THE EFFECTS OF AGING ON MUSCLE LOSS AND NUCLEAR FACTOR
KAPPA-B IN RATS FED A DIET CONTAINING
SUBOPTIMAL LEUCINE LEVELS

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ABSTRACT

The Effects of Aging on Muscle Loss and Nuclear Factor Kappa-B Levels in Rats Fed a Diet Containing Suboptimal Leucine Levels

Corinne Rose Kohlen

Loss of muscle due to aging is often associated with significant detrimental effects. Therefore, it is crucial to understand signaling molecules that may trigger the muscle loss or prevent the process. The transcription factor, Nuclear Factor Kappa-B (NF- κ B), is associated with both catabolic and anabolic pathways of muscle metabolism and may be involved in age-related muscle loss. Leucine is an essential amino acid that is required for both protein synthesis and intracellular signaling pathways that regulate protein synthesis and degradation. The current study examined muscle NF- κ B levels in male Sprague-Dawley rats, aged 6 (adult) and 21 months (old) fed a diet containing suboptimal leucine levels for 10-17 days. We found that old rats consumed less grams of food per body weight (BW) each day than adult rats (1.45% g diet/g BW vs. 2.4% g diet/g BW). Weight loss during the study was not significantly different between age groups. However the average mass of gastrocnemius and soleus muscles (g muscle/g BW) was significantly lower in old rats. Reduction in gastrocnemius (g muscle/g BW*10²) was associated with 1.8 fold higher muscle cell NF- κ B in old vs. adult rats ($p = 0.0443$). There was also a higher level of ubiquitinated proteins in old gastrocnemius muscle cells relative to the adult gastrocnemius, however differences did not reach statistical significance. For tibialis anterior muscle, the average mass (g muscle/g BW*10²), NF- κ B levels and ubiquitinated proteins were not significantly different between adult and old rats. Our findings suggest that aging affects muscle loss and NF- κ B in a tissue-specific manner in rats fed a diet with suboptimal leucine levels.

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LITERATURE REVIEW

Aging, Physiologic Changes, and Muscle Metabolism

Age Demographics

The American population is graying. In the last census (2000) 12.4% of the population was 65 years of age or older. In the year 2030, this percentage is expected to grow to 20%, making one in five people over the age of 65 (Administration on Aging, 2005b). In just two years from now, there are expected to be 40 million people aged 65 and older. In America, it is the “baby boomers” that are making up the new aged population. The “baby boomer” population is educated, intelligent, and enjoys being active. Over 71 % of older Americans have high school diplomas, while 17% have a bachelor’s degree or higher. Twenty six percent engage in leisure physical activity. Older adults will demand independence, and they will be relying on younger generations for healthcare and novel innovations to let them move with ease through their later years (Administration on Aging, 2005a).

One of the leading factors contributing to the increase in older people is advancement in medical science, healthcare, and technology. Unfortunately, not all areas of medicine have advanced, and disparities exist for the older populations. One such disparity is the lack of attention that has been given to maintenance and preservation of muscle mass. For the elderly, having muscle is synonymous to having freedom. Functioning muscle allows the elderly to maintain independence and mobility, and carry out Activities of Daily Living (ADLs) such as bathing, dressing, transferring between bed and chair, toileting, and feeding one’s self (Katz, 1963). Unfortunately, aging and many diseases accompanying the aging process are associated with muscle loss and wasting,

leading to debilitation, disability, and inability to carry out ADLs. In 1997, 54.5% of noninstitutionalized people over 65 reported having at least one disability. Almost 38% reported a severe disability, and almost 20% had difficulties with ADLs. In those over 80 years old, disabilities are even more frequent with 73.6% of people reporting at least one disability, and 52.5 % reporting difficulties with ADLs (Administration on Aging, 2005a). Besides dementia, inability to perform ADLs is likely to land an older adult in nursing home facilities, or care giver situations. Over 90% of people with chronic disabilities receive formal and/or informal care (Administration on Aging, 2005a). Older adults are also more likely to visit doctors, stay longer in the hospital, and have higher medical costs. Disabilities related to muscle function/structure are not entirely to blame for this, but they are significant contributors. Often disability leads to lack of self hygiene/care, falls, breaks, or inability for older persons to seek help. Older persons visit doctors almost twice as often as people 45-64, and go to the hospital three times as often. In 2002, older adults spent \$3741 out of pocket for healthcare. This represents 17.8% of annual income for males, and 30.9 % annual income for females (Administration on Aging, 2005a). Clearly science has been successful at prolonging the years of life, now we need to find ways to prolong years of functional, enjoyable, independent living.

Examining the aging process is a good place to begin.

