle strength decrements. Paddon-Jones et al. provided a 16.5 g EAA supplement $3 \cdot d^{-1}$ between three daily meals during 14 d BR which resulted in complete protection of leg lean mass and an attenuated strength loss in young subjects (Paddon-Jones et al. 2004). Brooks et al. administered a similar EAA supplement (15 g) only once daily during 28 d BR and saw a 0.7 kg loss of leg lean mass (-7%) along with a 22% decrement in lower body strength (Brooks et al. 2008); together, these studies highlight the importance of repeated maximal stimulation of the muscle protein synthetic pathways during periods of disuse (Volpi et al. 1999). Although EAA supplements are smaller volume than whole protein supplements, but an EAA supplement of 15 g nevertheless represents ~30 pills to ingest at one sitting. This presents potential problems with compliance, dysphagia, and even satiety.

In the interest of identifying a low volume supplement that avoids these issues, work has focused on the anabolic effects of the branched-chain essential amino acid, leucine. Numerous studies have demonstrated the role of leucine in acutely stimulating MPS in rats (Anthony et al. 2000, Anthony et al. 2000, Bolster et al. 2004, Crozier et al. 2005, Hong and Layman 1984) and in humans (Dreyer et al. 2008, Katsanos et al. 2006, Tipton et al. 2009). In the elderly, leucine added to a normal protein meal ($0.04 \text{ g} \cdot \text{kg}^{-1}$) increased post-prandial MPS more than a non-supplemented meal alone (Rieu et al. 2006). Enrichment of a suboptimal EAA dose (6.7 g; leucine = 1.74 g) with an additional 1 g of leucine (total = 2.74 g) normalized the post-prandial anabolic response in a group of older adults (Katsanos et al. 2006). Further evidence for leucine's efficacy was provided by Casperson et al. who supplemented community-dwelling older men with 4 g leucine with each of their three daily meals that contained the RDA for protein (Casperson et al. 2012). Two weeks of leucine supplementation increased both basal and post-prandial MPS but elicited no changes in lean body mass, likely due to the brief intervention period (Casperson et al. 2012).

Longer duration studies (3-6 mo) show that leucine does not increase lean mass or muscle strength in either healthy or diabetic, community-dwelling older adults that consume more than the RDA for protein (Leenders et al. 2011, Verhoeven et al. 2009). Only one study has examined the effects of leucine supplementation in humans during complete inactivity. Trappe et al. provided 3.6 g leucine $\cdot$ d^{-1} to young women during 60 d BR in addition to a daily protein intake of 1.45 g $\cdot$ kg^{-1}; control subjects consumed ~1.0 g protein $\cdot$ kg^{-1} $\cdot$ d^{-1} (Trappe et al. 2007). The high protein diet/leucine supplement was not effective as losses in calf muscle volume and leg strength were similar to controls (Trappe et al. 2007).

Muscle strength is a fundamental component of functional performance and by extension, of independent living. The chronic loss of strength associated with aging as well as the acute loss of strength characteristic of inactivity have a negative synergistic effect on functional performance. Research suggests that the chronic loss of strength related to aging is largely attributable to the chronic loss of muscle mass (i.e., sarcopenia) (Frontera et al. 2000, Frontera et al. 2000) although others have found this relationship more tenuous (Hughes et al. 2001, Metter et al. 1999). Thus, although absolute strength is lost with aging, muscle quality or relative strength (strength/unit muscle) may be maintained. This appears to differ from inactivity-induced strength loss in which strength is lost disproportionately to the change in muscle mass; this results in a loss of both absolute strength and muscle quality (relative strength) (Alkner and Tesch 2004, Kortebein et al. 2007, LeBlanc et al. 1992).

Therefore, this study investigated the effectiveness of a low volume leucine supplement to prevent or attenuate decrements in muscle mass, muscle strength, muscle quality, and aerobic capacity during 14 d BR inactivity and to facilitate recovery during 7 d of rehabilitation in healthy middle-aged adults who consumed an isocaloric diet and slightly more than the RDA for protein.

METHODS

MUSCLE MASS AND BODY COMPOSITION

Changes in body composition were determined by dual energy x-ray absorptiometry (DEXA; Lunar iDEXA, GE Medical Systems, Madison, WI). Subjects wore light clothing and no metal (e.g., zippers, underwire bra, or jewelry) and were positioned on the scanning surface per the manufacturer's instructions. Subjects were required to lay supine for 10 min prior to scanning. Subsequently, the technician verified that the subject's full body was appropriately captured and analyzed the scan by delineating all body segments per manufacturer's instructions. All scans for a given subject were analyzed by a single technician. Dependent variables were whole body lean mass (WBLM), leg lean mass (LLM), whole body fat mass (WBFM), and body fat percentage.

Testing for body composition was performed four times: pre-BR (Day 3), mid-BR (Day 10), post-BR (Day 17), and post-rehabilitation (Day 24). Post-BR and post-rehabilitation body composition testing were conducted on these days to not interfere with metabolic stable isotope tracer studies which were performed on Days 4, 18 (the actual first and last days of BR), and 25.

MUSCLE STRENGTH AND ENDURANCE

Changes in muscle strength and endurance of the knee and ankle extensor/flexors were determined using an isokinetic dynamometer (Biodex System 4, Biodex Medical Systems, Inc., Shirley, NY) which was calibrated per manufacturer's instructions. Muscle strength was measured thrice: pre-BR (Day 2), post-BR (Day 19), and post-rehabilitation (Day 26). Subjects completed a familiarization session on Day 1 during which they were fitted to the dynamometer, and position settings were recorded to be replicated for all future test sessions; subjects also performed sub-maximal and maximal repetitions at all

testing speeds to insure familiarity with both the device and giving maximal effort. Prior to isokinetic testing, an anatomic reference (90° and 0° for the knee and ankle, respectively) was measured with a hand-held goniometer during subject set–up. Knee testing was conducted in the seated position over a range of 95° (flexion) to 20° (extension). Ankle testing was also conducted in the seated position with the hip and knee joints of the tested leg flexed and positioned at ~85° and ~65°, respectively; the lower leg shank was parallel to the ground. Ankle testing was conducted over a range of 20° (extension) to -10° (flexion). Subjects wore their own athletic shoes (the same pair) for all three testing sessions and all testing was performed on subjects' right leg. This insured that the post-BR and post-rehabilitation strength tests were 15 d and 22 d, respectively, after Day 4 muscle biopsies of the right leg vastus lateralis.

Subjects completed 6-8 sub-maximal repetitions (50-75% of perceived maximum effort) and 1-2 maximal repetitions as warmup prior to testing the knee and ankle at each session. Muscle strength and endurance tests of the knee and ankle extensors/flexors consisted of: 1) 5 repetitions at $60° \cdot sec^{-1}$ (peak torque—knee); 2) 20 repetitions at $180° \cdot sec^{-1}$ (peak torque and total work—knee); 3) 5 repetitions at $60° \cdot sec^{-1}$ (peak torque—ankle; Table 1).

Table 1. Isokinetic testing protocol.

Joint	Movement	Velocity	Repetitions	Outcome
Knee	extension/flexion	$60° \cdot sec^{-1}$	5	peak torque (Nm)
Knee	extension/flexion	$180° \cdot sec^{-1}$	20	peak torque (Nm), total work (Nm)
Ankle	extension/flexion	$60° \cdot sec^{-1}$	5	peak torque (Nm)

Prior to the second knee testing set, subjects also performed 2-3 sub-maximal repetitions at approximately 50% of maximum effort to familiarize themselves with the movement velocity of that set. Subjects rested 2 min between testing sets. All testing was conducted by the same technician.

MAXIMAL AEROBIC CAPACITY

Maximal aerobic capacity (VO2max) was assessed via a graded exercise test conducted on a cycle ergometer (Monark Ergomedic 828E, Monark Exercise, Sweden). Handlebar and saddle height were adjusted to fit each subject and the position settings recorded for replication during all future tests. Total ventilation and expired gases were measured breath by breath by a metabolic cart (VMax Encore 29, Care Fusion, Yorba Linda, CA). The exercise protocol consisted of two, 3-min stages followed by 1-min stages until test termination. A "light" protocol, with the first two workloads set at 25 W and 50 W was used for smaller subjects; the "nominal" protocol employed workloads of 50 W and 100 W for the first two stages (Table 2).

During the subsequent 1-min stages for both protocols, intensity was increased 25 W each min until test termination. Subjects were considered to have achieved VO_{2max} when two of the following criteria were satisfied: 1) respiratory exchange ratio (RER) > 1.09; 2) a plateau in maximal oxygen uptake; 3) subject's volitional fatigue. All subjects pedaled at 75 revolutions $\cdot$ min^{-1}. Blood pressure, heart rate, and ratings of perceived exertion were monitored with a manual sphygmomanometer and stethoscope, 3-lead electrocardiogram (Critikon Dinamap Plus, Johnson & Johnson Medical, Inc., Tampa, FL), and Borg's RPE scale, respectively. Metabolic and ventilatory data were averaged every 30 s with the highest values for a 30-s period representing maximal aerobic capacity.

Table 2. Light and nominal VO_2max testing protocols.

Stage	Stage time (min)	Elapsed time (min)	Light (W)	Nominal (W)
1	3	3	25	50
2	3	6	50	100
3	1	7	75	125
4	1	8	100	150
5	1	9	125	175
6	1	10	150	200
7	1	11	175	225
8	1	12	200	250

Maximal aerobic capacity was measured thrice: pre-BR (Day 2), post-BR (Day 19), and post-rehabilitation (Day 26); data were expressed absolutely ($L \cdot min^{-1}$), relative to bodyweight ($ml \cdot kg^{-1} \cdot min^{-1}$), and relative to whole body lean mass ($ml \cdot kg\ lean\ mass^{-1} \cdot min^{-1}$).

RESULTS

MUSCLE MASS AND BODY COMPOSITION

Whole body lean mass was significantly decreased in both CON and LEU at mid- and post-BR time points; neither group differed from pre-BR values following rehabilitation. A significant interaction effect ($p=0.05$) was detected at mid-BR which

indicated an attenuated loss of WBLM in LEU (Figure 3).

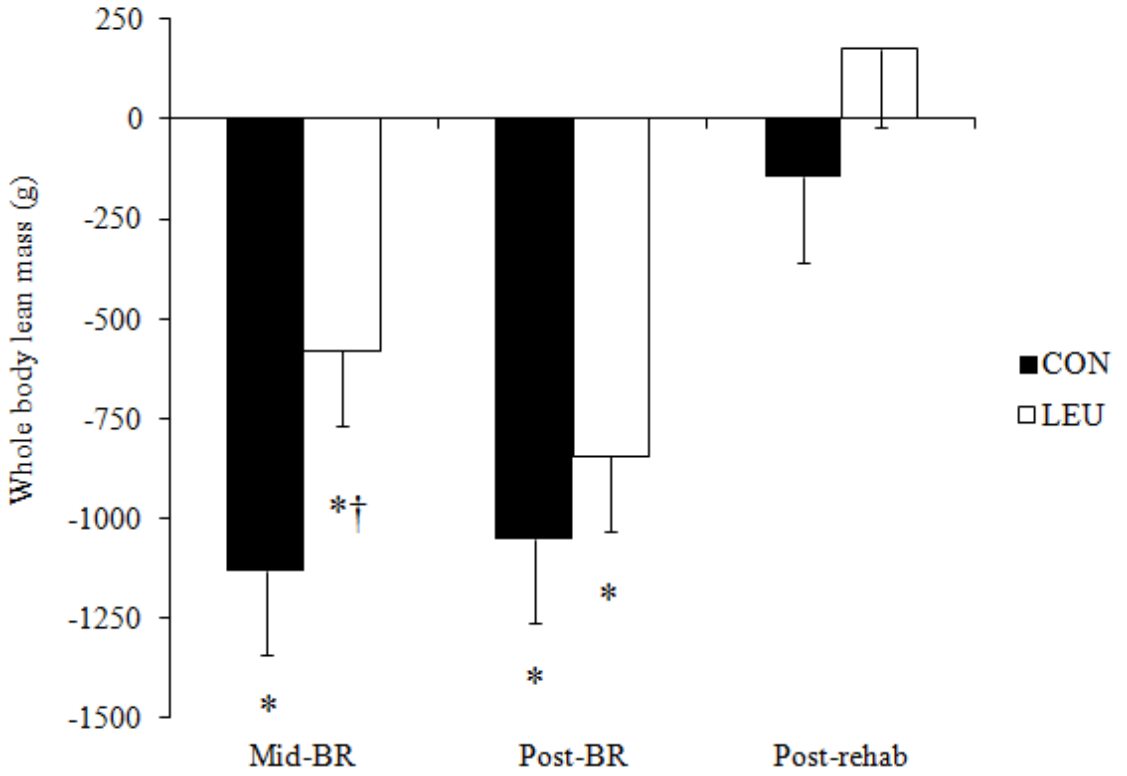


Figure 3. Changes in whole body lean mass (g) for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (DEXA; mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p=0.05$).

Leg lean mass (LLM) was significantly decreased in CON and LEU at mid- and post-BR ($p<0.05$; Figure 4). However, post-rehab, LLM was restored to pre-BR values in LEU while it remained depressed in CON ($p=0.02$).

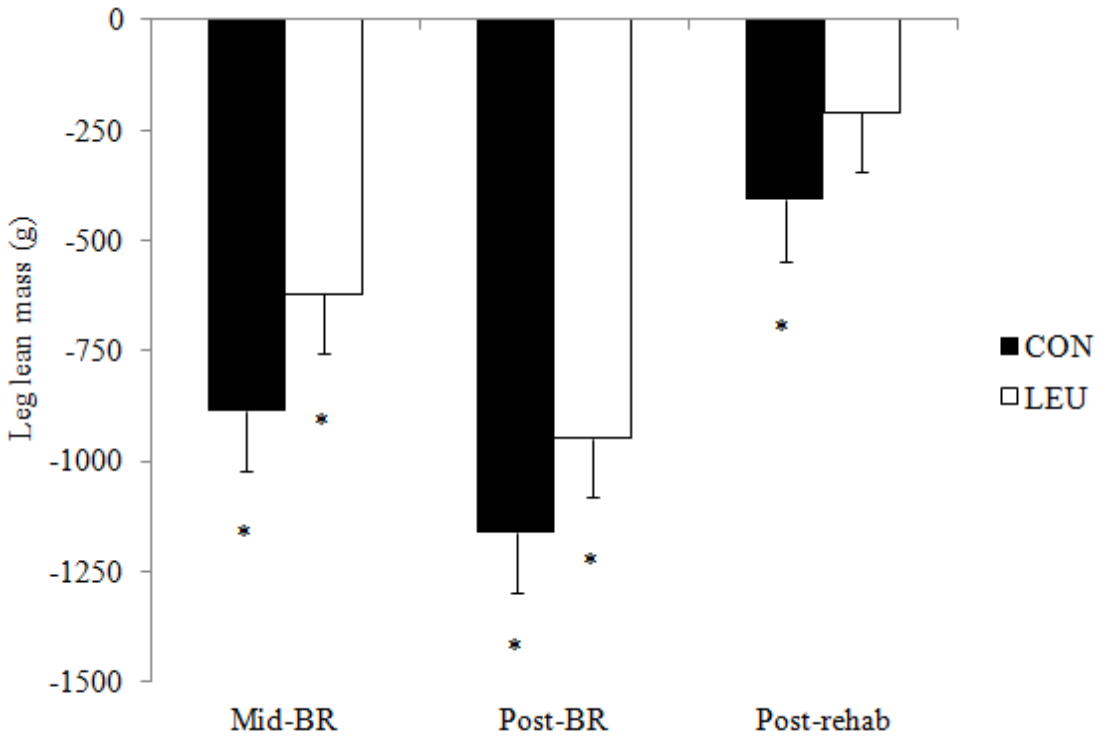


Figure 4. Changes in leg lean mass (g) for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (DEXA; mean±SE); * significant difference from pre-BR ($p<0.05$).

Whole body fat mass (WBFM) did not change in LEU, but was significantly increased in CON post-BR ($p=0.02$; Figure 5). Also, there was an interaction effect (group x time; $p=0.02$) at mid-BR which indicated a differential change in WBFM at that time point.

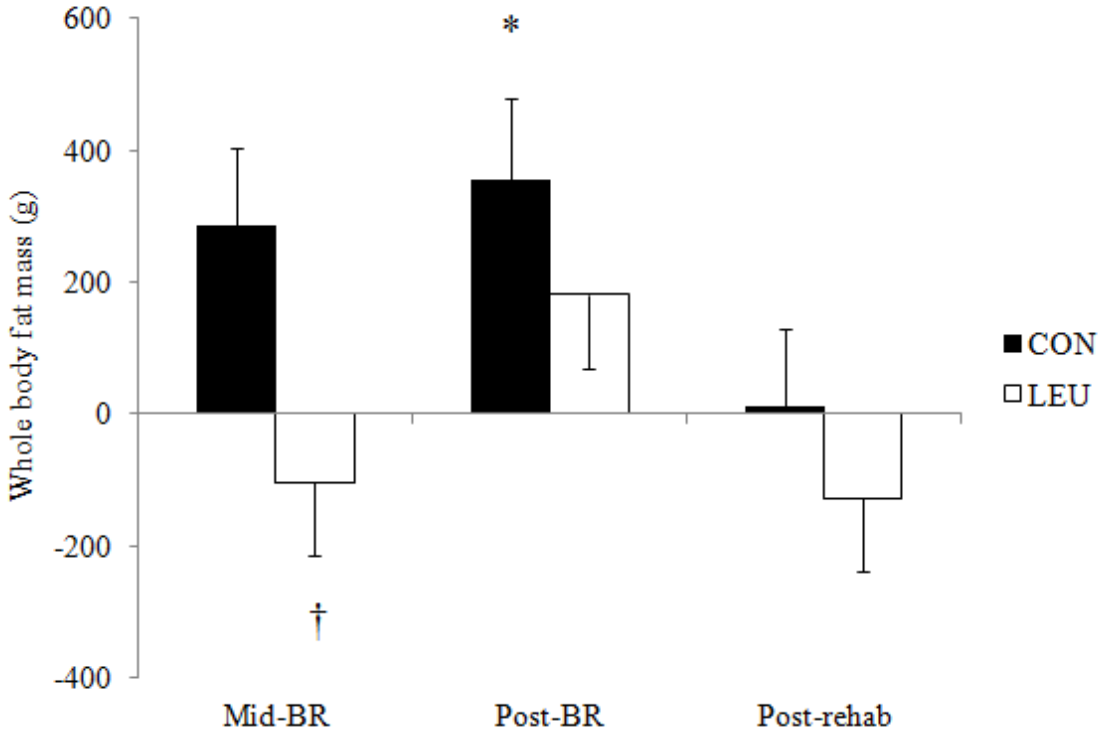


Figure 5. Changes in whole body fat mass (g) for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (DEXA; mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p=0.02$).

Body fat percentage (BF%) did not differ between groups pre-BR. For LEU, body composition did not change throughout the study. In CON, however, BF% was increased mid-BR and post-BR. Also, an interaction effect was detected mid- and post-BR which indicated a differential change in body composition during BR between the two groups (Table 3).

Table 3. Changes in body composition for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p<0.05$).

	Pre-BR		Mid-BR (Δ)		Post-BR (Δ)		Post-rehab (Δ)	
	CON	LEU	CON	LEU	CON	LEU	CON	LEU
Body mass (kg)	75.7±3.9	73.0±3.7	-0.93±0.23*	-0.89±0.22*	-1.24±0.23*	-1.00±0.22*	-0.75±0.23*	-0.37±0.22
WBLM (kg)	51.0±2.9	49.4±2.7	-1.13±0.21*	-0.58±0.19*†	-1.05±0.21*	-0.84±0.19*	-0.14±0.22	0.18±0.19
LLM (kg)	17.3±1.1	17.2±1.1	-0.88±0.14*	-0.62±0.13*	-1.16±0.14*	-0.95±0.13*	-0.41±0.14*	-0.21±0.13
WBFM (kg)	22.4±2.7	20.9±2.5	0.29±0.12	-0.10±0.11†	0.36±0.12*	0.18±0.11	0.11±0.12	-0.13±0.11
BF%	29.8±3.2	30.0±3.1	0.8±0.2*	0.0±0.2†	0.9±0.2*	0.4±0.2†	0.2±0.2	-0.3±0.2†

MUSCLE STRENGTH AND ENDURANCE

Isokinetic knee extensor strength ($60° \cdot s^{-1}$) was significantly decreased from pre-BR values at post-BR and post-rehab in both CON and LEU ($p<0.05$; Figure 6). However, there was an interaction effect post-BR ($p=0.01$) and post-rehab ($p=0.03$) which indicated that knee extensor strength loss was attenuated in LEU.