Theories of Aging

Aging involves changes that increase our chances of disease, and shorten the amount of time we live. At birth, most humans are expected to live around 85 years. Some, however, are “lucky” enough to reach 122 years of age (the maximum life span) (Harman, 2006). Many theories exist which try to explain the aging process. These

include evolutionary, molecular, cellular, and systemic theories of aging. Evolutionary theories include the mutation accumulation theory and the disposable soma theory. The mutation accumulation theory states that mutations accumulate over time and are not selected against. The soma theory is based on the idea that the sole purpose of life is reproduction. Once a species has reproduced (or comes to reproductive age), they are no longer needed, and the aging process begins. Molecular theories of aging include gene regulation, codon restriction, error catastrophe, somatic mutation, and dysdifferentiation theories. These theories focus on genes beneficial to increasing longevity, genetic mutations that decrease longevity, and molecular and translational damage that occurs to genetic material. Cellular theories of aging include the cellular senescence-telomere theory, the free radical theory, the wear and tear theory, and the apoptosis theory. The cellular senescence-telomere theory of aging focuses on the process by which cells lose their ability to replicate, and their telomeres become shorter and shorter. Telomeres are repeating DNA sequences that are located on the ends of chromosomes (Weinert & Timiras, 2003). The free radical theory of aging states that the reaction of free radicals with cellular components is responsible for ageing. These radicals are primarily superoxide radicals, which are formed during normal metabolism. These free radicals act to damage the mitochondria, and the rate of mitochondrial damage determines the life span (Harman, 2003 & Harman, 2006). The free radical theory of aging has received a lot of recent attention as a target to prolonging the life span. It is highly likely though, that it is not one theory that is responsible for the aging process, but many theories working together. Despite the difficulty assigning direct responsibility to one theory of aging, it is not difficult to recognize the physiologic changes that occur with age.

Physiologic changes

In the broad sense of the word, aging can be used to describe changes that occur over the course of the lifespan. During infancy, childhood, adolescence, and early adulthood these changes are usually celebrated and anticipated with joy. Unfortunately, the changes that follow early adulthood, and occur during adulthood and the transition over the age of 65 are not always pleasurable experiences. The following text will focus on physiologic changes that may have implications in preserving the ability to perform the ADLs, and are related to muscle metabolism.

Hormonal Changes with Age

Growth Hormone:

Many hormone levels decrease with age. These hormones include growth hormone, testosterone, estrogen, and dehydroepiandrosterone (DHEA). Hormone decreases may play a protective effect for overall health, such as with estrogen and breast cancer. Concerning muscle metabolism however, decreases in certain hormones appear detrimental. Growth hormone normally helps stimulate muscle and bone growth, and increases production of insulin-like growth factor 1 (IGF-1). IGF-1 causes muscle cells to increase in size and number. In aging, IGF-1 production is reduced, and muscle and bone growth are not stimulated by growth hormone (National Institute on Aging, 2007). In addition to decreased growth hormone levels affecting IGF-1 in aging, IGF-1 levels may be reduced due to other age related conditions. These include malnutrition, decreased physical activity, vitamin D deficiency, and loss of nerve innervating elderly muscle. In addition, elevated Tumor Necrosis Factor alpha (TNF- α) may act to inhibit IGF-1 signaling (Grounds, 2002). IGF-1 is involved in protein degradation pathways.

Decreased levels of IGF-1 (as in aging), lead to activation of Forkhead box O (FOXO), which increases expression of proteolytic ligases necessary for muscle loss (described in detail below) (Glass, 2005). Low levels of IGF-1 can also serve as a marker of protein-energy malnutrition and sarcopenia (Axelsson et al., 2006). Decreased levels of growth factor and IGF-1 with age are accompanied by decline of another growth related hormone, testosterone.

Testosterone:

Testosterone is important to bone and muscle functions and structure. With aging, decreases in testosterone may be correlated with reduced muscle and bone strength. In elderly men, muscle mass and physical activities are inversely correlated to testosterone levels (Baumgartner et al., 1999). Testosterone supplementation has improved muscle strength and decreased leptin in elderly men (Sih et al., 1997). DHEA is a precursor to testosterone and estrogen which declines sharply after the age of 20. Supplementation benefits from DHEA have yet to be established (National Institute on Aging, 2007). Perhaps further studies will reveal if replacing depleted hormones can provide any benefit during the elderly years.

Insulin:

While aging may cause a clear decrease in some levels of hormones, it can also deem hormones less functional, or less potent. This appears to be the case for insulin – a hormone produced by the beta cells of the pancreas. Insulin is a major anabolic hormone that allows glucose to be taken up from the blood stream, and be utilized by cells. Insulin also promotes activity of numerous anabolic processes and decreases protein breakdown. In skeletal muscle, insulin stimulates mitochondrial synthesis rates (Short et al., 2003).

Insulin acts to stimulate muscle protein synthesis in vitro. In vivo, insulin's main role may be to prevent proteolysis (Guillet & Boirie, 2005), but this is disputable. Age related changes in insulin action are most apparent during hyperinsulinemic states, and when comparing levels of insulin sensitivity.