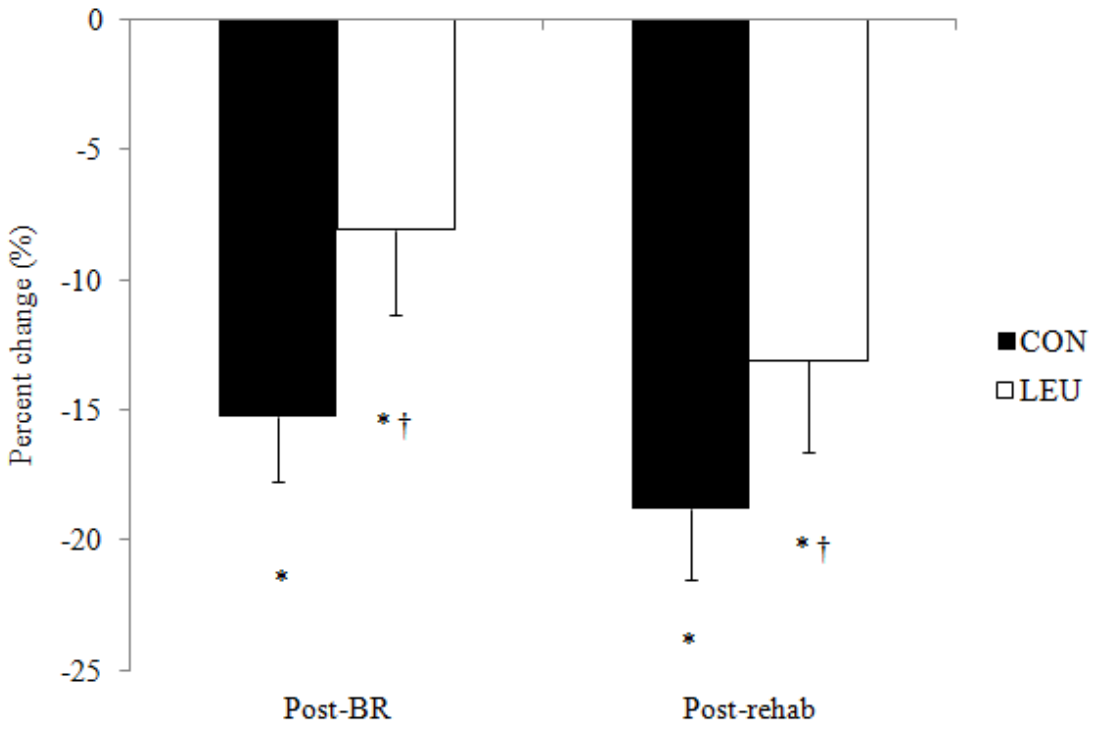


Figure 6. Changes in isokinetic knee extensor strength ($60° \cdot s^{-1}$; percent) for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p<0.05$).

Isokinetic knee extensor strength ($180° \cdot s^{-1}$) was also decreased in CON and LEU post-BR and post-rehab ($p<0.05$; Figure 7). Again however, interaction effects at both post-BR and post-rehab time points revealed that the strength losses were mitigated by LEU supplementation ($p<0.05$).

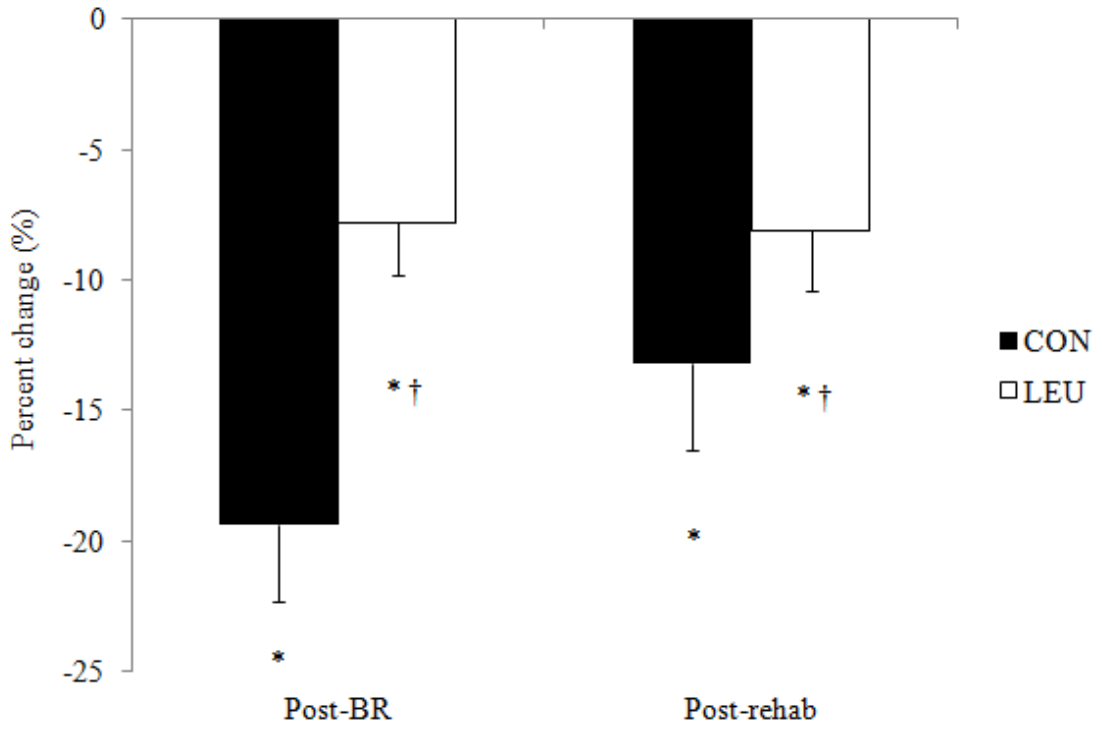


Figure 7. Changes in isokinetic knee extensor strength ($180° \cdot s^{-1}$; percent) for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p<0.05$).

Isokinetic ankle extensor strength ($60° \cdot s^{-1}$) was reduced in CON and LEU post-BR ($p<0.05$) and in CON only post-rehab ($p<0.05$; Figure 8). Also, an interaction effect at post-BR showed an attenuation of strength loss in LEU ($p=0.04$).

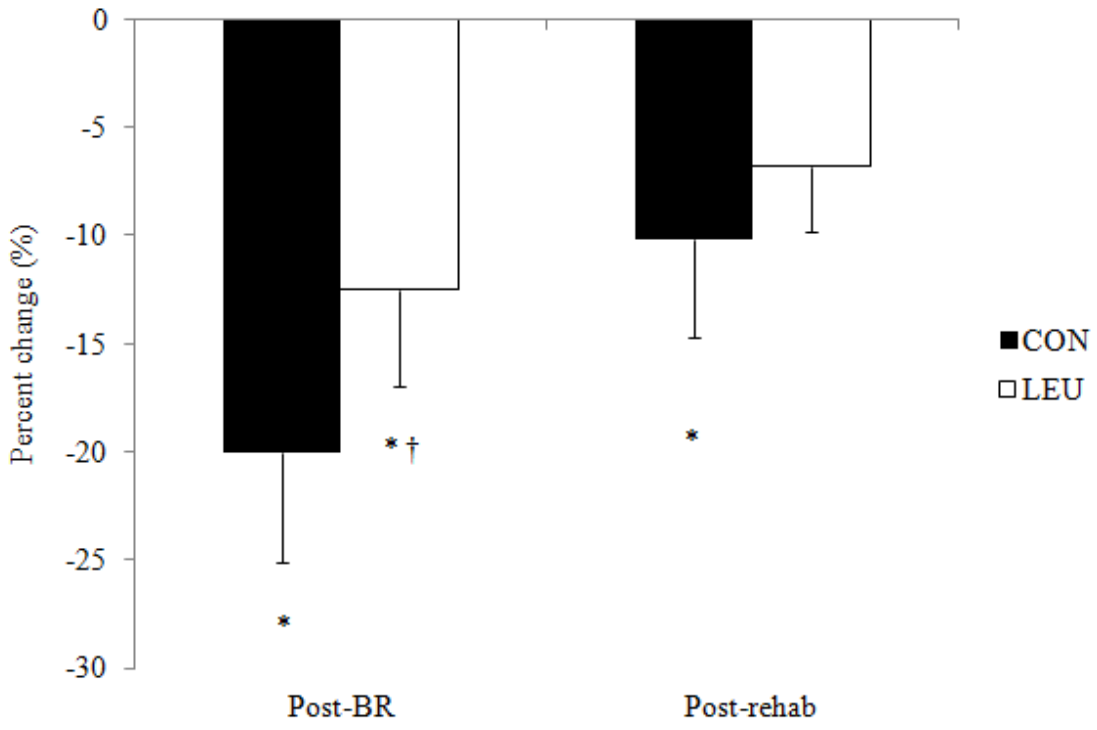


Figure 8. Changes in isokinetic ankle extensor strength (60° · s^{-1}; percent) for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p=0.04$).

Finally, isokinetic knee extensor endurance (total work at 180° · s^{-1}) was reduced only in CON post-BR and post-rehab ($p<0.05$; Figure 9). Also, a post-BR interaction effect revealed a differential change from pre-BR between the groups ($p=0.005$). Table 4 shows absolute values for all isokinetic strength and endurance variables.

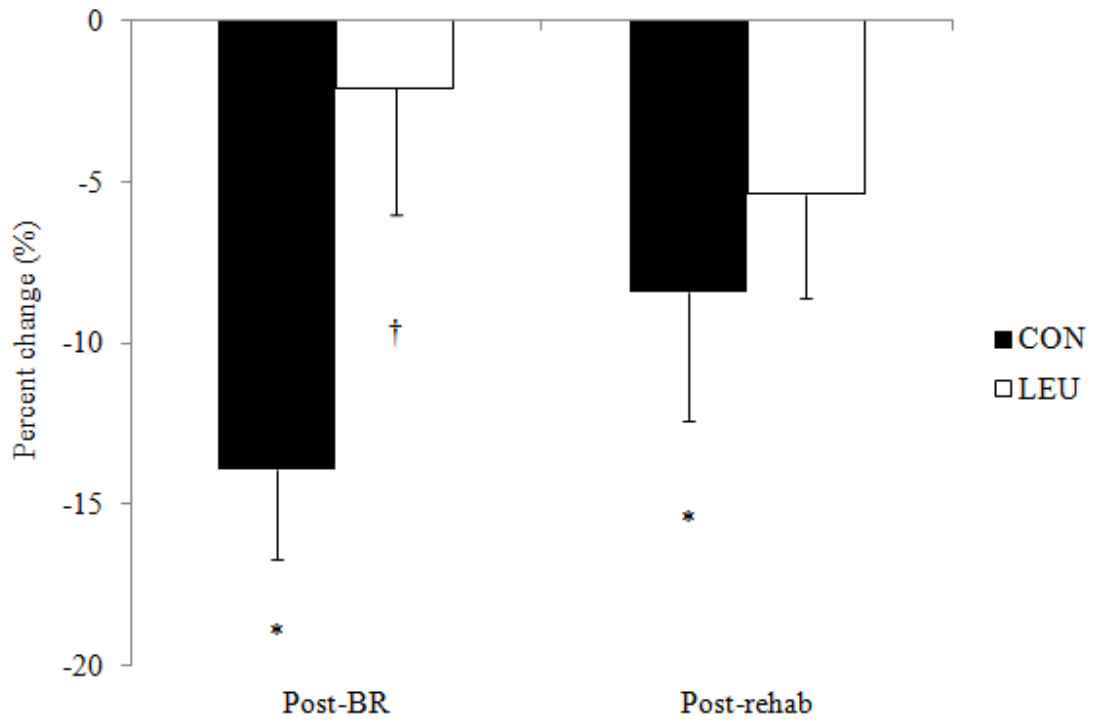


Figure 9. Changes in isokinetic knee extensor endurance (180° · s^{-1}; percent) for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p=0.005$).

Table 4. Muscle strength and endurance for CON and LEU before and after 14 d BR and after 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p<0.05$).

	Pre-BR		Post-BR (Δ)		Post-rehab (Δ)	
	CON	LEU	CON	LEU	CON	LEU
Knee extensor strength (60° · s^{-1}; Nm)	159±12	146±11	-24±4*	-10±4*†	-29±4*	-18±4*†
Knee extensor strength (180° · s^{-1}; Nm)	104±9	103±9	-20±3*	-7±3*†	-16±3*	-8±3*†
Ankle extensor strength (60° · s^{-1}; Nm)	66±5	57±4	-14±3*	-7±2*†	-8±3*	-4±2
Knee extensor endurance (180° · s^{-1}; Nm)	1693±204	1685±182	-251±62*	-20±56†	-179±62*	-74±58

MUSCLE QUALITY

Muscle quality, or relative strength, was reduced only in CON post-BR (p=0.004); this resulted in a significant interaction effect indicative of a differential change from pre-BR between the groups (p=0.01; Figure 10). Paradoxically, muscle quality was decreased in both groups after rehabilitation (p<0.05).

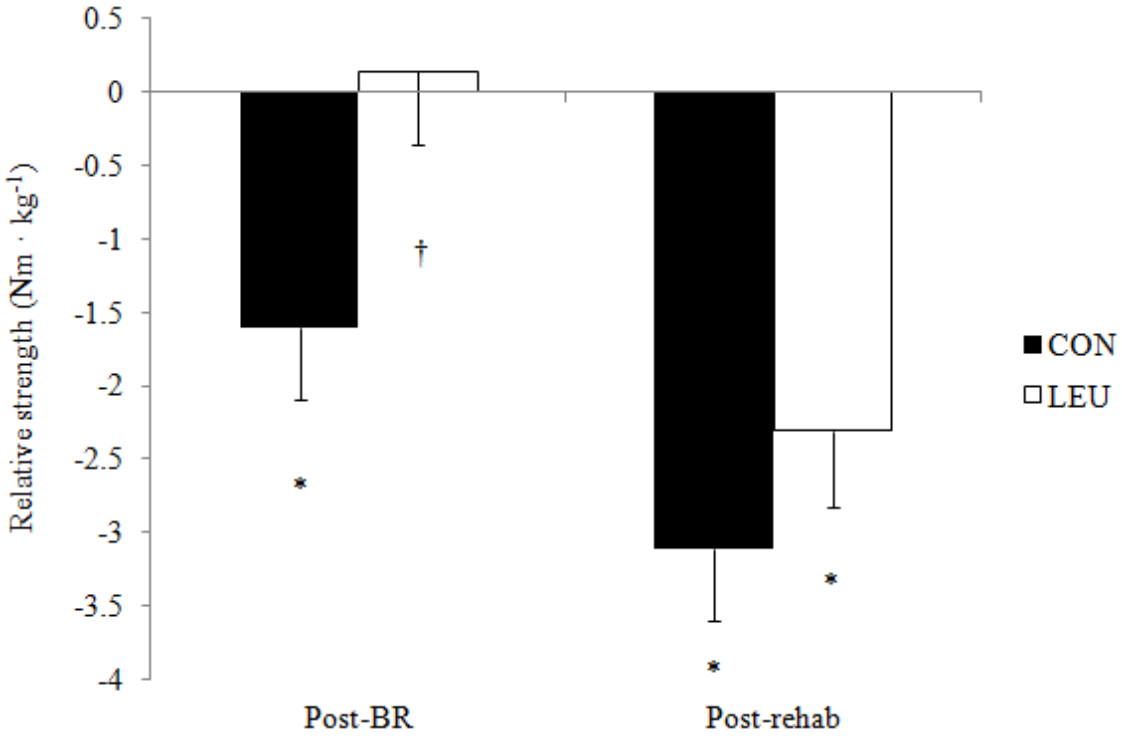


Figure 10. Changes in muscle quality, or relative strength (knee extensor peak torque at 60° · s^{-1}/right leg lean mass; Nm · kg^{-1}) for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR (p<0.05); † significant interaction effect (group x time compared to pre-BR, p=0.01).

MAXIMAL AEROBIC CAPACITY

Relative VO2max (ml · kg^{-1} · min^{-1}) was decreased post-BR in CON only (p=0.04; Figure 11). After rehabilitation, neither group differed from pre-BR mean values. Absolute VO2max was significantly decreased in both groups (p<0.05) after BR

but returned to pre-BR values following rehabilitation. Relative VO2max (ml · kg lean mass^{-1} · min^{-1}) was unchanged in either group at any time point (Table 5).

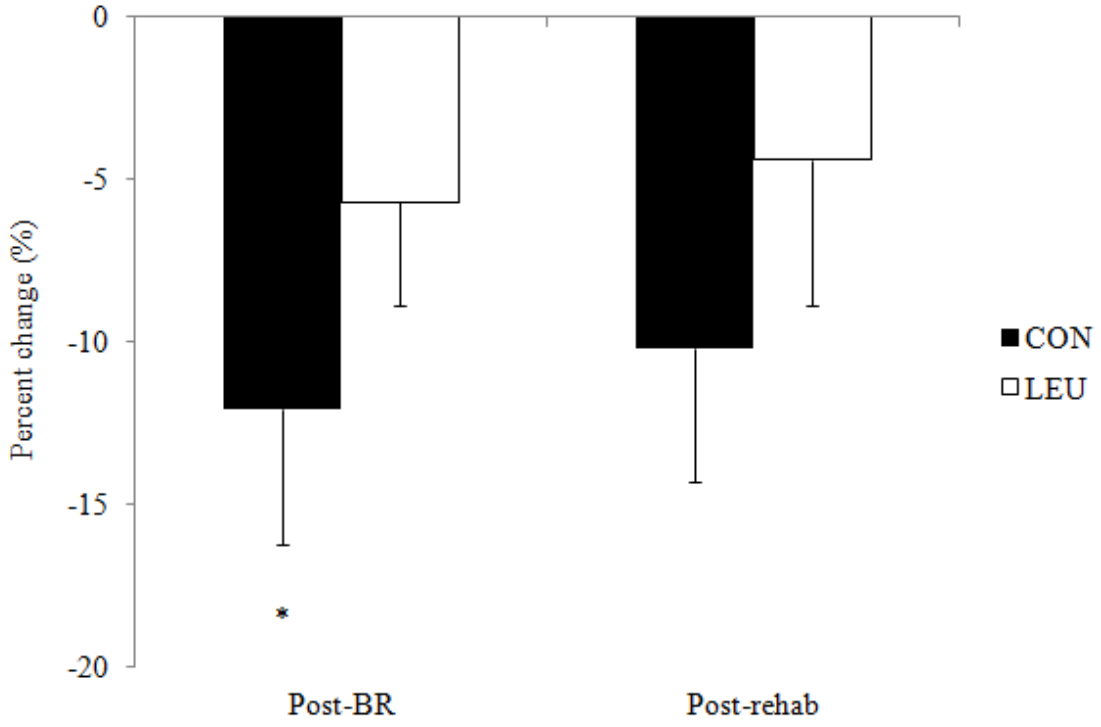


Figure 11. Changes in relative maximal aerobic capacity (ml · kg^{-1} · min^{-1}; percent) for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR (p=0.04).

Table 5. Changes in absolute and relative aerobic capacity for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$).

	Pre-BR		Post-BR (Δ)		Post-rehab (Δ)	
	CON	LEU	CON	LEU	CON	LEU
VO_2max ($L \cdot min^{-1}$)	2.07±0.19	2.20±0.17	-0.27±0.08*	-0.21±0.08*	-0.18±0.08	-0.14±0.07
VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)	27.4±2.2	30.3±2.0	-2.7±1.0*	-2.2±0.9	-1.9±1.1	-1.6±0.9
VO_2max ($ml \cdot kg\ lean\ mass^{-1} \cdot min^{-1}$)	39.9±2.4	44.8±2.2	-3.6±1.7	-3.2±1.5	-2.0±1.7	-3.3±1.5

DISCUSSION

The primary findings of this study were that leucine supplementation during 14 d BR attenuated the loss of whole body lean mass after 7 d BR, prevented changes in body fat, body fat percentage, knee extensor endurance, muscle quality, and aerobic capacity, and reduced losses of knee and ankle extensor strength. After 7 d rehabilitation, leucine restored whole body lean mass and ankle extensor strength and promoted changes in knee extensor strength that were superior to those in CON.

Previous BR studies have almost exclusively examined young adults with a mean age < 40 y (Alkner and Tesch 2004, Bamman et al. 1998, Dudley et al. 1989, Ferrando et al. 1996, Ferrando et al. 1997, LeBlanc et al. 1992, Paddon-Jones et al. 2004, Shackelford et al. 2004, Trappe et al. 2007); only one study to date has employed older adults (Kortebein et al. 2007, Kortebein et al. 2008). In the current study, a group of middle-aged adults was evaluated, a group whose age range and mean fall squarely between those of previous BR study cohorts. Thus, these data are able to provide unique insight into the effects of inactivity on the "pre-elderly," a group that is relevant both to spaceflight, as it reflects the age of most astronauts, and to the general population as it represents the current age range of Baby Boomers.