Insulin Insensitivity in Aging:

In a hyperinsulinemic state, insulin levels are greatly elevated in the blood stream. For young adults, hyperinsulinemia results in faster utilization of amino acids by muscle, increased amino acid disappearance from the blood stream, and increased amino acid incorporation directly into muscles for synthesis. In older people, hyperinsulinemia does not have an anabolic effect and does not increase protein synthesis (Rasmussen et al., 2006). Unfortunately, as we age our cells' sensitivity to insulin decreases, and insulin resistance increases. Insulin sensitivity decreases up to 8% per decade during aging (Short et al., 2003). In the elderly, insulin insensitivity is increased by increased Interleukin-6 (IL-6), TNF- α , physical inactivity, and abdominal obesity (Roubenoff, 2003a). This can lead to changes in muscle metabolism and protein utilization that are detrimental to the elderly population.

Impaired insulin sensitivity causes negatively balanced muscle metabolism patterns. In a study on women with impaired insulin sensitivity, 29% more protein was broken down than was synthesized (Chevalier et al., 2005). In another study which used insulin insensitive rats, protein synthesis was decreased 37%, and protein degradation was increased 141% (Rodriguez et al., 1998). This may be in part to decreased branched chain amino acid (BCAA) utilization. Under normal metabolic circumstances, postprandial insulin released to the blood causes a decrease in plasma glucose, and

plasma amino acid levels. In aged men, this mechanism is impaired and postprandial plasma BCAA levels do not decrease to the levels expected. This is especially significant when considering the BCAAs are important to muscle synthesis. If postprandial plasma levels of BCAAs are not decreasing as much, one can assume that they are not being used for protein metabolism as much (Marchesini et al., 1987). In summary, age related insulin changes significantly influence muscle and protein metabolism. Another metabolic issue influenced by aging is the intake of nutrients.

Food intake and GI function

Food intake peaks during young adulthood, and slowly declines thereafter. This decline is referred to as the anorexia of aging and leads to weight loss (Morley, 2001). Elderly persons consume up to 1000 less calories than teenagers. This difference is mostly comprised of a reduction of calories coming from dietary fats. The elderly consume significantly less fat than younger persons (MMWR, 1994). Elderly have less desire to eat, and feel less hunger. In addition, they have slower gastric emptying which may cause longer periods of satiety (Clarkston et al., 1997). The sense of smell and taste decline with age. This may lead to less pleasure when eating, and hence, less consumption of food (Morley et al., 1999). Depression is the most significant cause of anorexia in the elderly, and occurs in over one-third of elder persons who have weight loss (Morley, 2001). Whatever the cause of anorexia, malnutrition is the result. Malnutrition leads to weight loss, decreased ability to heal, decreased cognitive function, increased risk of dementia, decreased quality of life, decreased muscle mass, and eventually can lead to death (Morley, 1997).

Cachexia

Loss of skeletal muscle mass is accelerated with aging and is due to an imbalance between protein synthesis and protein degradation. Muscle loss may be categorized as part of either sarcopenia or cachexia. Cachexia is muscle loss which develops simultaneously with disease. Diseases intimately linked to cachexia include cancer, adult respiratory distress syndrome, diabetes, chronic obstructive pulmonary disease, and kidney disease. During disease states, muscle wasting is associated with a reduction of amino acid stores (Wolfe, 2006). Cachectic disease states are accompanied by increases in inflammatory cytokines such as IL-6 and TNF- α , and increases in tumor derived compounds such as proteolysis-inducing factor (PIF), lipid mobilizing factor (LMF), and anemia-inducing substances (AIS) (Siddiqui et al., 2006). Cachexia is associated with a loss of ability to perform daily activities, a decreased quality of life, an increase in other diseases such as diabetes and obesity, and an increased debilitation and weakness (Wolfe, 2006).

Sarcopenia

Defining Sarcopenia

Sarcopenia is a term coined in the late 1980's to describe the decline in muscle mass and strength that occurs with healthy aging. Sarcopenia has been defined in terms of appendicular skeletal muscle mass (ASM) divided by height (meters squared). This value is referred to as the relative skeletal muscle index (RSMI). A person's RSMI is compared against mean values for adults aged 18-40. If a person's RSMI value is more than two standard deviations below the young-normal mean, they are considered sarcopenic. RSMI values that represent sarcopenia for men and women are 7.26 and 5.45

respectively (Baumgartner et al., 1998). Sarcopenic muscle loss represents a loss in number of all muscle fibers, and a reduction in size of type 2 (fast twitch) fibers. This loss occurs at an accelerated pace, beginning after age 25. Between age 20 and age 50, 10% of muscle area is lost. By the time a person is 80, a 40% reduction in muscle area is likely to have occurred (Lexell et al., 1988). It is during these later years that the most rapid reductions occur with the most significant consequences.

Prevalence of Sarcopenia

The occurrence of sarcopenia increases with age. Depending on how it is measured, the prevalence of sarcopenia in people 65 and older ranges from 6-14.6% (Melton et al., 2000), occurs more frequently in elderly men than women, and may exist in more than 50% of people above age eighty (Baumgartner et al., 1998). Thirty five percent of disabilities (in people over 60) can be attributed to sarcopenia, and disability is expensive (Janssen et al., 2004b). Costs related to sarcopenia represent 1.5% of yearly medical spending and are greater than costs related to more “known diseases” such as osteoporosis (18.5 billion vs. 16.3 billion). The cost per case is \$860 per sarcopenic man, and \$933 for every sarcopenic woman. If sarcopenia rates were reduced just 10%, the healthcare system would save 1.1 billion dollars (Jansen et al., 2004a).

Anabolic vs. Catabolic Stimuli

Sarcopenic muscle decline may be due to a decrease in the anabolic muscle stimuli with aging, or possibly an increase in catabolic stimuli. Catabolic stimuli may induce muscle loss through apoptotic processes, amplified insulin resistance, or by acting directly on amino acid metabolism (Roubenoff, 2003a).

Catabolic Pathways

Inflammation:

One catabolic stimulus that increases with age is inflammation. In obese elderly, increased inflammatory markers C-reactive protein (CRP), IL-6, and Plasminogen Activator Inhibitor type 1 (PAI-1) associated with decreased muscle mass (Cesari et al., 2005). In healthy elderly men and women, the inflammatory cytokines TNF- α and Interleukin-1 (IL-1) levels are elevated. In this elderly population, increased IL-6 and TNF- α are associated with decreased muscle mass (Visser et al., 2002, Pedersen et al., 2003), decreased muscle area, decreased grip strength, and decreased knee extension strength (Visser et al., 2002). TNF- α plays not only an inflammatory role, but also a role in apoptotic signaling.

TNF- α , NF- κ B, and apoptotic signaling:

TNF- α levels are increased in muscle of elderly rats and correlate with changes in apoptotic signaling. In superficial vastus lateralis muscle (type 2) of 26 month-old rats TNF- α levels are two-fold higher than in 6 month-old rats. This coincides with a reduction of cross sectional area, reduction in 15% muscle mass, reduction in muscle fiber number, and an increase in apoptotic signaling (caspase-8) in 26 month old rats. In soleus muscle (type 1), TNF- α expression, and apoptotic signaling was not up-regulated, and only a reduction in muscle area and fiber number was seen. However, NF- κ B activation was up-regulated 56% (in soleus) when compared to vastus lateralis muscle. NF- κ B is a eukaryotic transcription factor which is affected by TNF- α levels, and is involved in both anabolic and catabolic pathways of muscle metabolism. Because NF- κ B is not uniformly upregulated in all muscle types, it is possible that proteolysis

pathways in aging are muscle fiber specific (Phillips & Leeuwenburgh, 2005). The potential roles of NF- κ B will be discussed thoroughly in later sections of the paper. For now, the focus shifts to TNF- α , and other catabolic signaling that is age affected.

Other catabolic pathways:

In obese elderly, TNF- α is elevated and TNF- α has catabolic properties (Reviewed in Roubenoff, 2003b). During conditions where TNF- α is high, proteolysis is activated through the NF- κ B pathway (Ladner et al., 2003). Increasing catabolic stimuli may be responsible for up-regulation of proteolytic pathways in aging. In ageing, components of the ubiquitin dependent proteasome (UDP) pathway are up-regulated (Attaix et al., 2005, Pattison et al., 2003, Cai et al., 2004b, Husom et al., 2004). This up-regulation may lead to increased protein catabolism. Increased protein catabolism clearly contributes to age related muscle loss. It is not the sole culprit however and decreases in anabolic pathways (protein synthesis) also play a significant role.

Anabolic Pathways and Aging

Exercise:

Anabolic factors decrease with age and contribute to sarcopenia. One anabolic factor that affects sarcopenia is exercise. Exercise, especially resistance exercise, builds muscle strength, increases muscle fiber number, and increases muscle diameter. For the elderly, however, exercise is not always part of a usual routine. Lack of exercise and often lack of activity leads to muscle atrophy, decreased strength, and decreased mobility. Luckily though, the anabolic effects of exercise appear to affect the elderly the same way they do the young. Through prescribed exercise an elderly person can increase their rate

of protein synthesis, increase their muscle mass, and increase their muscle power (Parise & Yarasheski, 2000).

Insulin:

As mentioned earlier, the effect insulin has on muscle synthesis is blunted in older adults. This insulin blunting may contribute to a similar amino acid blunting. Amino acids given to older adults do not act as efficiently to stimulate muscle anabolism.

Amino acid and insulin anabolism may be decreased through the p70 S6 Kinase 1 (p70S6K1) pathway. In elderly adults, amino acids and insulin phosphorylation of S6K1 is reduced (Guillet et al., 2004). In young adults, dietary intake, especially protein intake acts as an anabolic stimulus, and as the building blocks for muscle. It is possible that the elderly do not respond to dietary intake the same way younger people do. For example, eight month old rats respond to feeding by increasing protein synthesis and decreasing protein degradation (in the postprandial state). Twenty two month old rats however do not have decreases in protein degradation after feeding. It seems that the ubiquitin dependent proteasome system in skeletal muscle of aged rats is insensitive to meal-induced anabolic stimuli (Combaret et al., 2005).