MUSCLE MASS AND BODY COMPOSITION

The changes in muscle mass seen in the control group of these middle-aged subjects narrate a telling story, as per day decrements in LLM are 2-6 times greater than those reported for young subjects by other investigators. The per day loss for middle-aged subjects is actually very similar (~87%) to that of elderly subjects (Table 6). Given the insidious onset of age-related muscle loss (sarcopenia) around age 40, the rapid loss of muscle mass that this age group experiences with short-term unloading underscores the necessity of swift and effective interventions for both clinical and special (e.g., spaceflight) populations. Indeed, the data from this study strongly corroborate the conceptual notions put forth in the Catabolic Crisis model (English and Paddon-Jones 2010).

Table 6. Comparison of leg lean mass (LLM) loss per day during BR across age ([a]Paddon-Jones et al. 2004, [b]Ferrando et al. 1996, [c]current study, [d]Kortebein et al. 2007).

	BR (d)	Age (y)	Δ LLM (kg)	Δ LLM · d^{-1} (g)
Young[a]	28	38±8	-0.40±0.10	-14
Young[b]	14	30±6	-0.62±0.05	-44
Middle-aged[c]	14	52±4	-1.16±0.14	-83
Older[d]	10	67±5	-0.95±0.15	-95

The leucine supplement did have a muscle sparing effect over the first 7 d of BR. Unfortunately, no mechanistic data (e.g., measures of muscle protein synthesis or cell signaling) were collected at mid-BR to provide insight into the underlying cause of this protective effect. Regardless, leucine shows promise as a simple, stand alone intervention that can attenuate muscle loss during the first 7 d of inactivity. Currently, astronauts on the ISS are not scheduled to exercise during the first 7 d on station; leucine is an easily

implemented countermeasure that could mitigate muscle loss during the first week of spaceflight. Similarly, one week is a common duration for a hospital stay for an acute illness such as community-acquired pneumonia. Leucine could be easily added to the meals of such patients.

Some have suggested that the efficacy of EAA supplementation is due to the net increase that it provides in total protein intake and not to any inherent effect of the essential amino acids *per se* (Stein and Blanc 2011). Stein argues that BR studies that showed a protective effect with EAA supplementation all employed low (≤ 0.8 g $\cdot$ kg^{-1} $\cdot$ d^{-1}) protein intakes while studies that found no effect for EAA supplementation used baseline protein intakes ≥ 1.0 g $\cdot$ kg^{-1} $\cdot$ d^{-1}. The present results provide some refutation to this hypothesis as subjects in this study consumed 1.05 g $\cdot$ kg^{-1} $\cdot$ d^{-1}, an amount well above the RDA.

Stein appropriately highlights the importance of meeting daily energy needs (something lacking in many patient populations, and, historically during spaceflight; (Smith and Zwart 2008, Smith et al. 2005)) and notes that successful BR EAA supplementation involved adequate energy intake, i.e., no loss of total body mass (weight). These contentions appear to hold true for the studies he cites. For instance, Paddon-Jones' EAA/CHO supplement provided subjects with an additional 558 kcal $\cdot$ d^{-1}, such that supplemented subjects saw no change in total body mass while control subjects lost 2.4 kg, making it difficult to separate the effects of the amino acid supplement vs. the extra energy (Paddon-Jones et al. 2004). However, the leucine supplement in the present study provided only ~55 kcal $\cdot$ d^{-1} of additional energy, yet exerted a protective effect in subjects who incurred a small but significant loss of body mass (1.00 kg) and no change in fat mass; control subjects lost a similar amount of body mass (1.24 kg) but increased fat mass.

A potential reconciliation of Stein's thoughts and the current results might be had by examining the differences in leucine content of the supplements and the total energy

intake during BR. Paddon-Jones' effective EAA supplement included 9.3 g leucine · d^{-1} with no change in total body mass (likely due at least in part to additional carbohydrate supplementation)(Paddon-Jones et al. 2004); the ineffective supplements of Brooks et al. and Trappe et al. provided 2.8 g and 3.6 g leucine · d^{-1}, respectively, with total body mass losses of 3.7 and 2.7 kg, respectively, over the BR period (Brooks et al. 2008, Trappe et al. 2007). Total body mass losses in the present study were statistically significant (1.00-1.24 kg), but much less than in these other studies. Taken together, it appears that total energy intake during BR (whether from a supplement or regular food) sufficient to limit decreases in body weight to ~1 kg, is essential to the maintenance of lean tissue mass. With protein and total energy needs met, leucine is an effective intervention to mitigate lean tissue loss.

On the other end of the spectrum, excessive protein/energy intake can also be harmful to muscle mass during inactivity. Trappe et al. found a detrimental effect (i.e., greater muscle mass and strength loss than control) for a high protein diet (~1.45 g · kg^{-1} · d^{-1}) supplemented with 1.2 g leucine · $meal^{-1}$ (total daily protein intake = ~1.6 g · kg^{-1}) in young women during BR (Trappe et al. 2007). The mechanism for this accelerated loss is not certain, but the authors reasonably surmised that it may have been caused by enhanced protein breakdown secondary to increased amino acid stimulation in the absence of mechanical loading (Trappe et al. 2007); this explanation is supported by others' findings although the combined effects of various levels of energy and protein intake on protein turnover during inactivity are complex and remain not fully elucidated (Motil et al. 1981, Steffee et al. 1976, Stuart et al. 1990, Yang et al. 1986). Similarly, Biolo et al. found that young male subjects who gained more body fat during BR due to excess energy intake also lost more lean mass, an effect mediated by increased systemic inflammation and antioxidant defense activity (Biolo et al. 2008). This finding is consistent with other work that has shown increased muscle loss associated with elevated levels of cortisol (Paddon-Jones et al. 2006) and interleukin-6 (Haddad et al. 2005, Schols

and Sidossis 2011). Interestingly, control subjects in the current study had a significant increase in body fat while LEU subjects did not. Also, because this study supplemented with only leucine (~13-14 g · d^{-1}) and not a full complement of EAAs, energy intake was only increased a modest ~55 kcal · $d^{-1.}$ This may represent an ideal with regards to the balance between anabolic stimulus and excess energy as others that supplemented with EAA/CHO mixtures have reported significant increases in fat mass (Paddon-Jones et al. 2004). Moderate protein intakes of 1.0-1.2 g · kg^{-1} · d^{-1}—slightly above the RDA and equivalent to the 25-30 g protein · $meal^{-1}$ that have been recommended for optimal stimulation of MPS (Paddon-Jones and Rasmussen 2009, Symons et al. 2009)—may be important to facilitate the preservation of muscle mass during inactivity. It appears that a narrow range of both protein and energy intake is required to minimize muscle loss/fat gain during physical inactivity as excesses or deficits in either lead to greater changes.

One further nuance of leucine supplementation during inactivity is, not surprisingly, the frequency and volume of the supplement. Brooks et al. reported poor outcomes for an EAA supplement in BR, but their supplement was only provided once per day and contained only 2.8 g leucine (Brooks et al. 2008). Thus, it seems that the protective effect of the leucine supplement in this study is attributable to the fulfillment of several requisite co-factors: 1) adequate total energy intake (i.e., no change in fat mass, small loss of total body mass); 2) moderate protein intake (neither too high nor too low); 3) supplement provided 3 · d^{-1} in sufficient quantity.

MUSCLE STRENGTH, ENDURANCE, AND QUALITY

Leucine supplementation significantly attenuated the loss of knee and ankle extensor strength as well as knee extensor endurance during BR. The losses for knee extensor strength (15-19%), knee extensor endurance (14%), and ankle extensor strength (20%) in the control group were comparable to those seen in other BR studies (Table 7).

In contrast, LEU experienced attenuated losses of only 8%, 2%, and 13%, respectively, for the same strength and endurance measures.

Similar to the discussion above, protein and total energy intake appear to mediate the strength loss experienced during BR (Biolo et al. 2008, Brooks et al. 2008, Trappe et al. 2007). This is not surprising as alterations in mass and strength during inactivity correlate to one another, although changes in strength are often double or more the loss in mass (Table 7). This relationship was also documented in a review by Adams et al. across a variety of unloading conditions (i.e., spaceflight, bed rest, unilateral limb suspension, and immobilization)(Adams et al. 2003). In aging, data at the whole muscle level suggest that the loss of strength in both upper and lower body musculature is due to decreased muscle mass (Frontera et al. 2000, Frontera et al. 1991). Others, however, found a 30% decrease in specific tension (force/CSA) in elderly subjects compared to young controls (Morse et al. 2005); in old rats, Haddad and Adams documented a reduction in normalized force in comparison to young controls (Haddad and Adams 2006). Similarly, at the single fiber level, findings in both rodents (Thompson and Brown 1999) and humans (Frontera et al. 2000) suggest that aging-related changes in strength are not fully explained by decreases in cross-sectional area (CSA) and may be due to dysfunctional cross-bridge formation and myofilament sliding (Frontera et al. 2000).

The coupled relationship between mass and strength also holds true during positive adaptations (i.e., hypertrophy), but with a steeper slope; i.e., increases in muscle strength during resistance exercise training are proportionately greater, but correlated to, gains in muscle mass CSA or volume (Hakkinen et al. 1985, Kawakami et al. 2001, Ploutz et al. 1994). This phenomenon has even been measured dynamically using magnetic resonance imaging (MRI). Ploutz et al. found that, after a 9 week unilateral (one leg train, one leg untrained) knee extensor training program, CSA of the trained leg increased 5% with, predictably, no change in the untrained leg; however, 1-repetition maximum strength increased in both legs (14% in the trained leg and 7% in the

untrained). They also examined muscle activation in the quadriceps via MRI pre- and post-training and found that less muscle was activated during knee extension at set loads; this effect was seen in both the trained and untrained legs. The authors attribute this to enhanced neural activation, which resulted not only in an increase in absolute strength, but also in an increase in strength per unit muscle (Ploutz et al. 1994).

In the current study, LEU lost only 1.6 times more strength than leg lean mass in contrast to the ratio for CON which was 2.3:1. This differential is seen even more clearly in the muscle quality index which indicated no change in relative strength for LEU but a 9% decrease in CON after BR. Unfortunately, muscle quality cannot be calculated for others' studies due to the unavailability of the raw data; however, the ratio of changes in strength to changes in muscle mass functions as a serviceable adjunct to indicate the relationship between changes in these two key measures (Table 7).

Table 7. Changes in mean leg lean mass (LLM), knee extension strength, LLM/knee strength, and ankle extension strength in BR studies of various durations; some studies employed nutritional interventions. Vol = volume, Pro = protein, Con = control, EAA = essential amino acids, CHO = carbohydrate, CSA = cross-sectional area.

Study	BR duration (d)	Group	Δ LLM, CSA, or volume (%)	Δ Knee ext strength (%)	Δ LLM/Δ Knee strength	Δ Ankle ext strength (%)
Alkner 2004	29	CON	-10.0 (vol)			
	89	CON	-18.0 (vol)	-60.0	3.3	
Bamman 1998	14	CON		-14.5		
Berry 1993	30	CON	-11.0 (CSA)			
Current study	14	CON	-6.8 (mass)	-15.3	2.3	-20.0
	14	LEU	-5.0 (mass)	-8.1	1.6	-12.5
Dudley 1989	30	CON		-24		
Ferrando 1996	14	CON	-3.9 (mass)			
Gogia 1988	35	CON		-19.0		-24.4
Kawakami 2001	20	CON	-7.8 (CSA)	-10.9	1.4	
Kortebein 2007	10	CON	-6.3 (mass)	-15.6	2.5	
LeBlanc 1988	35	CON				-26

LeBlanc 1992	7	CON		-14.7		-7.2
	35	CON		-25.2		-12.5
	119	CON	-11.9 (vol)	-30.7	2.6	-19.9
Mulder 2009	60	CON	-13.5 (CSA)	-21.3	1.6	-24.9
Paddon-Jones	28	CON		-17.8		
2004	28	EAA+CHO		-8.8		
Suzuki 1996	20	CON	-10.6 (vol)	-23.6	2.2	
Trappe 2007	29	CON	-16.8 (vol)			
	60	CON	-21 (vol)	-33.7	1.6	-42.1
	29	PRO/LEU	-16.8 (vol)			
	60	PRO/LEU	-24 (vol)	-32.1	1.3	-44.0

(Alkner and Tesch 2004, Bamman et al. 1998, Berry et al. 1993, Dudley et al. 1989, Ferrando et al. 1996, Gogia et al. 1988, Kawakami et al. 2001, Kortebein et al. 2007, LeBlanc et al. 1988, LeBlanc et al. 1992, Mulder et al. 2009, Paddon-Jones et al. 2004, Suzuki et al. 1996, Trappe et al. 2007)

In the present study, it is difficult to ascertain the mechanism of maintained muscle quality, although neural factors may play a role. During the early weeks of strength training or conversely, the first weeks of detraining (while still maintaining normal 1-g ambulation), neural adaptations are rapid while muscle hypertrophy or atrophy is lesser in magnitude and delayed in time course (Berg et al. 1997, Hakkinen and Komi 1985, Ploutz et al. 1994). With acute disuse, such as in BR, atrophy too, is rapid (LeBlanc et al. 1988). During the first several weeks of disuse, these alterations in neural function (e.g., decreased activation and conduction velocity), along with muscle atrophy, together drive decreases in strength (Berg et al. 1997, Duchateau 1995, Kawakami et al. 2001, LeBlanc et al. 1988, Suzuki et al. 1994). Although it is tempting to speculate that neural factors are responsible for the preservation of relative strength and attenuation of strength loss in the current study, there is no clear mechanism by which leucine would affect these.

The most likely explanation for the observed effects of leucine is changes at the single fiber level. Widrick et al. observed a 10% decrement in single soleus fiber CSA after 17 d BR and half of their subjects exhibited a 9-12% reduction in single fiber normalized force, suggesting a loss of contractile protein greater than that of fiber atrophy (Widrick et al. 1997). They further describe a 20-24% decrease in thin filament density at the myofibril level (Riley et al. 1998, Widrick et al. 1997); maintenance of the ratio and spatial orientation of thin to thick filaments via leucine supplementation could possibly explain the preservation of relative strength seen in the present study. Similarly, Larsson et al. noted a 40% decrease in single fiber specific tension (peak force/CSA) in the vastus lateralis with a correlated decline in myofibrillar protein content per muscle cell volume after 42 d BR (Larsson et al. 1996). In a leucine-supplemented group that completed 60 d of BR (previously discussed), Trappe et al. documented significant decreases in force and diameter for myosin heavy chain I (MHC I) fibers (Trappe et al. 2007). However, unlike the control group, there was no decrement in specific tension in the leucine-supplemented

group. This finding, which was in spite of significant losses in total body mass, lean body mass, and muscle strength, may be indicative of leucine's protective influence on functional properties of skeletal muscle single fibers. Although single fiber properties were not measured in the present study, they provide a likely account for the protection of relative strength in LEU, particularly given the more optimal nutritional intake (adequate total energy and moderate protein) of the current subjects.

While complete protection of both muscle mass and strength during inactivity is the aim of any countermeasure, this goal is unrealistic with only nutritional interventions (i.e., without mechanical loading or a heretofore unknown pharmaceutical). The current finding of relative strength maintenance after BR is highly meaningful as it indicates protection of the force-generating capability of subjects' remaining muscle mass with leucine supplementation. Thus, the effect of the supplement extended beyond the protection of strength as mediated by the protection of mass to an actual preservation of the force development capacity of the conserved lean tissue.

MAXIMAL AEROBIC CAPACITY

Both CON and LEU significantly decreased absolute VO_2max after BR (CON: -14%; LEU: -8%). However, only CON experienced a significant decline in VO_2max relative to whole body mass (-12%); LEU did not (-6%). Both relative and absolute aerobic capacity returned to pre-BR levels after rehabilitation in CON and LEU.

Schneider et al. evaluated the effects of leucine supplementation during BR on aerobic capacity; they hypothesized that leucine would reduce the loss of VO_2max by maintaining lean tissue mass, leg strength, and cardiac muscle mass (Schneider et al. 2009). As discussed above, leucine did not maintain lean mass or leg strength (Trappe et al. 2007); although there was no change in ventricular mass, aerobic capacity declined similar to controls (Schneider et al. 2009). An exercise group that did maintain aerobic

capacity saw an increase in cardiac mass and wall thickness (Schneider et al. 2009). Although others have fully (Lee et al. 2009, Watenpaugh et al. 2000) or partially (Stenger et al. 2012) protected aerobic capacity during BR with exercise or artificial gravity, no studies have reported positive outcomes for this parameter with only a nutritional countermeasure (Schneider et al. 2009).

REHABILITATION

Seven day rehabilitation restored WBLM in both groups; however, LLM remained reduced in CON only. Knee extensor strength remained depressed below pre-BR levels in both groups after rehabilitation although the diminution was attenuated in LEU. Knee extensor endurance and ankle plantar flexor strength were both recovered in LEU after 7 d rehabilitation, but still significantly decreased in CON. Aerobic capacity was restored in both groups.

Rehabilitation outcomes are often not a focus of BR studies (Stein and Bolster 2006), perhaps because many are conducted as spaceflight analogs and muscle mass, strength, and aerobic capacity are largely recovered within 30 d of return from spaceflight (English et al. in review, Moore et al. 2010, Trappe et al. 2006). However, 14 d after landing, deficits in muscle size and function do persist (LeBlanc et al. 1995), or in some cases, arise (Narici et al. 2003). Similarly, several BR studies that have reported recovery data found that neither muscle mass, muscle strength (Brooks et al. 2008), nor vertical jump performance (Rittweger et al. 2007) were recovered within 14 d, in some cases despite exercise or EAA supplementation (Brooks et al. 2008). Conversely, with focused reconditioning, lumbar muscle mass is largely restored by 14 d post-BR (Hides et al. 2011).

In the present study, LLM was recovered during rehabilitation in LEU, but not in CON. However, knee extensor strength was not regained in either group. These results

are in concordance with data from spaceflight (English et al. in review, Narici et al. 2003) and BR (English et al. 2011) which indicate that muscle strength after ~14 d of rehabilitation is similar to, if not worse than, muscle strength immediately following prolonged unloading. In addition to focused exercise regimens, rehabilitation from spaceflight or BR is perhaps chiefly characterized by resumption of upright 1-g loading. The effects of such a stimulus are likely as or more profound than those experienced by a novice weight lifter; i.e., while there are many positive adaptations (e.g., acute increases in muscle protein synthesis), concomitantly, the unfamiliarity of gravity-induced muscle contractions also cause elevated inflammatory markers, structural muscle damage, and its associated soreness and discomfort (Clarkson et al. 2006). Narici et al. observed as much after a 17 d spaceflight from which crewmembers returned with no change in muscle function due to exercise performed during the mission, but who subsequently developed increased fatigability and reductions in CSA-normalized ankle extensor torques and other measures of muscle function during 15 d of follow-up testing (Narici et al. 2003). These findings, along with those of National Aeronautics and Space Administration (NASA) personnel responsible for standard post-flight testing and rehabilitation of ISS crewmembers (Kirk English and Mark Guilliams, personal communication) demonstrate the refractory nature of muscle strength recovery from extended periods of mechanical unloading.

Knee extensor endurance and ankle extensor strength were restored to pre-BR values after rehabilitation in LEU, but they remained depressed in CON. These results are indicative of a beneficial effect of leucine supplementation during recovery. Changes in calf muscle size and strength are particularly difficult to prevent during unloading (Trappe et al. 2007, Trappe et al. 2007), or even rehabilitation (Narici et al. 2003); that LEU was able to facilitate recovery in this particularly difficult muscle group is encouraging.

CONCLUSIONS

After 7 d BR, WBLM losses were reduced in LEU, although decreases were similar between CON and LEU after 14 d BR. WBFM and body fat percentage were maintained in LEU but increased in CON after BR. Isokinetic knee and ankle extensor strength loss was attenuated in LEU after BR. Unlike CON, knee extensor endurance, muscle quality, and aerobic capacity were preserved in LEU during BR.

Following 7 d rehabilitation, WBLM and LLM were both recovered in LEU, but LLM remained below pre-BR levels in CON. Body composition was also different between the groups as LEU tended to have decreased WBFM compared to pre-BR and CON tended to be slightly increased. Knee extensor strength after rehabilitation remained decreased in both groups, but the deficit was attenuated in LEU. Knee extensor endurance remained at pre-BR levels in LEU, but was not recovered in CON. Ankle extensor strength was recovered in LEU but remained depressed in CON. Aerobic capacity was returned (CON) or remained (LEU) at pre-BR baseline values after rehabilitation.

Leucine supplementation facilitated improved outcomes in muscle mass, muscle strength and endurance, muscle quality, and aerobic capacity after 14 d BR and 7 d rehabilitation in middle-aged adults. In the absence of mechanical loading, leucine can maintain, or attenuate decrements in, muscle performance outcomes.

Chapter 4: Leucine supplementation maintains muscle protein synthesis and anabolic cell signaling during 14 d bed rest in middle-aged adults

INTRODUCTION

Human skeletal muscle is essential to health and functional performance as both a contractile tissue responsible for mechanical force production and a metabolically active system with an indispensable role in glucose and amino acid metabolism. Skeletal muscle is lost at a rate of 3-8% per decade after the age of 40 in a multifactorial process called sarcopenia which causes decreases in muscle strength, leads to disability (Janssen 2006), and ultimately, to increased mortality (Manini et al. 2007). In light of the mushrooming population of older Americans (84 million people > 65 y by 2050, U.S. Census Bureau), sarcopenia, as an aging disease, has been identified as perhaps the most significant translational science target of our generation (Matthews et al. 2011).

Although aging *per se* results in chronic muscle loss, an even greater danger for older adults is the muscle loss that accompanies even brief periods of disuse (e.g., hospitalization). This phenomenon has been called "Catabolic Crisis" and is characterized by rapid, inactivity-mediated muscle loss and incomplete recovery on resumption of normal activity (English and Paddon-Jones 2010). Fortunately, the acute, episodic nature of these catabolic crises lends them to the implementation of focused interventions that can attenuate or prevent their harmful progression. Although young adults do not suffer from the insidious effects of sarcopenia, they are not immune to inactivity-induced muscle loss. Advanced age, on the other hand, exacerbates the decreases in lean tissue mass associated with mechanical unloading. Paddon-Jones et al. found that young subjects lost 400 g of leg lean mass during 28 d BR whereas a group of older subjects lost 950 g in only 10 d of BR (Kortebein et al. 2007, Paddon-Jones et al.

2004). Underlying the loss in the elderly was a 30% reduction in 24 h muscle protein fractional synthetic rate (FSR).

The acute loss of muscle mass during periods of inactivity is the result of a disturbance in the otherwise homeostatic balance between MPS and protein breakdown. Research evidence indicates that reductions in synthesis and not increases in breakdown are largely to blame for these losses (Ferrando et al. 1996, Kortebein et al. 2007, Paddon-Jones et al. 2006, Stuart et al. 1990). For instance, Paddon-Jones et al. found that phenylalanine rate of appearance in the blood, indicative of protein breakdown, was unchanged after 28 d BR (Paddon-Jones et al. 2004). In contrast, a significant decrease in 16 h muscle FSR was observed. FSR measurements made over prolonged time periods, e.g., 16 h (Paddon-Jones et al. 2004) or 24 h (Kortebein et al. 2007), encapsulate both post-absorptive and post-prandial states as subjects eat several times over the course of these studies. To address the relative contributions of these distinct metabolic states to MPS, others have discretely examined basal and fed state MPS and found that the disuse-associated deficit is due to both a blunting of the otherwise robust post-prandial muscle protein synthetic response and to a decrease in basal MPS (Glover et al. 2008). Specifically, after 14 d of unilateral knee immobilization, Glover et al. found that basal MPS was decreased 27% in the immobilized limb and post-prandial MPS was 54-68% higher in the non-immobilized limb (Glover et al. 2008); these maladaptations are, together, likely implicit in inactivity-mediated lean tissue losses. Thus, interventions that focus on preserving protein anabolism have, appropriately, received notable attention.

Exercise, which preserves muscle mass and strength during disuse (Alkner and Tesch 2004, Shackelford et al. 2004, Trappe et al. 2007), represents a potent countermeasure to alterations in MPS due to hospitalized BR and the mechanical unloading of space flight. Unfortunately, obstacles unique to each environment diminish the practical usefulness of exercise as a viable intervention. During hospitalization, patients are often unwilling to engage in physical activity due to fatigue, illness-induced

malaise, orthopedic issues, fear of falling, lack of support personnel, etc. During long-duration space flight, crew members participate in regular exercise, but still lose muscle mass and strength (Trappe et al. 2009). Exercise on future exploration missions will be restricted by spatial limitations on both the exerciser and the hardware. This may result in an exercise device that is unable to provide the high loading that has been shown to be protective during bed rest unloading and to improve musculoskeletal outcomes in ISS crewmembers (Smith et al. 2012).

Nutrition holds significant promise as a practical countermeasure in both the hospital and space flight environments. In hospital settings, nutritional supplementation can be provided with little or no effort on the part of the patient. In space flight, supplements can be taken in addition to regular exercise; evidence suggests that exercise combined with EAA supplementation synergistically increases MPS (Drummond and Rasmussen 2008, Symons et al. 2011) and better protects muscle mass during unloading (Brooks et al. 2008). Protein supplementation can be used in inactive, elderly inpatient populations, but this results in a reduction in voluntary food intake, rendering the added protein a replacement instead of a supplement (Fiatarone et al. 1994, Fiatarone Singh et al. 2000, Pupovac and Anderson 2002). EAAs increase MPS in both the young and the elderly (Paddon-Jones et al. 2004), but the post-prandial response in the elderly is blunted when a small dose is given (~7 g, (Katsanos et al. 2005)). Much work has focused on differences in muscle metabolism between young and old adults (Paddon-Jones et al. 2004, Volpi et al. 2000, Volpi et al. 1999, Volpi et al. 2001). Unexplored to date has been the muscle metabolism of middle-aged adults; data on this group will deepen understanding of age-related metabolic changes and facilitate the development of better interventions that can be implemented earlier in the lifespan—all with the goal of reducing age- and inactivity-associated muscle mass and performance losses.

Similar to a full complement of EAA, the BCAA leucine has been shown to stimulate MPS and anabolic cell signaling in the young (Glynn et al. 2010) and, in

adequate quantity, to restore the anabolic response to a small EAA serving (Katsanos et al. 2006) and a full meal (Rieu et al. 2006) in the elderly. Further, chronic leucine supplementation in healthy, ambulatory older adults has been shown to increase both basal and post-prandial MPS (Casperson et al. 2012). Therefore, the present study evaluated leucine as a countermeasure to decreases in post-absorptive and post-prandial MPS and in phosphorylation of cellular signaling proteins after 14 d BR and 7 d rehabilitation in healthy middle-aged adults.

METHODS

STABLE ISOTOPE INFUSION STUDIES

At 0700 on Days 4, 18, and 25, an 18-guage polyethylene catheter (Insyte-W, BD Biosciences, Sandy, UT) was inserted into an antecubital vein. Baseline blood samples were drawn for analysis of background amino acid enrichment. A second 18-guage polyethylene catheter was placed in the contralateral antecubital vein and used to maintain a primed (2 µmol/kg), continuous infusion (0.06 µmol/kg/min) of L-[ring-$^{13}C_6$] phenylalanine throughout the study (Figure 12).

Muscle biopsies were obtained from the vastus lateralis muscle approximately 10-15 cm proximal to the knee using a 5 mm Bergstrom biopsy needle; biopsies were performed under local anesthesia (1% lidocaine). The first two biopsies were obtained from a single incision; the third and fourth biopsies were obtained from a second incision made distal to the first.

An 11.8 g EAA drink (Table 8) was dissolved in 250 ml diet soda and consumed in a bolus fashion immediately after the second biopsy. A small amount of L-[ring-$^{13}C_6$] phenylalanine tracer was added to the drink in order to maintain plasma phenylalanine enrichments.

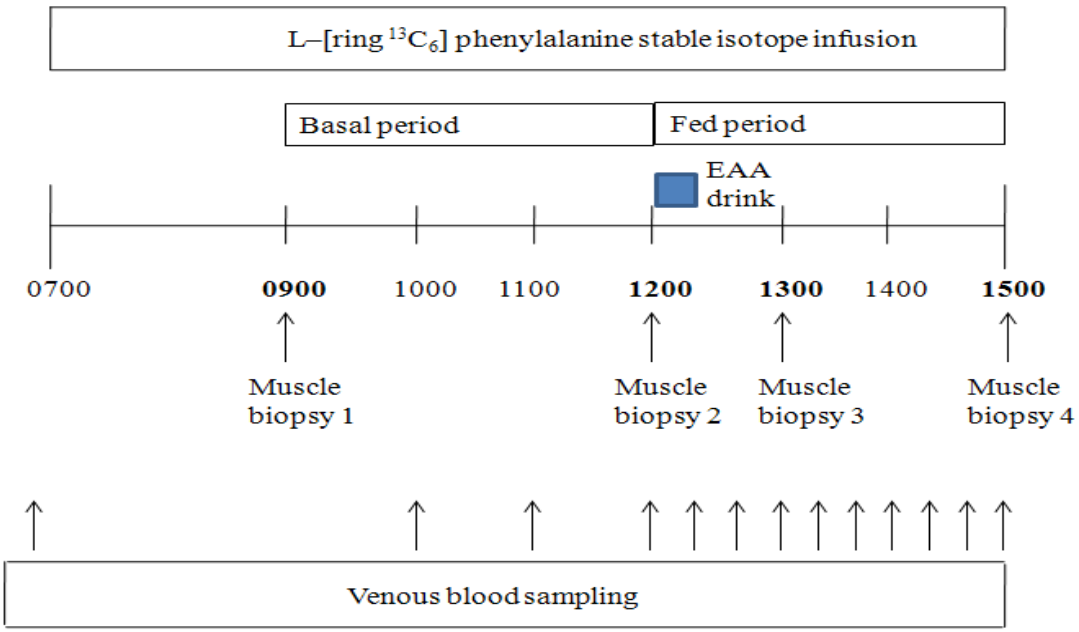


Figure 12. Timeline for stable isotope tracer studies.

Table 8. EAA drink composition.

	Weight (g)	%
Histidine	1.2	10.2
Isoleucine	1.0	8.5
Leucine	2.5	21.2
Lysine	2.5	21.2
Methionine	0.8	6.8
Phenylalanine	1.0	8.5
Threonine	1.2	10.2
Valine	1.5	12.7
L-[ring-$^{13}C_6$] phenylalanine	0.1	0.9
Total EAA	11.8	100.0

***MUSCLE PROTEIN SYNTHESIS*[2]**

Venous blood samples were immediately mixed and precipitated in tubes containing 1 ml sulfosalicylic acid solution. Samples were centrifuged and the supernatant was removed and frozen (-80 C) until analysis. After thawing, blood amino acids were extracted from 500 µl supernatant by cation exchange chromatography (Dowex AG 50W-8X, 100-200 mesh H+ form; Bio-Rad Laboratories, Richmond, NY). Phenylalanine enrichments were determined on the *tert*-butyldimethylsilyl derivative using gas chromatography-mass spectrometry (HP model 5973, Hewlett-Packard Co., Palo Alto, CA) with electron impact ionization. Ions 336 and 342 were monitored.

Muscle biopsy samples from the vastus lateralis were immediately rinsed in ice cold saline, blotted, and frozen in liquid nitrogen until analysis. Frozen samples were cut on dry ice (~25 mg), weighed, and protein was precipitated with 800 µl 10% perchloric acid. Approximately 1.5 ml supernatant were collected after tissue homogenization and centrifugation and processed in the same manner as the supernatant from the blood samples (Dowex AG 50W-8X, 200-400 mesh H+ form; Bio-Rad Laboratories, Richmond, NY). Intracellular phenylalanine enrichments were determined using the *tert*-butyldimethylsilyl derivative. The remaining muscle pellet was washed and dried, and the proteins were hydrolyzed in 3 ml of 6 N HCl at 110° C for 24 h. The protein-bound L-[ring-$^{13}C_6$] phenylalanine enrichments were determined using GCMS with electron impact ionization. Ions 238 and 240 were monitored for bound protein enrichments; ions 336 and 342 were monitored for intracellular enrichments.

[2] These methods have been previously published (42. Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E, Rasmussen BB. Resistance exercise increases ampk activity and reduces 4e-bp1 phosphorylation and protein synthesis in human skeletal muscle. *J Physiol* 2006, 576:613-624, 43. Dreyer HC, Glynn EL, Lujan HL, Fry CS, DiCarlo SE, Rasmussen BB. Chronic paraplegia-induced muscle atrophy downregulates the mtor/s6k1 signaling pathway. *J Appl Physiol* 2008, 104:27-33, 68. Fujita S, Dreyer HC, Drummond MJ, Glynn EL, Cadenas JG, Yoshizawa F, Volpi E, Rasmussen BB. Nutrient signalling in the regulation of human muscle protein synthesis. *J Physiol* 2007, 582:813-823).

The fractional synthetic rate (FSR; %/h) of mixed muscle protein was calculated by measuring the direct incorporation of L-[ring-$^{13}C_6$] phenylalanine into protein using the precursor-product model:

$$FSR = \frac{E_{P2} - E_{P1}}{E_m \, x \, t} x \, 60 \, x \, 100$$

E_{P1} and E_{P2} = bound enrichments of L-[ring-$^{13}C_6$] phenylalanine for two muscle biopsies

E_m = mean enrichment of L-[ring-$^{13}C_6$] phenylalanine in the muscle intracellular pool

t = time interval (min) between the two biopsies (e.g., 180 min)

Post-absorptive (basal) FSR was calculated using biopsy #1 and biopsy #2; FSR in the fed state was determined using biopsy #2 and biopsy #4.

CELL SIGNALING AND IMMUNOBLOTTING[3]

Muscle tissue from biopsy #1 and biopsy #3 was used to determine cellular signaling responses in the basal and fed (1 h post-EAA) states, respectively. Samples of 25-50 mg were homogenized (1:9, weight/volume) in a buffer containing: 50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM

[3] These methods have been previously published (42. Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E, Rasmussen BB. Resistance exercise increases ampk activity and reduces 4e-bp1 phosphorylation and protein synthesis in human skeletal muscle. Ibid. 2006, 576:613-624, 43. Dreyer HC, Glynn EL, Lujan HL, Fry CS, DiCarlo SE, Rasmussen BB. Chronic paraplegia-induced muscle atrophy downregulates the mtor/s6k1 signaling pathway. *J Appl Physiol* 2008, 104:27-33, 68. Fujita S, Dreyer HC, Drummond MJ, Glynn EL, Cadenas JG, Yoshizawa F, Volpi E, Rasmussen BB. Nutrient signalling in the regulation of human muscle protein synthesis. *J Physiol* 2007, 582:813-823).

EGTA, 1% Triton X-100, pH 7.4, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF and 5 μg ml^{-1} soybean trypsin inhibitor (SBTI). DTT, benzamidine, PMSF, and SBTI were added to the buffer immediately prior to use. Supernatant was collected after centrifugation at 3500 *g* for 10 min at 4°C. Then, total protein concentrations were determined using the Bradford assay (Bio-Rad Smartspec plus spectrophotometer).

Aliquots from homogenates were boiled at 100° C for 3 min in 2x sample buffer (SB) containing 125 mM Tris, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol, and 0.002% bromophenol blue. Samples (50 μg total protein per lane) were loaded in duplicate and separated by SDS-PAGE. For mTOR and p70 S6K1 separation, 7.5% gels were run for 60 min at 150 V; 4E-BP1 separation was accomplished with 15% gels also run for 60 min at 150 V. All samples for each subject were run together on one 7.5% gel and one 15% gel. A ladder and internal loading control of rat muscle (10 μg total protein) were also loaded on each gel. Following SDS PAGE, proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Hybond-P; Amersham Biosciences, Piscataway, NJ, USA) at 50 V for 1 h. Once transferred, PVDF membranes were placed in blocking buffer (5% non-fat dry milk (NFDM) in TBST (Tris-buffered saline and 0.1% Tween-20) for 1 h. Blots were then serially washed two times in deionized water and two more times in TBST, and incubated with primary antibody in 5% NFDM in TBST overnight at 4° C with constant agitation. The next morning, the blots were washed in TBST twice, and incubated with secondary antibody for 1 h in 5% NFDM in TBST at room temperature, with constant agitation. After secondary incubation, the blots were washed for 15 min, and then serially washed (4 x 5 min) with TBST. Blots were then incubated for 5 min with enhanced chemiluminescence reagent (ECL plus Western Blotting Detection System; Amersham Biosciences) to detect horseradish peroxidase activity. Optical density measurements were obtained with a CCD camera mounted in a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA, USA). Once the appropriate image was captured, densitometric

analysis was performed using Quantity One 1-D analysis Software (Version 4.5.2; Bio-Rad). The average of the duplicate samples was taken to represent each time point. The value for each sample (arbitrary units) was obtained as follows: (sample density – background density)/(rat standard density – background density). Activity of each protein was expressed as: phosphorylated/total. Subsequently, fold change was calculated as post-prandial activation/post-absorptive activation.

The primary antibodies used were all purchased from Cell Signaling (Beverly, MA, USA): phospho-mTOR (Ser2448; 1:1000), phospho-p70 S6K1 (Thr389; 1:250), and phospho-4E-BP1 (Thr37/46; 1:1000). Anti-rabbit IgG horseradish-peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

RESULTS

Plasma L-[ring-$^{13}C_6$] phenylalanine enrichments during the basal (blood draws 1-3) and the post-prandial periods (blood draws 4-12) did not differ between groups within studies; thus, combined plasma enrichments are presented in Figure 13. Plasma enrichments by group for the basal and post-prandial periods are displayed in Table 9.

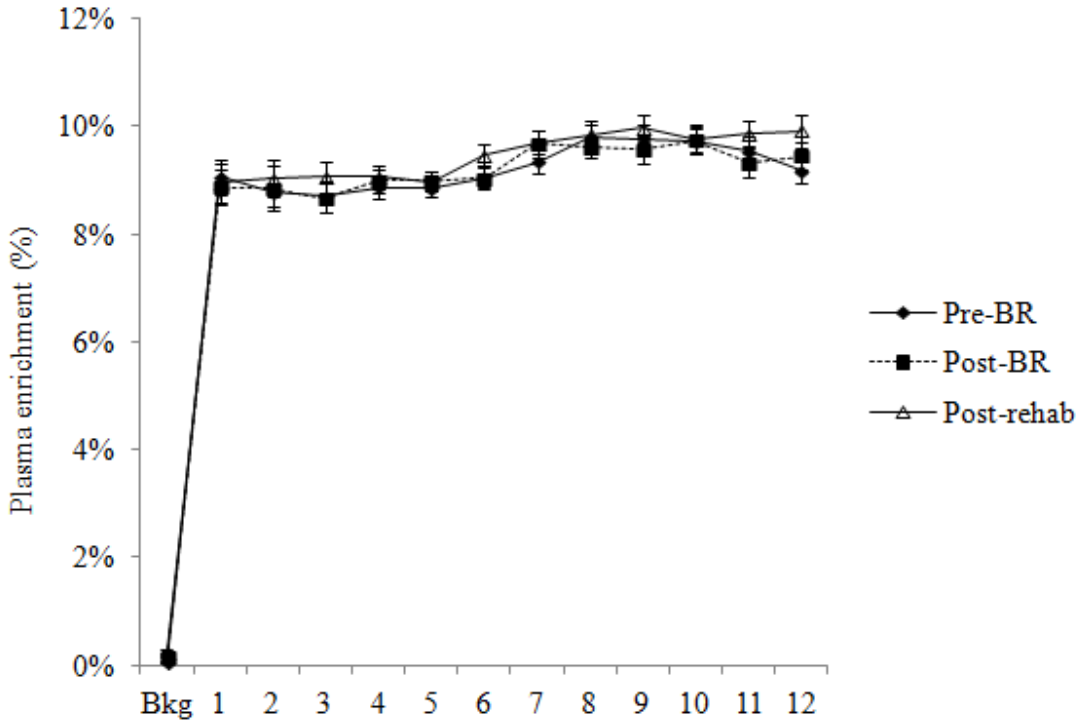


Figure 13. Plasma enrichment of L-[ring-$^{13}C_6$] phenylalanine (tracer/tracee ratio) during metabolic studies prior to and after 14 d BR and 7 d rehabilitation (mean±SE).

Table 9. Plasma enrichment of L-[ring-$^{13}C_6$] phenylalanine (tracer/tracee ratio) during metabolic studies prior to and after 14 d BR and 7 d rehabilitation (mean±SE).

	Pre-BR		Post-BR		Post-rehab	
	CON	LEU	CON	LEU	CON	LEU
Basal plasma enrichment (%)	8.7±0.3	8.7±0.3	9.0±0.3	8.3±0.3	9.3±0.3	8.3±0.3
Post-prandial plasma enrichment (%)	9.7±0.3	9.1±0.3	9.6±0.3	9.1±0.3	9.9±0.3	9.3±0.3

MUSCLE PROTEIN SYNTHESIS

At pre-BR baseline, neither post-absorptive nor post-prandial FSR values differed between groups ($p>0.05$). Fourteen d BR caused significant reductions in post-absorptive FSR in CON (pre-BR: 0.061 ± 0.005 vs. post-BR: 0.043 ± 0.004; $p<0.05$), but post-absorptive FSR was unchanged in LEU (pre-BR: 0.077 ± 0.007 vs. post-BR: 0.067 ± 0.006; Figure 14). BR did not alter post-prandial FSR in either group (CON: pre-BR, 0.084 ± 0.014 vs. post-BR, 0.079 ±0.013; LEU: pre-BR, 0.084 ± 0.014 vs. post-BR, 0.090 ± 0.014; Figure 15).