Biomarkers of anabolism:

Although some anabolic stimuli decrease with age, some biomarkers of anabolism continue to increase or remain stable even during sarcopenia. Quantification of the biomarkers mammalian target of rapamycin (mTOR), eukaryotic initiation factor 4E (eIF4E), eukaryotic initiation factor 4G (eIF4G), p70S6K1, and eukaryotic initiation factor 2 α (eIF2 α) is indicative of anabolic metabolic activity because together these biomarkers participate in a pathway which acts to initiate translation of proteins (Anthony

et al, 2001). Sprague-Dawley rats begin to experience sarcopenia around 21 months of age. When the anabolic biomarkers mTOR, eIF4E, eIF4G, p70S6K1, eIF2 α are measured in sarcopenic 21, 24, and 27 month-old rats they are just as high as in young rats. Anabolic biomarkers are maintained despite reduction in gastrocnemius muscle weight (wt.) (50% smaller gastrocnemius weight/body weight in 27 vs. 12 month old rats). This indicates that muscle loss is occurring without the complete reduction in muscle synthesis potential, and that sarcopenia cannot be completely explained by changes in anabolic conditions (Kimball et al., 2004). Therefore, it is likely that sarcopenia may be due to increased proteolysis.

Sarcopenic muscle loss has similar implications to cachexic muscle loss; debilitation, weakness, decreased mobility, and increase in injury and disease. Whatever the cause, the endpoint of muscle wasting is death and asphyxiation due to collapse of the respiratory muscles. A loss of 40% of lean body mass is typically fatal. Therefore the importance of halting muscle loss in the elderly is urgent, and undeniable.

Anabolic Pathways in Normal Muscle Metabolism

mTOR/Akt/p70S6K Pathway

Protein synthesis or anabolism is dependent on the number of ribosomes present in a cell, and the translational efficiencies of each ribosome (Anthony et al., 2001). Regulation of protein synthesis occurs through many pathways, one of which is the phosphatidylinositol-3 kinase (PI3K)/ protein kinase B (PKB/Akt) signaling pathway. **(Figure 1)** PI3K is phosphorylated as a result of signals such as IGF-1 binding to the insulin receptor. Once PI3K is phosphorylated it phosphorylates Akt, and forms Akt-P (Glass, 2005). Akt-P is responsible for inhibiting multiple molecules. Akt-P inhibits GSK3 β (Glycogen synthase kinase 3 beta). If GSK3 β is not inhibited by Akt-P, it acts to block protein translation through the eukaryotic initiation factor 2B (eIF2B) protein. When GSK3 β is inhibited, eIF2B is able to increase protein synthesis (Hardt & Sadoshima, 2002). Akt-P phosphorylates FOXO transcription factors, causing their inhibition and preventing protein degradation from occurring (Lecker et al., 2006). Akt-P disrupts the tuberous sclerosis complex-1 & complex-2(Tsc1/2) by phosphorylating Tsc2. Normally the Tsc1/2 complex acts to inhibit Rheb - a Ras like GTPase which is a positive regulator of mTOR. When Rheb is inhibited by Tsc1/2, mTOR is inhibited and the activation of p70S6K is blocked (Norton et al., 2006). However, when Akt-P phosphorylates Tsc2, mTOR can become activated (Glass, 2005). The Tsc1/2 complex is also influenced by AMP kinase (AMPK) concentration. AMPK is activated by rising AMP levels which result under conditions of hypoxia, low glucose, or during exercise. When AMPK levels are high AMPK phosphorylates Tsc2, leading to increased formation

of the Tsc1/2 complex, increased inhibition of Rheb, and increased inhibition of mTOR (Norton & Layman, 2006). **(Figure 1)**

mTOR plays a central role in integrating signals leading to protein synthesis. It does this through two distinct pathways: the p70S6K pathway, and the PHAS-1/4E-binding protein 1 (4E-BP1) pathway. mTOR can be directly activated by many stimuli including IGF-1 (Glass, 2005), and possibly leucine (Anthony et al., 2001). Once activated, mTOR activates p70S6K by phosphorylation, and activation of p70S6K leads to preferential translation of mRNA that encode components of the protein synthesis machinery. These include eIF4G, eEF1, eEF2, and poly (A) binding protein. Exercise can indirectly activate p70S6K through the p38 mitogen-activated protein kinase (p38MAPK) (Blomstrand et al., 2006). PI3K is also capable of directly activating p70S6K (Norton & Layman, 2006). mTOR also inhibits PHAS-1/4E-BP. PHAS-1/4E-BP is a negative regulator of the protein initiation factor eIF-4e. If activated, PHAS-1/4E-BP decreases protein synthesis (Glass, 2005). **(Figure 1)**

Figure 1: Pathways involved in protein synthesis. Protein synthesis occurs through activation of PKB/Akt pathway, activation of mTOR, or activation of p70S6K. Protein synthesis is initiated by presence of insulin, insulin like growth factor, exercise, hypoxia, low glucose, and possibly leucine. Diagram adapted from: Norton & Layman, 2006; Glass, 2005; Anthony et al., 2001; Kimball & Jefferson, 2006; and Blomstrand et al., 2006.