Seven d rehabilitation returned post-absorptive FSR to pre-BR levels in CON (0.073 ± 0.007) while post-prandial FSR remained unchanged from pre-BR values (0.091 ± 0.020). For LEU, both post-absorptive (0.077 ± 0.007) and post-prandial (0.107 ± 0.017) FSR remained at pre-BR levels following rehabilitation.

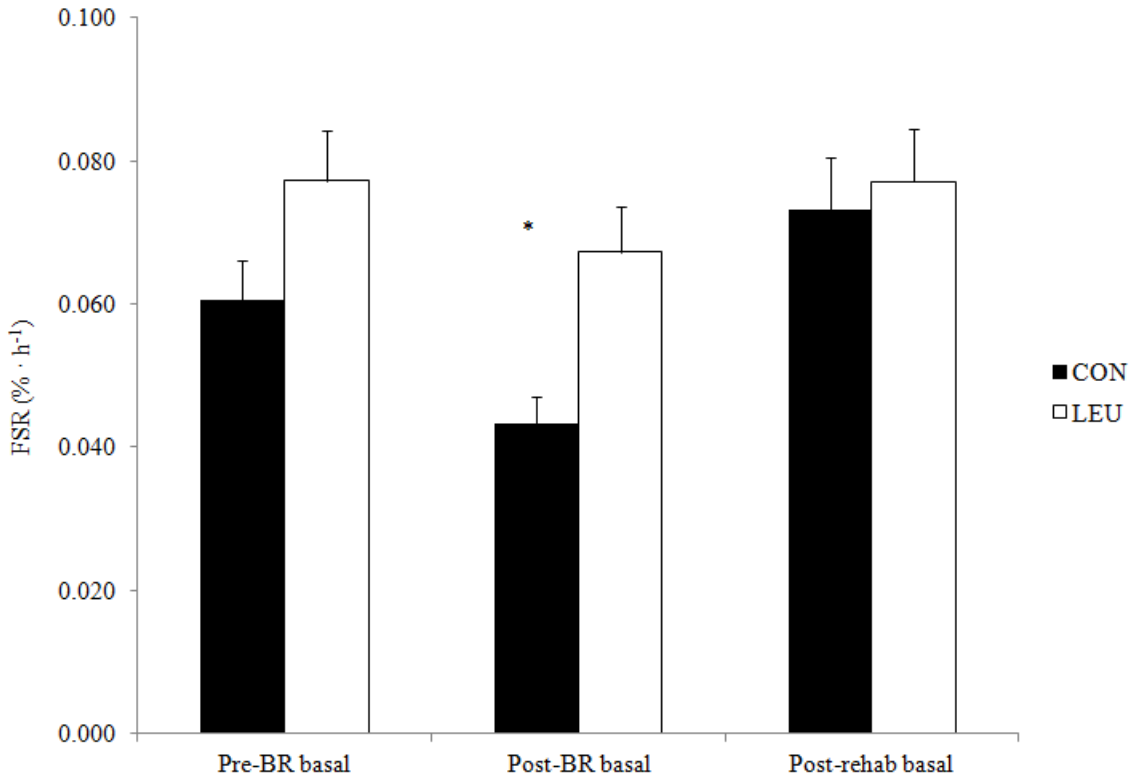


Figure 14. Post-absorptive FSR (% · h^{-1}) for CON and LEU prior to and after 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$).

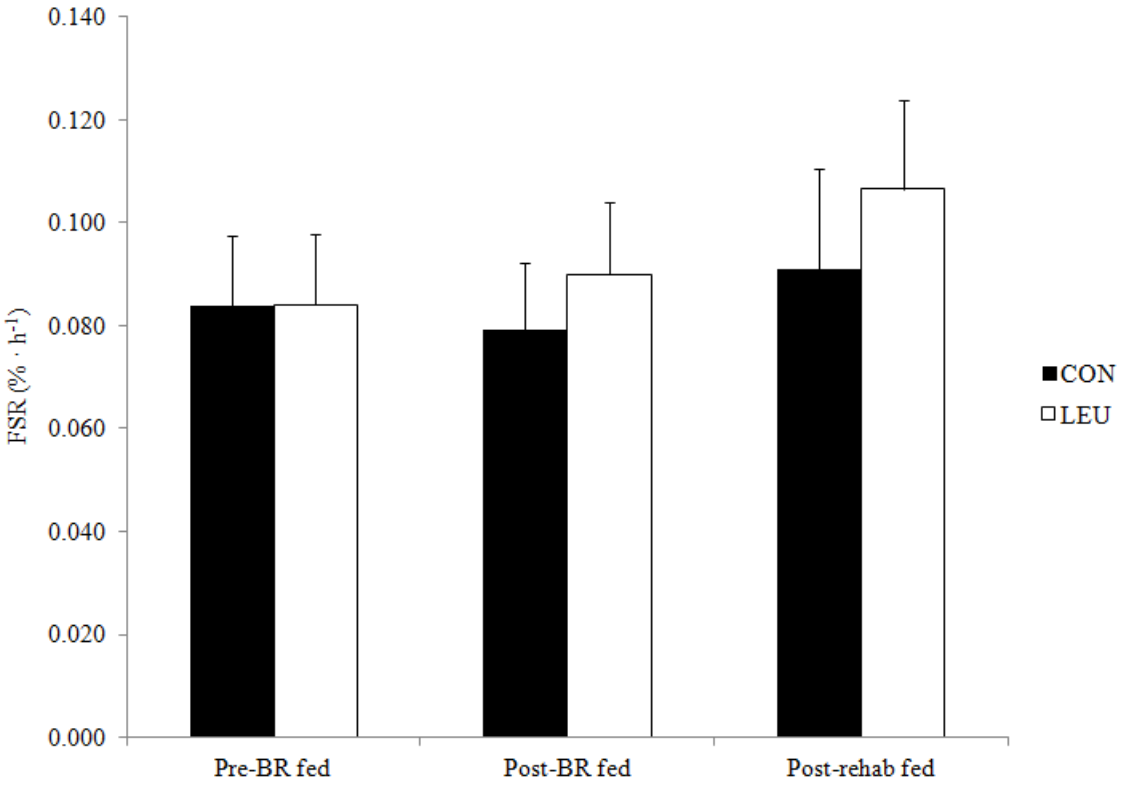


Figure 15. Post-prandial FSR (% · h^{-1}) for CON and LEU prior to and after 14 d BR and 7 d rehabilitation (mean±SE).

CELL SIGNALING

Pre-BR, EAA feeding induced a significant increase in phosphorylated mTOR activation (CON: 2.2 ± 0.3 fold above post-absorptive; LEU: 1.9 ± 0.3 fold above post-absorptive; both $p<0.05$; Figure 16). These responses were unchanged after BR (CON: 2.3 ± 0.4 fold above post-absorptive; LEU: 2.2 ± 0.3 fold above post-absorptive) and rehabilitation (CON: 2.0 ± 0.3 fold above post-absorptive; LEU: 2.1 ± 0.3 fold above post-absorptive).

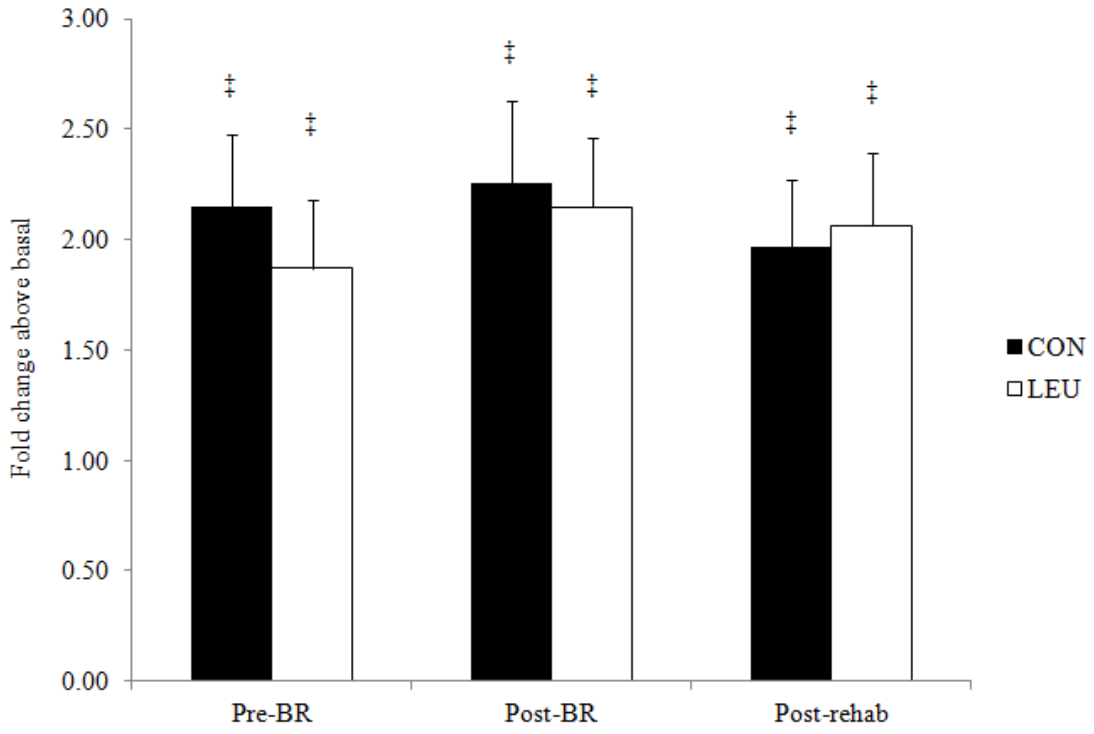


Figure 16. Fold changes (post-prandial/post-absorptive) in phosphorylated mTOR expression relative to total protein for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); ‡ significant effect of feeding ($p<0.05$).

Pre-BR, S6K1 phosphorylation was significantly increased by EAA feeding (CON: 3.2 ± 0.9 fold above post-absorptive; LEU: 3.7 ± 0.8 fold above post-absorptive; both $p<0.05$; Figure 17). Post-BR, this EAA-induced increase in S6K1 expression was maintained in LEU (3.2 ± 0.8 fold above post-absorptive, $p<0.05$), but not in CON (1.5 ± 0.9 fold above post-absorptive; $p>0.05$). After rehabilitation, a significant feeding response was present in both CON (4.5 ± 0.8 fold above post-absorptive; $p<0.05$) and LEU (3.2 ± 1.0 fold above post-absorptive; $p<0.05$).

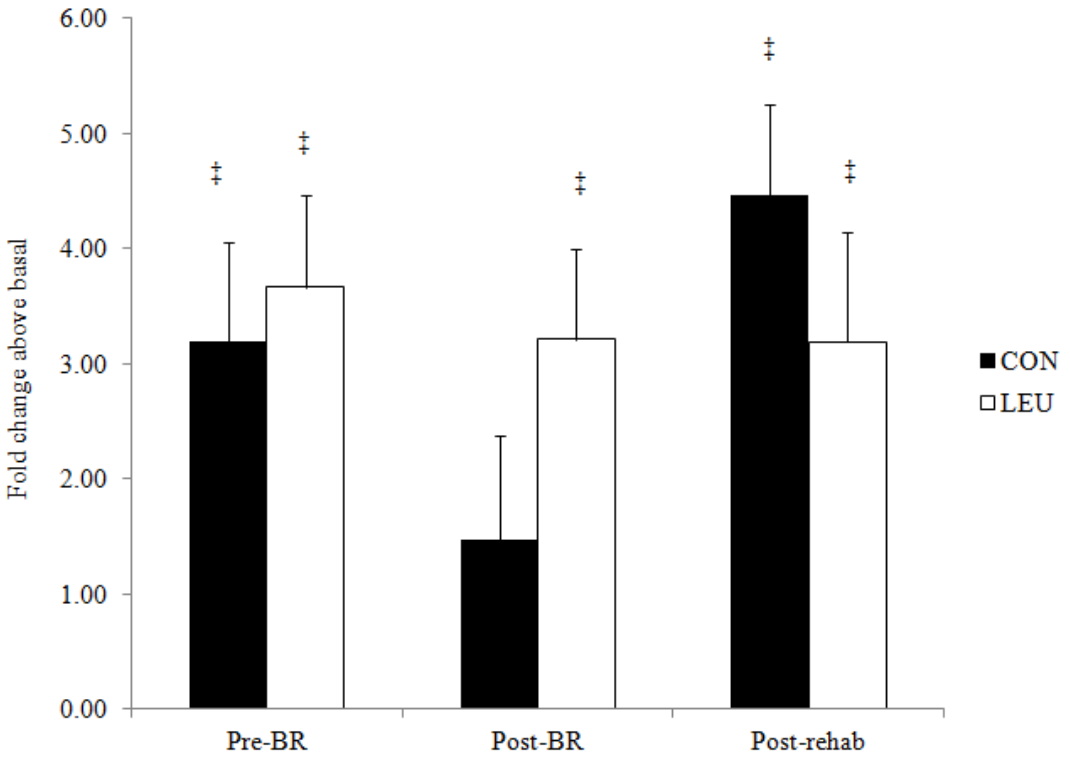


Figure 17. Fold changes (post-prandial/post-absorptive) in phosphorylated S6K1 expression relative to total protein for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); ‡ significant effect of feeding ($p<0.05$).

Prior to BR, 4E-BP1 phosphorylation was significantly increased by EAA feeding (CON: 1.4 ± 0.1 fold above post-absorptive; LEU: 1.5 ± 0.1 fold above post-absorptive; both $p<0.05$; Figure 18). Post-BR, this EAA-induced increase in 4E-BP1 expression was maintained in LEU (1.4 ± 0.1 fold above post-absorptive, $p < 0.05$), but not in CON (1.1 ± 0.1 fold above post-absorptive; $p>0.05$). After rehabilitation, a significant fed response was present in CON (1.3 ± 0.1 fold above post-absorptive; $p < 0.05$) but not LEU (1.3 ± 0.1 fold above post-absorptive; $p<0.05$).

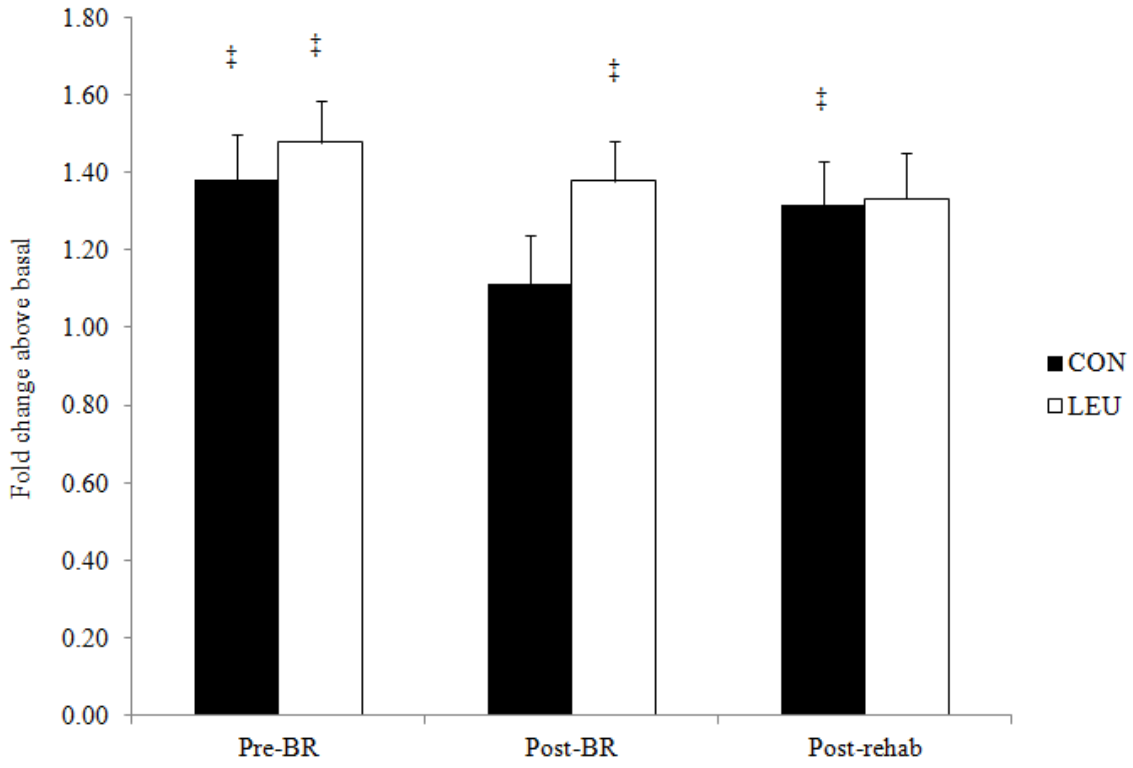


Figure 18. Fold changes (post-prandial/post-absorptive) in phosphorylated 4E-BP1 expression relative to total protein for CON and LEU after 14 d BR and 7 d rehabilitation (mean±SE); ‡ significant effect of feeding ($p<0.05$).

DISCUSSION

Fourteen d BR inactivity decreased basal MPS in CON while leucine supplementation preserved both post-absorptive and post-prandial FSR. Phosphorylation responses of S6K1 and 4E-BP1 to EAA feeding were diminished post-BR. After 7 d rehabilitation, CON returned to, and LEU remained at, pre-BR values for post-absorptive and post-prandial FSR, and cellular signaling responses to feeding.

This study focused its examination of muscle protein kinetics following inactivity on changes in protein synthesis as alterations in muscle protein breakdown are negligible following short-duration BR (Ferrando et al. 1996) and spaceflight (Stein and Schluter 1997). A key premise of acute metabolic studies is that they represent a complete and

accurate portrait of chronic metabolic processes. Thus, a critical question that must be answered when employing metabolic tracer studies to evaluate the effectiveness of a nutritional intervention is: should the post-intervention metabolic study be conducted identical to the pre-intervention study (e.g., provide only a standard meal or EAA drink) or, should the experimental group be studied post-intervention while continuing the experimental treatment (e.g., consuming additional leucine with a standard meal or EAA drink)? To choose the former is to risk missing acute effects of the nutritional intervention that were present during the intervention period but absent during a metabolic study that excluded the nutritional intervention. Conversely, including the nutritional intervention with the post-intervention metabolic study potentially obfuscates any chronic effects of the supplement; i.e., one is unable to differentiate between the acute effect of the supplement during the metabolic study and any chronic effects resulting from the actual intervention period.

In the present investigation, subjects were studied under standardized conditions both pre- and post-nutritional intervention period (i.e., BR and rehabilitation). LEU maintained post-absorptive and post-prandial mixed muscle FSR after BR, but CON experienced a post-BR decrease in post-absorptive FSR. It is possible that had LEU subjects been supplemented during the post-BR and post-rehabilitation metabolic studies that the results would have differed. The 11.8 g EAA drink that was provided at each of the metabolic studies contained 2.5 g leucine. The regular meals served to all subjects throughout this investigation contained ~25-30 g protein (including ~2-3 g leucine) while LEU subjects received an additional ~4.5 g leucine with each of their three daily meals. It is possible that the acute synthetic response during BR and rehabilitation to the regular meals (~2-3 g leucine) plus ~4.5 g supplemental leucine (total = ~7 g leucine) was greater than that observed during the post-BR and post-rehabilitation metabolic studies. Unlike young individuals (31 y), older adults (68 y), do not significantly increase muscle protein accretion above post-absorptive levels in response to small doses of EAA (EAA = 6.7 g,

including 1.7 g leucine) (Katsanos et al. 2005). However, when additional leucine is provided (1.7 + 1.1 g = 2.8 g leucine), older adults' synthetic response is normalized (Katsanos et al. 2006). These studies were conducted in ambulatory individuals; corresponding data are not available for unloaded populations. However, one might expect that additional leucine may be necessary to fully stimulate post-prandial MPS in non-young (≥ 45 y) individuals during disuse due to the associated anabolic resistance to feeding (Glover et al. 2008). In a study that very closely mimicked the daily meals served to both CON and LEU subjects in the present investigation, Rieu et al. demonstrated that a mixed meal (~755 kcal with 30 g protein (leucine: 2.4 g)) supplemented with leucine (~3.9 g; total leucine = 6.3 g) increased MPS in older adults while the same meal with supplementary alanine failed to do so (Rieu et al. 2006). The 2.4 g of leucine in Rieu et al.'s control meal is almost identical to the leucine content of the present study's regular meals as well as the EAA drink provided during each metabolic study; in light of these data, it is perhaps unsurprising that the present subjects did not exhibit robust post-prandial MPS responses. Nevertheless, regardless of the magnitude of the post-prandial MPS response, fed FSR values were maintained in both groups after BR and rehabilitation.

Only a few studies have reported FSR after disuse. Ferrando et al. observed a 45% decrease in basal FSR in young men after 14 d BR; post-prandial MPS was not assessed (Ferrando et al. 1996). In young adults, Glover et al. noted a 27% decrease in basal FSR after 14 d knee immobilization and a 54-68% greater post-prandial FSR in the non-immobilized vs. immobilized leg (Glover et al. 2008). After 7 d BR in older adults, Drummond et al. reported an insignificant decrease in basal FSR and a significant decrease in post-prandial FSR following ingestion of an 11.8 g EAA (2.5 g leucine) drink identical to the one used in the present study (Drummond et al. 2012). Ferrando et al. measured 24 h FSR (which included post-absorptive and post-prandial time periods) in older adults after 10 d BR; subjects consumed normal meals throughout the 24 h period

and the experimental group also ingested 3 x 15 g EAA (including 3 x 5.4 g leucine), the supplement regimen that they had followed throughout BR. FSR decreased 30% in control subjects but was maintained in those supplemented with EAA (Ferrando et al. 2010). It is noteworthy that 24 h FSR was similar between control and supplemented subjects pre-BR despite provision of additional EAA/leucine during the metabolic study; i.e., no effect of the supplement was apparent until a metabolic challenge (i.e., BR) reduced MPS in the control group. Employing a similar protocol in young individuals, Paddon-Jones et al. saw a trend ($p=0.09$) toward decreased 16 h FSR in controls after 28 d BR and no change in EAA-supplemented subjects; however, unlike older subjects, FSR was higher in EAA-supplemented subjects both pre- and post-BR (Paddon-Jones et al. 2004). None of these studies employed the same standardized, basal/fed metabolic study protocol after a supplemented vs. control period of BR like that used in the present investigation; the studies described above differ either in the metabolic study protocol or did not provide nutritional supplementation during BR. Regardless, the present study's finding of reduced basal FSR in control subjects is in common with the reports of others (Ferrando et al. 1996, Glover et al. 2008).

Muscle loss during the first 7 d of BR was significantly attenuated in LEU (Ch. 3); however, this effect was not present after 14 d BR although CON lost, on average, 200 g more WBLM than LEU subjects. These losses are perhaps accounted for by the reductions in basal FSR after 14 d BR although it is unclear why muscle mass was not better preserved in LEU during the second week of BR. No metabolic studies were performed after 7 d of BR; thus, we have no insight into the metabolic status that contributed to the significant attenuation of muscle loss in the LEU group during the first week of BR. The muscle losses seen in these middle-aged CON subjects are quite comparable to those observed in older adults (Kortebein et al. 2007, Paddon-Jones et al. 2004); thus, it is tempting to speculate that FSR results from previous investigations in the elderly (Drummond et al. 2012, Ferrando et al. 2010, Kortebein et al. 2007) should be

similar to our data. Regardless, it is essential to remember that leucine supplementation may very well have exerted beneficial effects throughout the bed rest period that were not captured by non-supplemented metabolic studies. Alternatively stated, the metabolic conditions that were employed presented a more stringent evaluation than had the LEU group been allowed to continue supplementation during the metabolic study.

Studies conducted in ambulatory, community-dwelling elderly also provide insight into the effects of chronic EAA/leucine supplementation on MPS. Dillon et al. supplemented older women with EAA (2 x 7.5 g · d^{-1}; leucine content: 2 x 1.4 g · d^{-1}) for three months and observed an increase in basal FSR. Casperson et al. evaluated a two week, leucine-only supplement regimen (3 x 4 g · d^{-1}) in older men and women and found that it increased both basal and post-prandial FSR (2012).

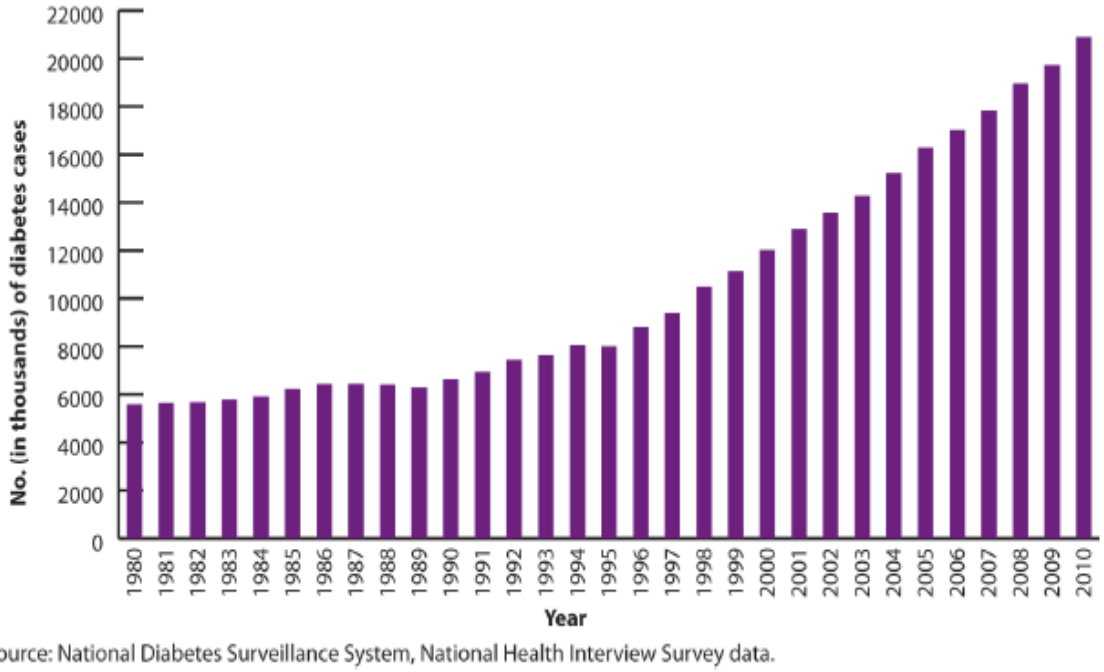
The cell signaling results of the present study compare favorably with those of Drummond et al. (Drummond et al. 2012). Both found that the robust feeding response of S6K1 and 4E-BP1 to EAA ingestion, present before BR, was absent post-BR. In contrast, leucine supplementation maintained the fed response of S6K1 and 4E-BP1 post-BR. Feeding-induced increases in mTOR phosphorylation were unchanged in both groups post-BR; this result differed from that of the older adults investigated by Drummond et al. (Drummond et al. 2012) and may explain the maintenance of post-prandial FSR values in the present study as mTOR activation is essential to feeding-induced increases in MPS (Dickinson et al. 2011). Anabolic cell signaling responses to EAA were restored after 7 d rehabilitation; this is the first report on these parameters after a recovery period from disuse in humans.

In summary, 14 d BR reduced post-absorptive MPS and EAA-induced increases in S6K1 and 4E-BP1 expression; these maladaptations were prevented with chronic leucine supplementation during BR. MPS and anabolic cell signaling responses returned to pre-BR levels in both groups after 7 d rehabilitation.

Chapter 5: Leucine maintains insulin sensitivity during 14 d bed rest and improves it after 7 d rehabilitation in middle-aged adults.

INTRODUCTION

The prevalence and incidence of Type II diabetes mellitus (DM2), which comprises ~95% of diabetes cases, and its ubiquitous set of risk factors, termed the 'metabolic syndrome', have been increasing for the last several decades (Figure 19Figure 1Figure 20). DM2 is defined by glucose intolerance which typically develops from impaired insulin sensitivity. Reduced insulin sensitivity is a hallmark of physical inactivity and thus a common feature of hospitalization; it has also been documented in the weightlessness of spaceflight (Stein et al. 1994, Tobin et al. 2002).



Source: National Diabetes Surveillance System, National Health Interview Survey data.

Figure 19. Diabetes prevalence in U.S. adults (18-79 y) from 1980-2010 (Centers for Disease Control and Prevention).

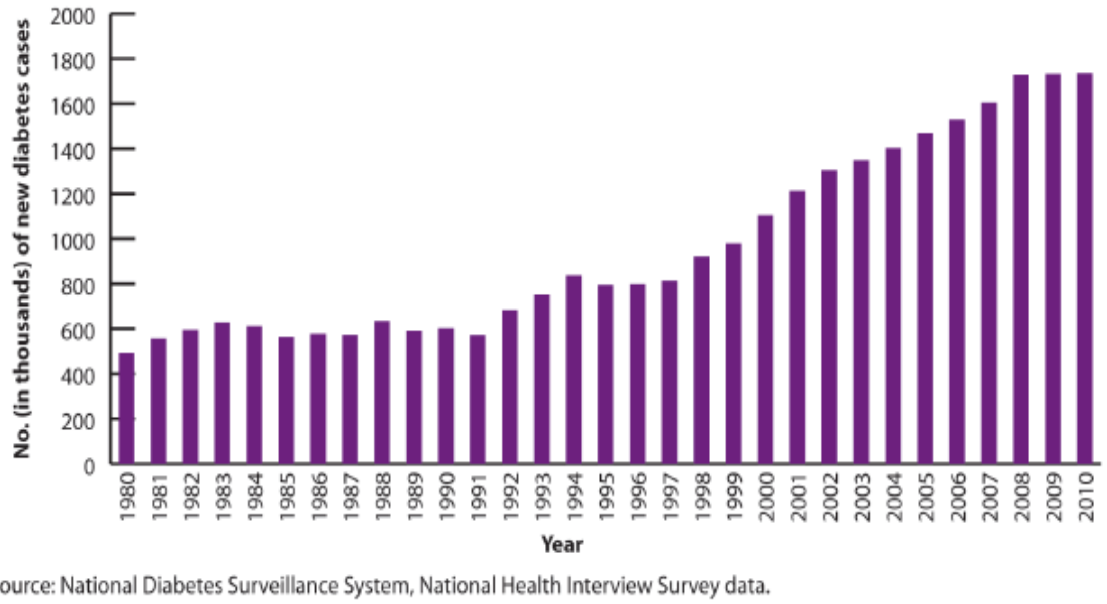


Figure 20. Diabetes incidence in U.S. adults (18-79 y) from 1980-2010 (Centers for Disease Control and Prevention).

Skeletal muscle is the primary site of glucose uptake both at rest and during exercise (Stump et al. 2006). A highly plastic tissue that is indispensable to movement and exercise, the positive effects of physical activity on insulin sensitivity in skeletal muscle are clear as even a single bout of exercise has been shown to improve insulin sensitivity (Magkos et al. 2008). With chronic exercise training, insulin sensitivity is improved secondary to increased leg lean mass (Nassis et al. 2005).

Unfortunately, a significant decrease in physical activity and/or mechanical loading is virtually inherent in both clinical (e.g., hospitalization) and applied (e.g., spaceflight) settings (Fisher et al. 2011). This disuse leads to a pronounced loss of muscle mass and strength. The EAA leucine has been shown to counteract these losses during

experimental bed rest (Ch 3). Thus, it follows that the effects of leucine supplementation on glucose tolerance and insulin sensitivity during BR would also be of considerable interest.

It is well-established that leucine acutely stimulates the release of insulin (Floyd et al. 1966) although other amino acids produce more dramatic elevations in plasma insulin. Indeed, intact beef and chicken protein with their full complement of EAAs elicit the greatest increases in insulin (Floyd et al. 1966). As a potent stimulator of mTOR and its downstream target, S6K1, leucine has been shown to impair insulin signaling in muscle cells via a reduction of phosphatidylinositol (PI) 3-kinase activity and to thus induce insulin resistance in L6 skeletal muscle cells (Tremblay and Marette 2001). In young rats supplemented with leucine, Balage et al. found a reduction in tyrosine phosphorylation of insulin receptor (IR), insulin receptor substrate 1 (IRS-1), and PI3K although S6K1 and 4E-BP1 phosphorylation was similar to controls (Balage et al. 2011). Further, insulin sensitivity was decreased following chronic leucine supplementation as evidenced by elevated $\text{insulin}_{\text{AUC}}$ during OGTT (Balage et al. 2011).

Others, however, have observed beneficial effects of leucine on glucose tolerance and insulin sensitivity. Zhang et al. demonstrated that leucine supplementation (delivered *ad libitum* via drinking water) attenuated deleterious changes in fat mass, hyperglycemia, and insulin sensitivity in mice fed a high fat diet, a model of metabolic stress/insulin resistance (Zhang et al. 2007). Using similar methodology, Macotela et al. reported that leucine supplementation improved insulin-stimulated phosphorylation of IR, IRS-1, and Akt despite a concomitant increase in p-70S6K1 phosphorylation greater than that seen in high fat diet-only mice (Macotela et al. 2011). Zeanandin et al. noted that chronic leucine supplementation in old rats did not alter mTOR activation or intracellular insulin signaling and actually improved insulin-mediated glucose uptake (Zeanandin et al. 2012).

Therefore, we evaluated leucine as a countermeasure to changes in glucose uptake and insulin sensitivity during 14 d of BR inactivity and 7 d of rehabilitation in healthy, glucose tolerant middle-aged adults.

METHODS

BLOOD GLUCOSE AND INSULIN

A standard oral glucose tolerance test (OGTT) was performed to assess changes in glucose and insulin kinetics. Subjects were excluded from the study if they displayed: 1) fasting blood glucose > 100 mg · dl^{-1}, 2) 2-h blood glucose > 200 mg · dl^{-1}, or 3) two blood glucose values during the 2-h OGTT > 200 mg · dl^{-1}; thus, all subjects exhibited normal glucose tolerance prior to BR (Table xx). On four mornings [pre-BR (Day 3), mid-BR (Day 10), post-BR (Day 17), and post-rehabilitation (Day 24)] following a 12 h overnight fast, a catheter was placed in an antecubital vein and a baseline blood sample obtained. Subjects then consumed a 75 g serving of glucose (Limeondex, Fisherbrand). Subsequent 5 ml blood samples were obtained 30, 60, 90, and 120 min after drink ingestion. Whole blood glucose concentrations were analyzed using an automated blood glucose analyzer (YSI 2300 STAT PLUS, YSI Inc., Yellow Springs, OH); fasting blood glucose values are displayed in Table 10. Serum insulin concentrations were measured via an IMMULITE 2000 (Siemens Healthcare Diagnostics, Deerfield, IL); fasting plasma insulin values are displayed in Table 11.

Table 10. Fasting whole blood glucose (mg · dl^{-1}) at baseline and after 7 and 14 d BR and 7 d rehabilitation (mean±SE) in control and leucine-supplemented subjects.

	Pre-BR	Mid-BR	Post-BR	Post-rehab
CON	78.2 ± 2.4	75.1 ± 2.4	76.3 ± 2.4	76.1 ± 2.4
LEU	81.6 ± 2.3	81.5 ± 2.3	80.9 ± 2.3	80.2 ± 2.3

Table 11. Fasting plasma insulin (µIU · ml^{-1}) at baseline and after 7 and 14 d BR and 7 d rehabilitation (mean±SE) in control and leucine-supplemented subjects.

	Pre-BR	Mid-BR	Post-BR	Post-rehab
CON	6.0 ± 3.5	5.4 ± 3.3	6.1 ± 4.1	5.9 ± 3.9
LEU	4.5 ± 1.0	4.1 ± 0.9	4.6 ± 1.0	3.3 ± 0.6

WHOLE BODY INSULIN SENSITIVITY INDEX

Whole body insulin sensitivity (WBISI) was calculated for each OGTT using the index developed by Matsuda and DeFronzo (Matsuda and DeFronzo 1999) (Figure 21):

$$\frac{10{,}000}{\sqrt{(fasting\ glucose \times fasting\ insulin) \times (mean\ OGTT\ glucose \times mean\ OGTT\ insulin)}}$$

Figure 21. Whole body sensitivity index (WBISI; (Matsuda and DeFronzo 1999)); higher values indicate superior insulin sensitivity.

RESULTS

BLOOD GLUCOSE AND INSULIN

Whole blood glucose area under the curve (AUC) during OGTT was elevated in CON and LEU at mid-BR ($p<0.05$); post-BR, glucose was increased above pre-BR levels only in LEU ($p<0.05$; Figure 22). After 7 d rehabilitation, glucose values returned to pre-BR baseline in LEU but remained elevated in CON ($p<0.05$); this resulted in a significant group x time interaction effect compared to post-BR glucose values ($p=0.02$).

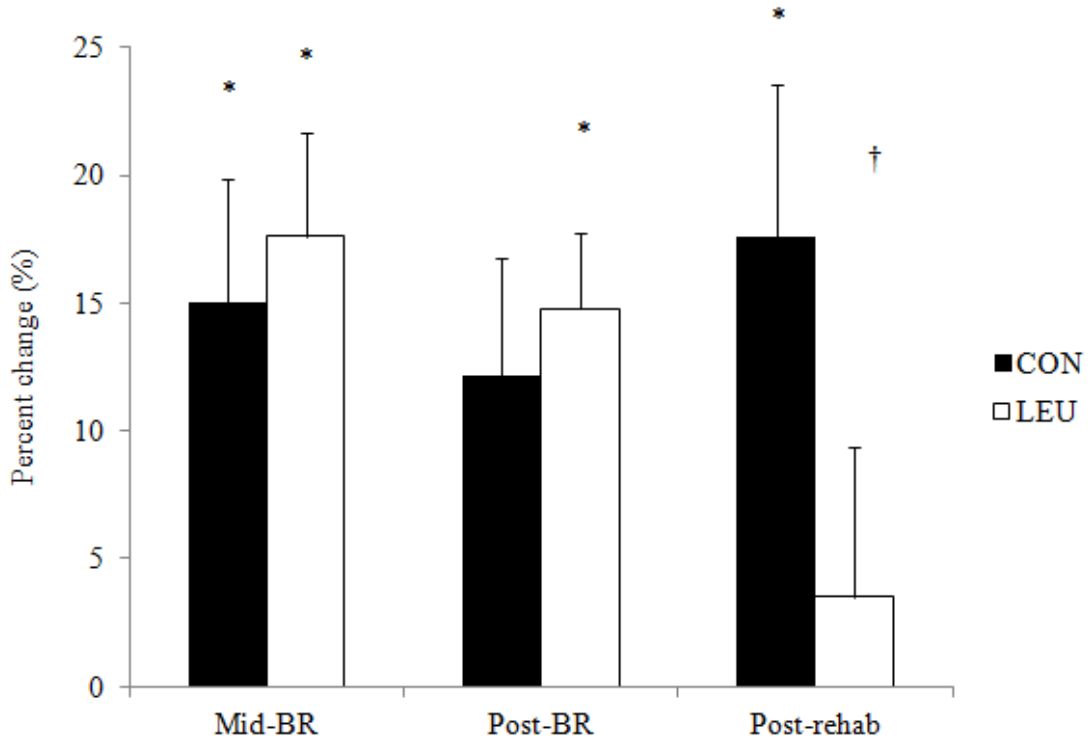


Figure 22. Changes in whole blood glucose area under the curve during OGTT for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to post-BR, $p=0.02$).

Plasma insulin AUC was significantly increased ($p<0.05$) mid-BR and post-BR in CON only; this resulted in a significant group x time interaction effect at mid-BR

(p=0.03; Figure 23). CON returned to, and LEU remained at, pre-BR levels after 7 d rehabilitation.

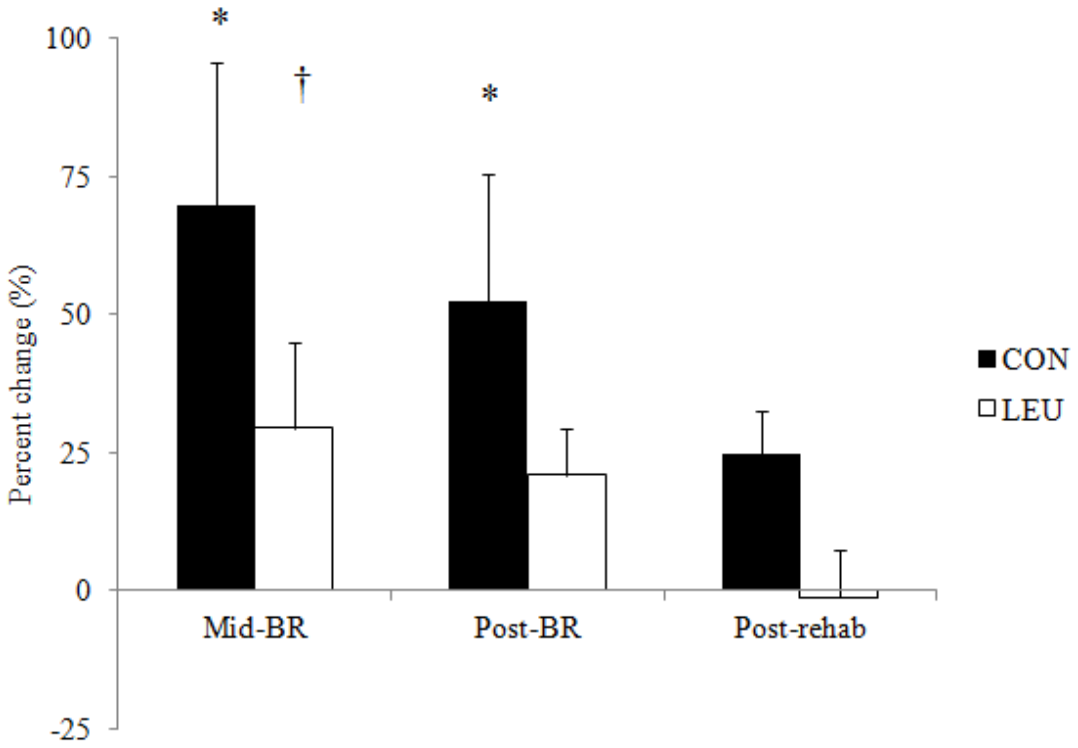


Figure 23. Changes in plasma insulin area under the curve during OGTT for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR ($p<0.05$); † significant interaction effect (group x time compared to pre-BR, $p=0.03$).