Protein synthesis occurs through many complex interactions and interwoven pathways. Continuous protein synthesis throughout the lifespan is a key component to adequate muscle mass and muscle function. It is through the pathways of protein breakdown (proteolysis), however, that muscle mass is lost when proteolysis exceeds protein synthesis.

Proteolysis Pathways

Muscle catabolism is due primarily to proteolysis, carried out by one of three pathways: the lysosomal pathway, the Ca²⁺ dependent pathway, and the UDP pathway (Attaix et al., 2005).

Lysosomal Pathway

The lysosomal pathway involves the degradation of a substrate by lysosomes. Lysosomes are membrane bound vesicles which contain hydrolytic enzymes. These enzymes may include proteases, glycases, lipases, and nucleases. In order for the lysosome to hydrolyze a substrate, that substrate must be transported into the lysosome through autophagy, either by endocytosis, pinocytosis, or phagocytosis (Bechet et al., 2005a). The physical separation from substrates in the cytoplasm isolates the lysosomal pathway from the other proteolytic systems.

Lysosomal proteases responsible for skeletal muscle proteolysis are primarily cathepsin B, H, L, or D (Attaix et al., 1998). Cathepsin B, H, L, and D are all endopeptidases. Cathepsin B is also a carboxypeptidase, and cathepsin D is only active below pH 6 (Turk et al., 2001). The cathepsins exist in high concentrations in tissues with rapid protein turnover rates (spleen, kidney, liver), and exist in lower concentrations in tissues with slow protein turnover (such as skeletal muscle). Cathepsins may even vary between muscle fiber types and various life stages. For example, in skeletal muscle, cathepsin concentration is higher in slow-twitch oxidative muscles than in fast-twitch glycolytic muscles (Bechet et al., 2005a).

Cathepsins are abundant in fetal muscle. Cathepsin B may play a role in the differentiation of myoblasts and fetal muscle formation. Adults have much lower levels of cathepsins; however their role in proteolysis is still thought to be significant.

Cathepsin L is believed to be most significant to adult muscle atrophy (Bechet et al., 2005a). Cathepsin L mRNA is up-regulated in skeletal muscle (specifically tibialis anterior and gastrocnemius) of rats treated with the glucocorticoid dexamethasone, and in tumor-bearing rats (Deval et al., 2001). Cathepsin L mRNA is also elevated in fasted mice, rats with induced diabetes mellitus, and rats with induced uremia (Lecker et al., 2004). In catabolism caused by sepsis, Cathepsin L is a stronger, and earlier, marker of proteolysis than components of the ubiquitin-proteasome-dependent pathway, which are only weakly expressed. Cathepsin L is up-regulated by glucocorticoids, and TNF- α (Deval et al., 2001). Cathepsins are up-regulated in aged liver, in the central nervous system, and in fibroblasts (Cuervo & Dice, 1997), and their gene expression appears to be up-regulated in aged soleus muscle (Pattison et al., 2003).

Lysosomal proteolysis is dependent on the ability of a substrate (skeletal muscle cell) to enter the lysosome. In skeletal muscle, macroautophagy is the primary process which allows lysosomal proteolysis to occur. Macroautophagy is partly regulated by cellular amino acid concentrations, possibly through PI3K activity (Bechet et al., 2005b). In myotubes deprived of leucine (for 24 hours), proteolysis rates are accelerated 11-15% per hour of leucine deprivation. These proteolysis rates correlate with increased autophagy and increased activation of the lysosome dependent pathway, without increases in cathepsin mRNA. Proteolysis during leucine deprivation persisted even when mTOR was inhibited (Mordier et al., 2000). Although it has already been proposed that

decreased leucine levels result in mTOR inhibition/down-regulation and increased proteolysis, leucine deprivation does not reduce levels of S6K – a primary mediator of protein synthesis. Therefore it seems that leucine may also work through other pathways besides mTOR activation to prevent protein degradation and promote protein synthesis (Mordier et al., 2000). As discussed with leucine, macroautosomes can also be induced by starvation. Macroautophagy is muscle cell number, and size dependent. It occurs faster in fast-twitch muscles (extensor digitorum longus) than in slow-twitch muscles (soleus). Macroautosomes are larger in hepatocytes than in skeletal muscle cells, and size may correlate to activity. The small size of macroautosomes in skeletal muscle often causes them to be overlooked, and brushed off as insignificant. The weight of the role macroautosomes play in protein degradation is still yet to be established (Bechet et al., 2005a).