WHOLE BODY INSULIN SENSITIVITY INDEX

WBISI was unchanged in both groups during BR. However, WBISI was significantly increased in LEU after 7 d rehabilitation ($p<0.05$) while it was unchanged in CON; this resulted in a group x time interaction effect ($p<0.05$; Figure 24).

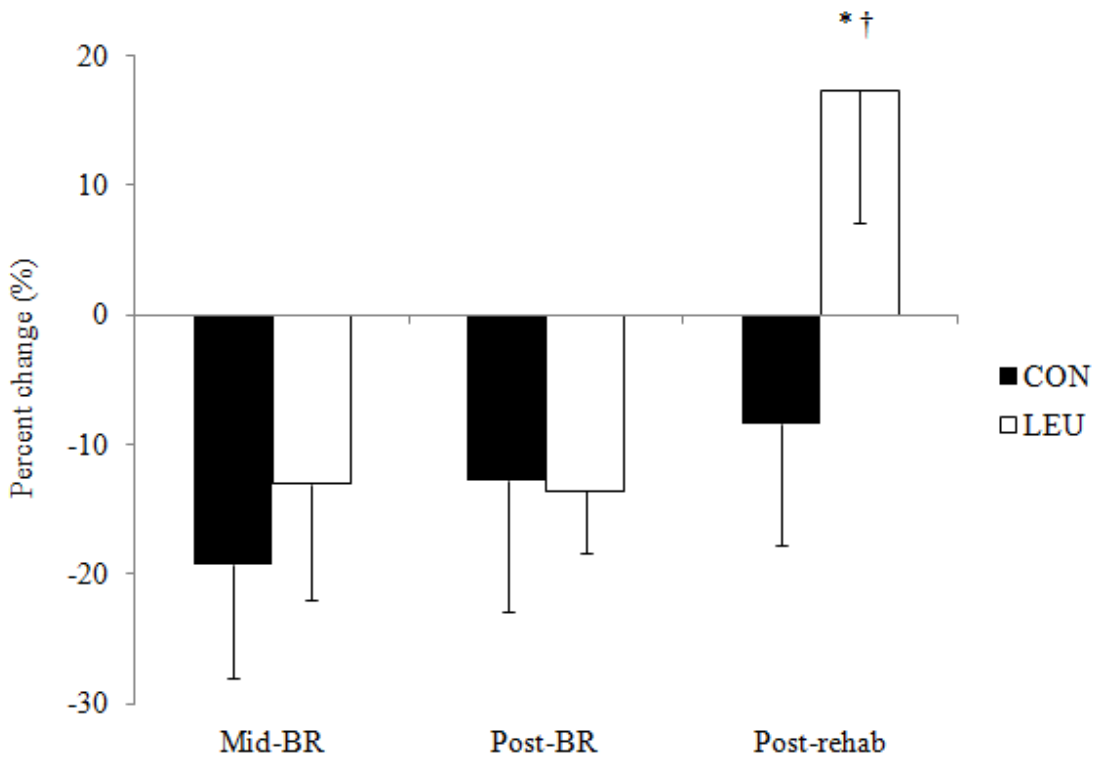


Figure 24. Changes in whole body insulin sensitivity (WBISI; %) during OGTT for CON and LEU after 7 and 14 d BR and 7 d rehabilitation (mean±SE); * significant difference from pre-BR (p=0.04); † significant interaction effect (group x time compared to pre-BR, p=0.05).

DISCUSSION

In middle-aged adults with normal glucose tolerance, BR did not alter post-absorptive glucose values, but elicited increases of 15-18% in $glucose_{AUC}$ during OGTT. After 7 d rehabilitation, $glucose_{AUC}$ returned to pre-BR baseline in LEU, but remained elevated in CON. BR did not change post-absorptive insulin values, but it did increase $insulin_{AUC}$ 52-70% in CON, while LEU remained at pre-BR baseline levels throughout BR. CON returned to, and LEU remained at, pre-BR values for $insulin_{AUC}$ after rehabilitation. Last, WBISI did not change during BR but increased above pre-BR values after rehabilitation in LEU only.

The finding of decreased insulin sensitivity/glucose tolerance with bed rest inactivity is not novel. Mechanical unloading is strongly associated with these maladaptations as they have been observed in both bed rest (Lipman et al. 1970, Paddon-Jones et al. 2004, Shangraw et al. 1988, Vernikos-Danellis et al. 1976) and indirectly, in spaceflight (Stein et al. 1994, Tobin et al. 2002). Similar to the present study, post-absorptive glucose and insulin concentrations remained unchanged after BR in young individuals with or without EAA/CHO supplementation (Paddon-Jones et al. 2004). Because fasted glucose levels are largely a function of hepatic insulin sensitivity (i.e., the liver's ability to appropriately decrease endogenous glucose production), maintenance of fasted glucose and insulin concentrations during and after BR indicates preservation of hepatic insulin sensitivity (Matsuda and DeFronzo 1999). It may thus be inferred that increases in glucose and insulin concentrations with OGTT during BR were the result of alterations in peripheral (i.e., largely muscle) insulin sensitivity (Matsuda and DeFronzo 1999). This contention is supported by others who have reported decreased insulin sensitivity and glucose uptake at the muscle level (Mikines et al. 1989, Mikines et al. 1991) facilitated by decreased glucose transporter 4 (GLUT-4)(Tabata et al. 1999). No alterations in hepatic glucose production were observed (Mikines et al. 1989, Mikines et al. 1991, Stuart et al. 1988), strengthening the notion that BR-induced changes in insulin sensitivity/glucose tolerance are mediated by changes at the muscle level (Stuart et al. 1988).

Inactivity-induced insulin resistance in the muscle is driven by maladaptations at the cellular level. Immobilization induces a host of derangements including reductions in insulin-stimulated phosphorylation of IR-β, subsequent phosphorylation of IRS-1 at tyrosine residues, and binding of IRS-1 to the p85 subunit of PI3K; interestingly, these reductions occur despite maintenance of IR-β and p85 protein levels (Hirose et al. 2000).

Some evidence suggests that leucine supplementation can also decrease insulin sensitivity (Balage et al. 2011). Mechanistically, this is thought to result from leucine's

stimulation of mTOR and its downstream target S6K1, and the latter's subsequent impairment of PI3K activity (Tremblay and Marette 2001) via increased phosphorylation of IRS-1 at serine residues (Harrington et al. 2004). Optimal insulin-mediated glucose uptake results from PI3K activation of Akt followed by increased translocation of GLUT4 to the cell membrane (Zanchi et al. 2012). This was demonstrated in an atrophy model (dexamethasone administration) in rats that performed resistance exercise, supplemented with leucine, or both (Nicastro et al. 2012). Resistance exercise maintained fasted glucose and insulin values while leucine increased these parameters or attenuated the positive effects of resistance exercise. These changes were facilitated by a reciprocal pattern in GLUT4 translocation to the cell membrane which was increased with resistance exercise, decreased with leucine supplementation alone, and increased but attenuated with resistance exercise plus leucine (Nicastro et al. 2012).

In two human trials of chronic leucine intake, one in healthy older men, the other in diabetic older men, no changes in glucose tolerance or insulin sensitivity were observed after three and six months of supplementation, respectively (2.5 g leucine · 3 meals · d^{-1})(Leenders et al. 2011, Verhoeven et al. 2009). In the present study, glucose levels at mid-BR in CON were elevated during OGTT along with increased insulin levels at mid- and post-BR; this is characteristic of impaired peripheral insulin sensitivity although the WBISI was not significantly decreased. Glucose levels were higher in LEU at mid- and post-BR, but insulin was not increased at any time point and WBISI was unchanged during and after BR. These findings are more difficult to interpret as neither fasted glucose/insulin values were altered in LEU with BR (indicative of maintained hepatic insulin sensitivity), nor was insulin production during OGTT increased (indicative of maintained peripheral/muscle insulin sensitivity).

Two factors provide possible explanations for the seemingly divergent findings in the literature on the effects of leucine supplementation on insulin resistance: 1) the frequency or pattern of leucine delivery (i.e., continuous vs. bolus) (Zanchi et al. 2012)

and 2) a predisposition to insulin resistance due to age (Zeanandin et al. 2012), glucocorticoid-induced muscle atrophy (Zanchi et al. 2012), or diet (Macotela et al. 2011). In dexamethasone-treated rats provided leucine via drinking water (continuously) or gavage (bolus), the continuously supplemented animals developed significantly higher fasting hyperglycemia. The authors speculate that this deleterious effect may be the result of chronic leucine stimulation of the insulin signaling pathways (Zanchi et al. 2012). In older rats supplemented with leucine for six months, blood glucose and insulin values during OGTT were not different from control and in fact, muscle glucose uptake was increased in leucine-supplemented animals (Zeanandin et al. 2012), results that suggest an overall positive effect of leucine on insulin sensitivity/glucose tolerance in skeletal muscle. Finally, in young mice fed a chronic high fat diet for eight weeks, leucine supplementation with high fat diet normalized muscle glucose tolerance and maintained insulin-stimulated IR, IRS-1, and Akt activation compared to high fat diet only (Macotela et al. 2011). The changes were observed in spite of greater insulin-stimulated S6K1 phosphorylation in leucine-supplemented animals. Thus, leucine appears to exert a neutral or even positive effect on insulin sensitivity/glucose tolerance when supplemented in a bolus fashion and/or in metabolically compromised situations such as aging or a high fat diet.

Finally, leucine exerted a positive effect on insulin sensitivity and glucose tolerance during 7 d rehabilitation. Unlike CON, $glucose_{AUC}$ returned to pre-BR levels in LEU and WBISI was greater than pre-BR baseline. These results are particularly meaningful in light of previous work that documented 14 d BR-induced glucose intolerance secondary to peripheral insulin resistance that persisted after 7 d recovery; only after 14 d did peripheral glucose uptake return to pre-BR levels (Lipman et al. 1970).

The findings from the present study are in agreement with existing literature and extend our understanding of the effects of leucine supplementation on glucose tolerance

and insulin resistance in humans. Specifically, in middle-aged adults subjected to 14 d BR inactivity, thrice daily pulsatile supplementation of leucine resulted in similar increases in $glucose_{AUC}$ during OGTT compared to CON while insulin values were maintained at pre-BR levels. Further, leucine supplementation improved whole body insulin sensitivity after 7 d rehabilitation. These data indicate that, in humans, chronic, bolus leucine supplementation during BR will not impair insulin sensitivity/glucose tolerance *per se*, and may in fact ameliorate decreases in insulin sensitivity during physical inactivity.

Chapter 6: Exercise and nutritional interventions for the preservation of muscle mass and muscle performance during exploration spaceflight

"The secret of a good sermon is to have a good beginning and a good ending and to have the two as close together as possible." –George Burns

INTRODUCTION

Human spaceflight began with Soviet Union cosmonaut Yuri Gagarin's 1961 orbital flight and has continued over the last 50+ years with missions to the Moon, countless flights to low Earth orbit, and currently, a continuous presence on the ISS. Beyond the substantive engineering issues associated with spaceflight (e.g., vehicle protection during the heat of re-entry), human physiology-related problems remain some of the most significant obstacles to long-duration spaceflight and particularly, to extended manned exploration missions (e.g., to Mars). These problems range from increased radiation exposure to musculoskeletal deterioration, the latter a product of the mechanical unloading inherent to the microgravity environment of space. Although there is currently a lack of clarity regarding the next US exploration destination (i.e., Moon, Mars, or an asteroid), the prevention—or at the least, mitigation—of these physiologic maladaptations is essential to the success of these future missions and possibly to the long-term health of the crewmembers.

Hans Selye described the Specific Adaptations to Imposed Demands principle (SAID), which states that systems and organisms adapt to the stimuli of their own actions (e.g., exercise) and of the surrounding environment (e.g., high temperature). In sharp contrast to Earth's 1-g environment which provides continuous acceleration of 9.81 $m \cdot s^{-2}$ towards the center of the Earth's rotating mass, space, free from this gravitational pull, features a microgravity environment that provides only the most miniscule gravitational

acceleration. Despite this, matter retains its inertial properties in the microgravity environment of space. Thus, unlike on Earth where the purpose of most force production in everyday human life is to overcome the acceleration of gravity (e.g., during walking or lifting an object), in space, force production is almost exclusively devoted to the acceleration or deceleration of objects, i.e., overcoming their inertial resistance. So although the microgravity environment occasionally requires human force production of similar magnitudes as on Earth, the frequency and thus, the volume of force production (i.e., total work or, impulse) is dramatically less in microgravity. From a muscular perspective, this reduction in loading volume evokes a potent downregulation of muscle mass and muscle strength (Gopalakrishnan et al. 2010, LeBlanc et al. 1995, Trappe et al. 2009). Although it may be argued that these alterations are unlikely to have negative functional impacts during time spent in microgravity, decrements in muscle performance have potential consequences ranging from minor (e.g., decreased work capacity) to catastrophic (e.g., inability to egress the vehicle in an emergency situation) during extraterrestrial exploration or following return to Earth.

Nutrition plays an essential role in the maintenance of muscle mass and muscle strength, both on Earth and, perhaps arguably, to a greater extent in the microgravity environment of space. Although exercise (specifically, the loading it provides) is the most potent stimulus currently available to protect muscle during microgravity exposure, poor nutritional status (Matsumoto et al. 2011, Stein et al. 1999) undermines the effects of exercise while proper nutrition combined with strategic supplementation offers promise to enhance the efficacy of exercise during long-duration spaceflight (Smith et al. 2012). Nutritional supplementation may also attenuate losses of muscle mass and strength during spaceflight when exercise hardware is either unavailable or limited in its capabilities. Thus, the purpose of this paper is to: 1) discuss the history of nutrition in spaceflight; 2) examine the role of exercise as a countermeasure to spaceflight-induced losses of muscle mass and strength; 3) ultimately focus on nutritional countermeasures to

these losses, particularly during exploration spaceflight, either employed alone or in combination with exercise.

NUTRITION IN SPACE: HISTORICAL PERSPECTIVES

Early US space missions of the 1960s lasted between 15 minutes and 14 days. The nutritional focus of these inaugural flights was in keeping crewmembers healthy, satiated, and fit to fulfill the objectives of the mission; this narrow focus was likely due to the brevity of the missions and perhaps to a measure of ignorance regarding the deconditioning effects of microgravity exposure on human muscle. During the Mercury, Gemini, and Apollo programs, foods were pre-packaged in individual serving sizes, often in squeezable tubes similar to those used for toothpaste (Lane et al. 1994). Only in later Apollo missions were crews afforded the ability to warm food via heated water, a feature available only in the command module, not the lunar module (Lane et al. 1994). During the 1970s, Skylab had onboard freezers and refrigerators which facilitated a wider range of food choices to offset the longer duration of the missions (28-84 d); later, a convection oven was included to facilitate food preparation on the Shuttle (Lane et al. 1994). Regardless of these improvements, the ability to store and prepare a wide variety of foods across a range of temperatures was largely lacking over the first four decades of US spaceflight.

Perhaps not surprisingly, these limitations resulted in lower energy intake, particularly in short-duration spaceflight (e.g., Apollo and Space shuttle; Table 12). It is important to note that a myriad of other factors beyond food availability, palatability, and ease of preparation may affect food intake during spaceflight; these include endocrine changes, space motion sickness, depressed appetite, decreased gastrointestinal motility, and increased transit time (Lane et al. 1994, Smith and Zwart 2008).

Table 12. Total energy and macronutrient intakes in crewmembers on Apollo, Skylab, Space shuttle, and ISS.

	Apollo (n=33)	Skylab (n=9)	Space shuttle (n=8)	ISS (n=11)
Energy				
kJ/d	7864 ± 1734	11906 ± 1300	8346 ± 1364	9563 ± 2625
WHO recom (%)	64.2 ± 13.6	99.1 ± 8.2	67.5 ± 19.3	80 ± 21
Protein				
g/d	76.1 ± 18.7	111.0 ± 18.4	75.7 ± 20.2	102 ± 29
% of kJ intake	16.2 ± 2.1	15.5 ± 1.2	15.0 ± 2.8	--
Carbohydrate				
g/d	268.8 ± 49.1	413.3 ± 59.3	282.9 ± 49.5	--
% of kJ intake	58.1 ± 7.1	58.1 ± 4.4	57.0 ± 5.1	--
Fat				
g/d	61.4 ± 21.4	83.2 ± 13.8	62.7 ± 13.8	--
% of kJ intake	28.8 ± 5.4	26.4 ± 3.8	28.2 ± 4.1	--

Mean ± SD; WHO = World Health Organization. Table adapted from (Lane et al. 1994, Smith et al. 2005). Used by permission.

Decreased dietary energy intake during long-duration Mir (Stein et al. 1999) and ISS spaceflight resulted in a significant reduction in body weight (-2.7 kg) with losses in both fat and lean mass (Smith et al. 2005). Energy expenditure during short-duration spaceflight appears to be similar to energy expenditure on Earth (Lane et al. 1997) and 28 d ground-based consumption of a Space Shuttle-only diet resulted in no changes in lean mass or muscle performance (Gretebeck et al. 1994). These findings, together with others that document a reduction in muscle protein synthesis during spaceflight (Stein et al. 1999) and a state of net protein catabolism during hypocaloric BR (Biolo et al. 2007), underscore the implicit role of reduced energy intake in spaceflight-induced losses of

body mass (Smith and Zwart 2008). Additionally, inflight exercise performed in an energy deficient state may exacerbate body mass losses (Lane et al. 1998).

Factors other than decreased energy intake are also likely to contribute to the loss of body mass during spaceflight as metabolic studies conducted on Skylab indicate that, despite intake of the recommended total energy, crewmembers still lost body mass (Rambaut et al. 1977). Chief among these other factors is likely exercise. Trappe et al. reported that ISS crewmembers performed aerobic exercise an average of ~45 min · d^{-1} (6 d · $week^{-1}$) in addition to resistance exercise which was performed 4-6 d · $week^{-1}$ (Trappe et al. 2009). Even at the moderate exercise intensities documented by Trappe et al., exercise likely created an additional daily caloric expenditure > 500 kcal. With voluntary dietary intake reduced during spaceflight, the performance of exercise countermeasures only widens the caloric deficit (Smith and Zwart 2008).

EXERCISE TO PROTECT MUSCLE IN SPACE

Early human spaceflight on Apollo did not incorporate inflight exercise (Thornton and Rummel 1977); similar to the nutritional provisions for these missions discussed previously, the near-singular focus of these initial exploration flights was operational success, i.e., landing on the Moon. Mission brevity (≤ 14 d) and perhaps a degree of uncertainty regarding the effects of microgravity exposure on muscle mass and strength were also likely contributors to the absence of any inflight exercise countermeasures. Despite this, crewmembers did use an Exer-Genie, a hand-held device that provided friction-based resistance, to perform exercise on their own time (Human Research Program 2008).

During the three, longer duration manned Skylab missions (Skylab 2, 3, and 4), exercise played a much more prominent role as a countermeasure to muscular deconditioning (Thornton and Rummel 1977). Each mission incrementally added

exercise hardware and increased exercise time such that by Skylab 4 (the third space mission—Skylab 1 was a ground-based experiment), a full complement of exercise hardware, which included a cycle ergometer, a stationary treadmill, and two low-load resistance exercise devices, was able to substantially attenuate losses in body weight, leg volume, and leg strength in comparison to the two earlier Skylab missions despite its 84 d duration (Skylab 2 and 3 were 28 d and 56 d, respectively) (Thornton and Rummel 1977).

The advent of the Space Transportation System (STS, or commonly, Shuttle) brought a return to shorter-duration spaceflight with most missions lasting 10-14 d. In light of the lessons learned from earlier flights, the Shuttle was equipped with a rower, a treadmill, and a cycle ergometer. Absent however, was any form of resistance exercise. Crewmembers performed inflight exercise with highly variable frequency ranging from none to daily. Shuttle exercise appeared to attenuate losses in muscle strength of the knee extensors/flexors but did not protect trunk extensor/flexors (Greenisen et al. 1999). Paradoxically, calf strength was modestly preserved (non-exercisers) if not increased (exercisers) during spaceflight (Greenisen et al. 1999); this finding is in conflict with other disuse research that has shown the muscle and performance of the lower leg to be among the most difficult to protect (Gopalakrishnan et al. 2010, Trappe et al. 2007).

The Russian space station, Mir, provided the next platform for long-duration human spaceflight, featuring the longest missions to that point in time with an average duration of 140 d (Human Research Program 2008). Losses in muscle mass were greater than those observed on short-duration missions, but reflected an apparently decreased rate of loss with increasing mission duration. This finding, with corroborating evidence from ground-based disuse models (de Boer et al. 2007) and the present study, has important operational implications for the prevention of muscle mass and strength loss during spaceflight as it suggests that the first 1-2 weeks are the most vulnerable and thus the most critical in terms of providing countermeasures to muscle maladaptations. Presently,

exercise on the ISS is not scheduled during the first 7 d of spaceflight and, anecdotally, may not begin until the third week of flight.

Current human spaceflight centers on the ISS which hosts 6-person crews of Russian, American, Canadian, Japanese, and European astronauts for stays of approximately 6 months. In continuous manned operation since October 2000, the ISS is inarguably the greatest space platform ever for the breadth of its science, the longevity of its operation, and the coordination required of its international partners. The ISS is outfitted with a full complement of exercise hardware, specifically, a cycle ergometer, treadmill, and a resistance exercise device. In the last 5 years, the original ISS treadmill (TVIS) and resistance exercise device (iRED) have been replaced by second generation versions (T2 and ARED, respectively)—a point that a recent review of ISS exercise hardware inexplicably failed to mention (Davis and Davis 2012). These new devices represent substantial upgrades over their predecessors and were developed both to enhance exercise equipment durability and to provide increased functionality, particularly in response to research that indicates that intensity (e.g., running velocity or resistance load) is a critical component to elicit optimal physiologic responses to exercise both in 1-g and in simulated microgravity (Campos et al. 2002, Shackelford et al. 2004, Tabata et al. 1996, Trappe et al. 2009, Trappe et al. 2007).

Changes to the ISS treadmill were rather singular, while those to the resistance exercise device were comprehensive. The primary enhancement for T2 over TVIS was an increased peak velocity (from 4.5 $m \cdot s^{-1}$ to 6.7 $m \cdot s^{-1}$) which enables crewmembers to perform high intensity intervals that provide high ground reaction forces that may be needed for optimal muscle maintenance (Lee et al. 2007, Trappe et al. 2007).

iRED, the original ISS resistance exercise device, had three significant shortcomings: 1) a low peak load, 2) a low eccentric:concentric resistance ratio, and 3) a distorted force-displacement curve (Loehr et al. 2011). The peak load iRED provides is 136 kg. In a microgravity environment, a crewmember's body mass provides no loading

(unlike in 1-g) with the exception of minimal inertial resistance. Thus, for a 90 kg crewmember performing a squat on iRED, the 136 kg maximum load represents an effective peak load of only 46 kg (~50% of bodyweight) after factoring in the "replacement" of bodyweight. Even for smaller crewmembers, iRED was unable to provide the high loads (i.e., > 75% 1-RM) recommended for the development of maximal strength (Campos et al. 2002). Second, iRED provides eccentric loading that is only ~72% of the corresponding concentric load (Amonette et al. 2004, Loehr et al. 2011); free weights, the "gold standard" of resistance exercise in 1-g, provide ~95% eccentric loading (Amonette et al. 2004). Although concentric-only training increases muscle size and strength, coupled eccentric-concentric training produces superior adaptations in these parameters (Colliander and Tesch 1990). Other studies have further underscored the importance of eccentric loading by demonstrating that eccentric overload (eccentric resistance > concentric resistance) can elicit greater adaptations in muscle mass (Norrbrand et al. 2008) and strength (Brandenburg and Docherty 2002) than the ~1:1 eccentric:concentric ratio provided by standard free weights and machines. Third, iRED produces a force curve that is notably different than the force curve of free weights in 1-g. iRED resistance is provided by elastomer bands that are alternately stretched during the concentric portion of a lift and then relaxed during the eccentric portion. Thus, during a squat, for example, the resistance is lightest at the bottom of the lift and increases as the crewmember stands more upright (Figure 25). This contrasts sharply with the force curve of free weights in 1-g that is characterized by a substantial force peak at the bottom of the lift due to free weights' high inertia and a decrease in force output as the weight is accelerated upward (Figure 26). A significant difference between Figure 25 and Figure 26 is the difference in peak force despite identical external loads; for iRED, the peak force is ~900 N whereas for free weights it is over 1200 N.

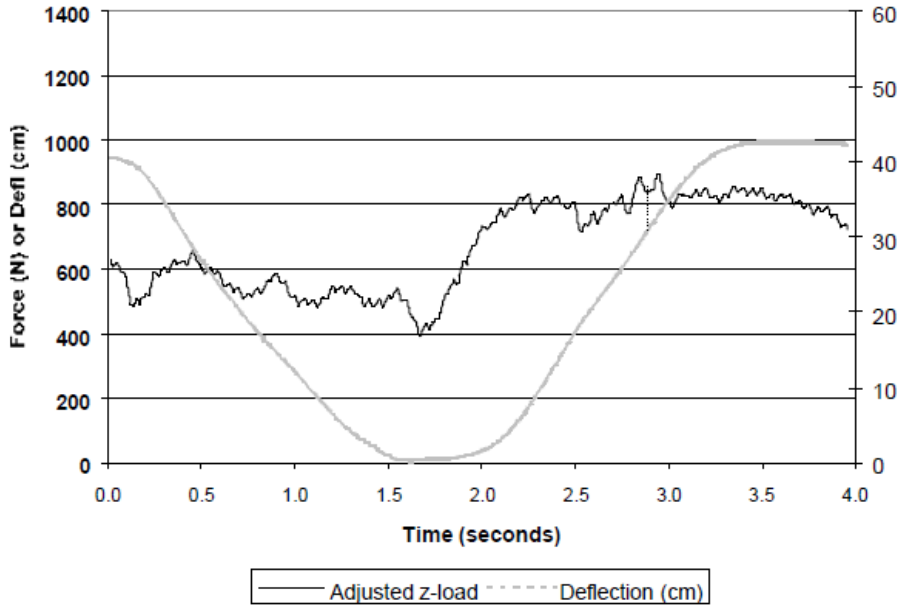


Figure 25. Ground reaction forces during an iRED squat in 1-g (Amonette et al. 2004).

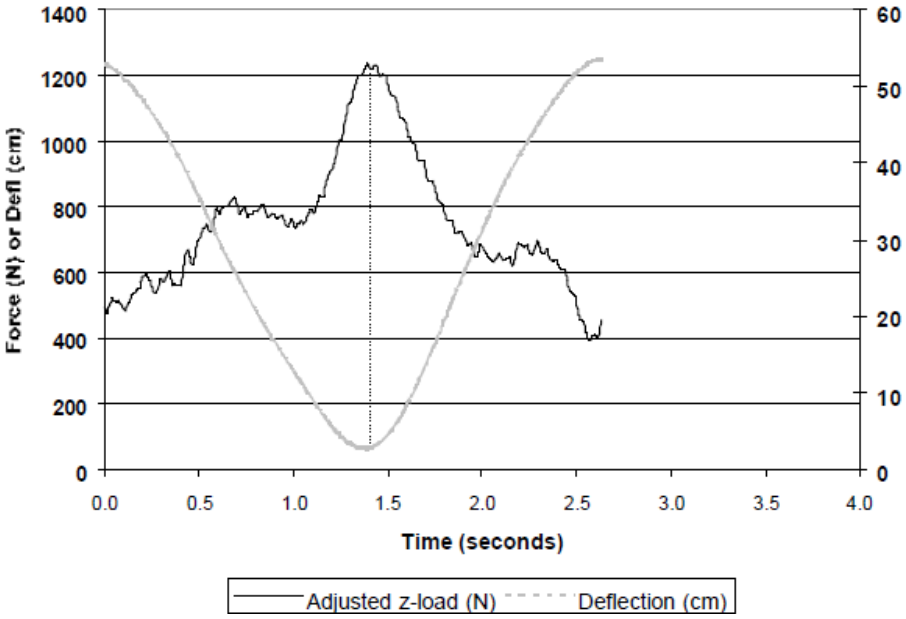


Figure 26. Ground reaction forces during a free weight (Smith machine) squat in 1-g (Amonette et al. 2004).

ARED, the current ISS resistance exercise device, addressed all of iRED's primary deficiencies. First, peak load was doubled to 273 kg. Using the previous example

of a 90 kg crewmember, ARED provides up to 183 kg of additional loading after "replacement" of the 90 kg bodyweight. Crewmembers can now perform multi-joint exercises such as the squat, heel raise, and deadlift at high loads of 85-100% 1-RM which are protective of muscle during BR and spaceflight (Shackelford et al. 2004, Trappe et al. 2001). Second, ARED provides loading during the eccentric (lowering) portion of a lift equivalent to ~90% of the concentric resistance (Loehr et al. 2011). Third, in combination with the constant force vacuum canisters, the inline flywheels on ARED provide velocity-dependent forces that mimic the inertial aspect of lifting free weights in 1-g. The flywheels spin more rapidly with increasing movement velocity which subsequently requires greater force input from the lifter to decelerate and then lift the load. The resultant force curve is thus similar to that of free weights depicted in Figure 26. A ground-based study demonstrated that physiological adapations (e.g., increased muscle mass and strength) to 16 weeks of training on ARED were comparable to those that resulted from identical training with free weights (Loehr et al. 2011), which was not the case when adaptations to iRED and free weights were compared (Schneider et al. 2003).

ARED has been deployed on the ISS since January 2009 and while early results from its use have been promising (Smith et al. 2012), ARED is unsuitable for exploration missions. The new crew capsule Orion, currently in development, has an internal volume of ~9 m^3 which precludes the use of a large device like ARED. Currently, NASA is working with its contractors and university groups in an effort to develop small, light exercise hardware that will fit in the restricted environment of the exploration capsule. Ideally, this hardware will retain capabilities similar to those of ARED; i.e., a high load capacity and a velocity-sensitive, inertial-like component coupled with a high eccentric:concentric resistance ratio. Also due to the limited capsule space, the exercise device will most likely need to provide both resistance and aerobic exercise. Although resistance exercise is most influential in the maintenance or development of muscle mass

and strength, aerobic exercise has been shown to act synergistically on these key outcomes in an unloaded environment (Trappe et al. 2007).

Presently, the most promising piece of hardware for exploration use is a flywheel exercise device (FWED). Relatively small and capable of producing very high resistance with eccentric loads greater than concentric ones (i.e., eccentric overload), the flywheel appears ideally suited to the spatial and power (i.e., energy) restrictions of an exercise device in a small capsule. It can also be used as a rower to provide aerobic exercise; this is ideal as aerobic exercise acts synergistically with resistance exercise to protect muscle during unloading (Trappe et al. 2007) and, due to limited space, a two-in-one device is highly preferable. Several flywheels have been built: one, by the European Space Agency (ESA) is currently onboard the ISS, although it is seldom used; the other was developed by Tesch et al. The Tesch flywheel has been well-researched in both ambulatory populations (Alkner et al. 2003) and disuse models with positive results. In 60 and 90 d BR, FWED prevented losses in muscle volume and strength of the quadriceps while attenuating decreases in these parameters for the triceps surae (Alkner and Tesch 2004, Trappe et al. 2007). In a shorter 35 d unilateral limb suspension study, FWED actually induced increases in muscle volume and strength in the quadriceps (Norrbrand et al. 2008, Tesch et al. 2004).

Despite the flywheel's efficacy, it remains to be seen whether it, or other prospective exercise devices, will be successfully integrated into the exploration capsule. This uncertainty, combined with the extended mission duration and the possibility of in-flight musculoskeletal injury or hardware failure, suggests that non-exercise countermeasures should also be considered for exploration spaceflight—particularly those that can be employed in conjunction with exercise.

NUTRITION TO PROTECT MUSCLE IN SPACE

As previously discussed, the frontline nutritional defense for muscle during long-term exposure to microgravity is adequate energy intake. Eucaloric diets on the final Skylab mission and in a recent cohort of ISS crewmembers attenuated (Thornton 1977) the loss of muscle mass and largely maintained muscle strength (Thornton and Rummel 1977) and bone mineral density (Smith et al. 2012).

Besides adequate energy intake, dietary protein is the most potent nutritional effecter of skeletal muscle with unloading. Although investigators have reported protein intake and examined the effects of micronutrients such as Vitamin D (Zwart et al. 2011) and calcium (Smith et al. 2012) on various physiologic outcomes (Smith et al. 2005) using a variety of experimental models, no controlled nutritional supplementation studies have been conducted in long-duration spaceflight. Because spaceflight is first an operational environment, the control and rigor necessary to test specific scientific hypotheses are often lacking. Thus, ground-based unloading models have been used to evaluate nutritional strategies for the protection of muscle mass and strength during spaceflight.

It is well-understood that during bed rest, low protein diets (0.06 $g \cdot kg^{-1} \cdot d^{-1}$) result in negative nitrogen balance, decreased whole body protein synthesis, and increased amino acid oxidation (Stuart et al. 1990); these undesirable changes are prevented with a protein intake of 1.0 $g \cdot kg^{-1} \cdot d^{-1}$. Although the ground-based recommended dietary allowance for protein is 0.8 $g \cdot kg^{-1} \cdot d^{-1}$, its validity has been called into question (Layman 2009, Paddon-Jones and Rasmussen 2009, Wolfe et al. 2008). Alternatively, some have advocated a meal-based approach supported by evidence which indicates that MPS is maximally stimulated with ~30 g of high quality protein (Layman 2009, Paddon-Jones and Rasmussen 2009, Symons et al. 2009). For an individual consuming three daily meals and weighing 75 kg, this equals 1.2 g protein $\cdot kg^{-1} \cdot d^{-1}$;

others have specifically recommended intakes of 1.5 g · kg^{-1} · d^{-1} to protect muscle in the elderly (Wolfe et al. 2008). These values are comparable to daily protein intakes reported on the ISS (~1.4 g · kg^{-1} · d^{-1}) (Smith et al. 2005).

Although total protein intake in spaceflight appears adequate, no data have been reported on the distribution of protein intake over the course of the day. Typically, Americans consume a high CHO/low protein (e.g., 10 g) breakfast, followed by a slightly higher protein lunch (e.g., 20 g), and finish the day with a high protein dinner (e.g., 60 g), i.e., a "skewed" protein distribution. Although this dietary pattern results in a seemingly acceptable daily protein intake, it may fail to maximally stimulate MPS at two of the three meals; theoretically this could result in the loss of lean tissue over a prolonged period of time, particularly in a metabolically antagonistic environment like spaceflight. Recent unpublished data from our lab appear to support the importance of this evenly distributed pattern of protein consumption as we observed a lower 24 h MPS response in individuals who consumed an isocaloric, isonitrogenous skewed protein diet compared to an evenly distributed intake pattern. Thus, it is advisable that crewmembers consume at least 30 g of high quality protein at each meal in order to maximize MPS. For most crewmembers, this will incidentally result in relative daily protein intakes of 1.0-1.5 g · kg^{-1} · d^{-1}.

In addition to adequate, distributed protein intake, supplementation of a single EAA may provide further protection for muscle against unloading-induced losses. The present study added ~4.5 g leucine to each meal of middle-aged adults during 14 d BR; protein intake was 1.05 g · kg^{-1} · d^{-1} and was evenly distributed across three daily meals in keeping with the recommendations discussed above. Weight loss during BR was limited to 1 kg, likely due in large part to fluid loss associated with BR; this, coupled with a small increase in fat mass indicated that total energy intake was adequate. Compared to control subjects, leucine supplementation attenuated the loss of muscle mass after 7 d BR and mitigated the decrease in muscle strength of the knee and ankle extensors after 14 d

BR (Ch 3). These results are particularly noteworthy in light of the optimization of other dietary factors (i.e., eucaloric energy intake and adequate protein provision and distribution); leucine appears to offer unique protection to muscle mass and strength during unloading that extends beyond its role as an amino acid substrate. This argues directly against those who have criticized amino acid supplementation during disuse and ascribed any observed efficacy to the simple additive effect of increased protein intake and not to effects of the supplementation *per se*; i.e., they assert that supplementation is only effective when dietary protein intake is relatively low (0.8 g · kg^{-1}) and the supplement rescues this to meet physiologic requirements (≥ 1.0 g · kg^{-1}) (Stein and Blanc 2011). Our data appear to refute this contention.

Another key factor that bears consideration for nutritional supplementation during exploration spaceflight is astronaut age. Although it is unclear whether exploration crewmembers will be significantly different in age from current astronauts who are typically 40-60 y old, it is clear that muscle responses to disuse worsen with aging. For instance, young men lost 400 g leg lean mass over a 28 d BR period (Paddon-Jones et al. 2004) while older adults (> 60 y) lost more than twice this amount (~950 g) after only 10 d BR (Kortebein et al. 2007). Middle-aged subjects similar in age to current crewmembers lost about 1150 g leg lean mass in 14 d BR (Ch 3). Obviously, this loss is virtually identical to that of older adults and notably greater than that of young individuals.

Older adults not only lose muscle mass more quickly during unloading, they are also less responsive to nutrition during normal 1-g activity. Thus, adequate EAA/leucine intake is critical as it is known that older adults (and possibly middle-aged individuals too) do not mount an anabolic response to small servings of EAA (6.7 g; leucine: 1.7 g)(Katsanos et al. 2005); fortunately, this blunting can be rescued with additional leucine (1.7 g + 1.1 g = 2.8 g total leucine) (Katsanos et al. 2006). Similarly, Rieu et al. demonstrated that a mixed meal (~755 kcal with 30 g protein (leucine: 2.4 g))

supplemented with leucine (~3.9 g; total leucine = 6.3 g) increased MPS in older adults while the same meal with supplementary alanine failed to do so (Rieu et al. 2006). Together, these data underscore the importance of adequate EAA/leucine intake in middle-aged/older individuals and the apparent utility of leucine supplementation to restore anabolic sensitivity.

CONCLUSIONS

Spaceflight has been characterized historically by reduced energy intake which exacerbates unloading-induced losses of muscle mass and strength. Adequate energy provision coupled with protein intake ≥ 1.0 g $\cdot$ kg^{-1} $\cdot$ d^{-1} and exercise appear to ameliorate these negative changes. Thus, based on a synthesis of current research evidence, it is recommended for current spaceflights and particularly for future long-duration exploration missions that crewmembers: 1) ingest a eucaloric diet that minimizes the loss of body mass; 2) consume $\geq$ 30 g protein $\cdot$ meal^{-1} at least thrice daily to maximize skeletal MPS; 3) supplement with ~4.5 g leucine at each of three daily meals; 4) have access to hardware capable of supporting both resistance and aerobic exercise; the resistance exercise device should provide high loads and an eccentric overload capability. Together, these countermeasures will protect muscle mass and strength during exploration spaceflight with minimal impacts to the limited volume and payload of an exploration capsule.

Appendices

APPENDIX A. NUTRITIONAL INTAKE FOR CON AND LEU DURING THE PRE-BR, BR, AND POST-BR PERIODS (MEAN±SE).

Group	Phase	Energy (kcal)	CHO (g)	Fat (g)	Protein (g)	Protein ($g \cdot kg \cdot d^{-1}$)
CON	Pre-BR	2542±71	344±10	85±2	93±4	1.23±0.05
	BR	1837±42	258±6	62±2	71±1	0.95±0.02
	Rehab	2223±84	326±18	75±3	86±3	1.14±0.04
LEU	Pre-BR	2396±96	337±14	80±3	92±4	1.25±0.03
	BR	1831±42	258±6	62±2	73±2	1.00±0.03
	Rehab	2178±83	307±11	73±3	85±3	1.15±0.03

APPENDIX B. NUTRITIONAL INTAKE FOR CON AND LEU FOR EACH OF THE THREE DAILY MEALS (MEAN±SE).

Group	Time	Energy (kcal)	CHO (g)	Fat (g)	Protein (g)	Protein ($g \cdot kg \cdot d^{-1}$)
CON	Breakfast	716±11	95±5	29±1	28±1	
	Lunch	651±14	93±2	22±1	26±1	
	Dinner	723±13	109±2	21±1	28±1	
	Total	2001±39	285±7	68±1	78±1	1.03±0.02
LEU	Breakfast	710±11	92±2	27±1	27±1	
	Lunch	647±14	92±2	22±1	27±1	
	Dinner	709±13	106±2	21±1	28±1	
	Total	1975±39	278±5	67±1	78±2	1.06±0.02

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PUBLICATIONS

Journal articles

English KL, Lee SMC, Loehr JA, Ploutz-Snyder RJ, Ploutz-Snyder LL. Isokinetic strength changes following long-duration spaceflight on the International Space Station. *Aviat Space Environ Med* (in review).

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INTELLECTUAL PROPERTY

Amonette WE, **English KL**, Buford W, Amonette BW. An apparatus to facilitate upright posture and improved gait velocity in the elderly and methods for making the same. US PSN: 61/20100318005 Provisional patent application filed June 08, 2009 (patent pending).

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