Ca²⁺ Dependent Pathway

The Ca²⁺ dependent proteolysis pathway consists of calpains: calcium-activated cysteine proteases. Two calpains are ubiquitous: μ -calpain, and m-calpain (Huang & Forsberg, 1998), and one calpain is specific to skeletal muscle: p94 (Attaix et al., 2005). Calpain levels are elevated during vitamin E deficiency, Duchenne muscular dystrophy, fasting, and muscle wasting (as reviewed in Huang & Forsberg, 1998). Calpains contribute to muscle wasting and proteolysis by disassembling the sarcomere. They specifically degrade nebulin, an important structural sarcomeric protein. In conditions such as serum withdrawal, where elevated protein degradation is induced, calpains are most active. Inhibiting calpains under conditions of induced proteolysis causes a reduction in protein degradation of 30-63%. It has been proposed that the Ca²⁺

dependent pathway is the preceding and rate limiting step leading to degradation of proteins by the 26S proteasome (Huang & Forsberg, 1998). The 26S proteasome is capable of degrading the muscle proteins myosin, actin, troponin, and tropomyosin. However when these proteins are complexed in intact myofibrils, they are not degraded at a significant rate (Solomon et al., 1996). The Ca^{2+} pathway could act to degrade the myofibrils into smaller components, which would then be capable of degradation by the 26S proteasome. This theory has yet to be confirmed in vivo (Huang & Forsberg, 1998).

Ubiquitin Dependent Proteasome Pathway

The ubiquitin-dependent proteasome pathway (UDPP) is responsible for the degradation of the majority of all cell proteins (Rock et al., 1994). The UDPP is responsible for protein degradation in the following conditions or disease states: eating disorders, renal tubular defects, diabetes, uremia, neuromuscular disease, immobilization, burn injuries, sepsis, cancer cachexia, hyperadrenocortisolism, and hyperthyroidism (Lecker et al., 1999). The UDPP marks proteins for degradation by enzymatically linking 5 or more ubiquitin (Ub) molecules to them. Once tagged by a Ub “tail”, proteins are recognized by the 26S proteasome complex where they are degraded to peptides, and released to the cytosol for breakdown into amino acids and antigens (Glickman & Ciechanover, 2002). **(Figure 2)**

Ubiquitin Description and Action

Ubiquitin is a 76-amino-acid long polypeptide which is found in many tissues and organisms, and was first identified in 1975. In 2004, the Nobel Prize in chemistry was awarded for the identification and explanation of Ub’s role in the proteasome pathway (Nobel Foundation, 2004). Important features of Ub include a C-terminus which is

crucial to conjugation, and lysine residues which are crucial to polyubiquitination. Ubiquitin exists in chains, or it is bound to ribosomal proteins. De-ubiquitinating enzymes act on bound and linked ubiquitin to produce ubiquitin monomers, which are essential for protein “tagging” (Lecker et al., 2006). In order for Ub to “tag” substrate proteins for degradation, multiple reactions occur involving three enzymes: E1, E2, and E3 (Glickman & Ciechanover, 2002). **(Figure 2)**

Steps in Ubiquitin Conjugation

The first step in Ub conjugation involves E1: the ubiquitin activating enzyme. E1 uses phosphate generated from ATP to form a strong Ub thioester bond (Haas & Rose, 1982) between glycine 76, and a conserved active site cysteine. This creates a Ub-thiol bond-E1 intermediate, and “activates” the Ub molecule. There is only one E1 enzyme active in humans, it is called Uba1 (Jackson et al., 2000).

The next step in Ub conjugation involves E2: the ubiquitin-conjugating enzyme. At least 13 different E2's exist in humans. Each E2 has a site capable of binding E3's, and each E2 interacts with multiple and different E3s and substrates. E2 transfer of Ub is dependent on E3s (Glickman & Ciechanover, 2002).

The E3 ligases are referred to as the “ubiquitin ligases”. Although many different E3 ligases exist, they can be categorized into two different groups based on prominent catalytic features. E3's either display homology to E6-AP C terminus (HECT) domains, or display really important new gene (RING) finger domains. E3's with HECT domains have extensive N-terminal domains which allow them to bind substrate, and C-terminal domains which transfer Ub to the substrate. In HECT E3s, Ub is transferred from E2 to E3 where it forms a thioester, and then from E3 to the substrate attached to E3 (Jackson

et al., 2000). In E3s with the RING finger domain, one less step occurs because Ub is transferred directly from E2 to the substrate bound to E3. After the Ub is transferred to the substrate, the cycle repeats itself until a polyubiquitin chain is formed on the protein. (Glickman & Ciechanover, 2002). **(Figure 2)**

Figure 2: The Ubiquitin Dependent Proteasome Pathway (UDPP). The UDPP is responsible for degradation of the majority of cellular proteins. It marks proteins for degradation by the 26 S proteasome by “tagging” them with the Ubiquitin polypeptide. This is done through a series of cellular ligases (E1, E2, and E3). E1 = Ub activating enzyme, E2 = Ub-carrier or conjugating protein, E3 = Ub-protein ligase. The end result is the production of peptides, tripeptides and amino acids. Adapted from Kisselev et al., 1999, Lecker et al., 2006; Tisdale, 2005

Ubiquitin Pathway Regulation in Aging

The ubiquitin pathway appears to be regulated both in a tissue-specific and age-dependent manner. For example, the number of ubiquitinated proteins increases in aged fast-twitch muscles. Tibialis Anterior of 24 month-old rats had 90% more ubiquitinated proteins than 6 month-old rats (Clavel et al., 2006). Ubiquitin expression is elevated in old (24 month) rat, and human (70-79 year old) Extensor Digitorum Longus (EDL) (fast twitch), and quadriceps muscle (fast & slow twitch), but not in soleus muscle (slow twitch), when analyzed using immunohistochemistry (Cai et al., 2004b). In addition, ubiquitin protein conjugates are elevated in adult senescent cells compared to younger cells. Ubiquitin mRNA, however does not appear to be elevated in aged cells (Pan et al., 1993). This indicates that UDPP activity is somehow increased without increased transcription, potentially increasing enzyme activity and potency during aging.

E3 Ligases

Although there are many different types of E3 ligases, the RING finger E3s represent the majority of all E3s. The RING finger motif is 30-40 residues long, and contains core cysteine and histidine amino acids in either a C3HC4 (RING) or a C3H2C3 (RING-H2) pattern (Deshaies, 1999). Of the many RING finger E3 ligases, muscle-specific ring finger-1 (MURF-1), and E3 α (Ubr1), play a key role in skeletal muscle atrophy (Lecker, 2003). Muscle atrophy F box (MAF bx)/atrogin-1 has also been linked to skeletal muscle atrophy (Bodine et al., 2001).

Ubr1 E3 Ligase:

E3 α (Ubr1) displays the RING-H2 domain and acts with E2_{14K} to recognize substrates with unblocked hydrophobic N terminus, or acidic or basic amino acids (Lecker, 2003). This mode of recognition is referred to as the N-rule pathway. (Varshavsky et al., 2000). Substrate recognition by the N-rule pathway is responsible for 60% of ATP-dependent degradation of soluble proteins from skeletal muscle extract. E2_{14K} is one of the most abundant Ub ligases in muscle protein. E2_{14K} is very abundant in muscle cells, and its expression increases in atrophying muscles, where subunits of the 26S proteasome and levels of polyubiquitin chains also increase (Solomon et al., 1996).

MURF-1 E3 Ligase:

MURF-1 contains a RING domain that plays a role in conjugating ubiquitin. MURF-1 is up-regulated during atrophy and expressed selectively in cardiac and skeletal muscle (Bodine et al., 2001). MURF-1 is also up-regulated in cachexic animals undergoing fasting states, diabetes, cancer, and uremia (Lecker, 2003). In septic rats, MURF-1 is up-regulated (10 fold) in extensor digitorum longus muscle, but not soleus muscle (Wray et al., 2002b). In a gene knockout experiment, mice were forced into an atrophy state by denervation, and subsequent disuse of gastrocnemius and soleus muscle. Mice that had the MURF-1 gene knocked out experienced 36% less muscle loss than control mice (no genes knocked out). This muscle sparing effect of MURF-1 gene knockout implicated MURF-1 as a key component of muscle atrophy (Bodine et al., 2001). MURF-1 was again implicated as a key component to muscle atrophy in an experiment focusing on NF- κ B activation. NF- κ B activation resulted in reduction in muscle fiber diameter (56%), reduction of muscle fiber area (32%), and reduction in

muscle force and velocity in Tibialis anterior, as well as reduction in whole body wt. These changes were coupled with a 3.3-fold increase in MURF-1 mRNA. The MURF-1 gene was subsequently knocked out (NF- κ B was still activated) and a 50% reversal of muscle wasting was observed. This knockout experiment proved that MURF-1 plays a key role in muscle atrophy and is up-regulated by NF- κ B. Since MURF-1 knockout mice only had a 50% reversal of muscle loss, MURF-1 must not be the only pathway by which NF- κ B promotes muscle loss. It is possible that NF- κ B works in an indirect manner to activate MAFbx/Atrogen-1 (Cai et al., 2004a). Upstream regulators of MURF-1, including NF- κ B are possible targets to control muscle atrophy.

MURF-1 mRNA is also up-regulated by aging. Aged rat tibialis anterior contains 2 X more MURF-1 mRNA than in 6 month olds. Aged rats had higher levels of oxidized proteins and tumor necrosis factor α . It is possible then that TNF- α , and oxidative stress cause the up-regulation of MURF-1 during aging. TNF- α and oxidative stress may also cause the up-regulation of MAFbx/atrogen-1 (Clavel et al., 2006).

MAFbx/atrogen-1 E3 Ligase:

MAFbx/atrogen-1 is an E3 in the Skp1 Cullin F-box (SCF) family of ligases and has a Skp1, Cullin, F-box, and atrogen-1 component (Cao et al., 2005). The Cullin component forms an extended structure which helps to organize substrate binding. The F-box binds protein substrates to Ub and the E3 platform, allowing the polyubiquitin chain to form. Skp 1 is involved in substrate binding (Jackson & Eldridge, 2004).

Like MURF-1, MAFbx/atrogen-1 is upregulated in muscles of diabetic, uremic, and tumor bearing rats, has been correlated with muscle atrophy, dexamethasone administration, and cachexic states (Lecker, 2003), and is expressed in skeletal and

cardiac muscle (Bodine et al., 2001). In septic rats, atrogen-1 is induced 16 fold in extensor digitorum longus muscles, but not soleus muscles (Wray et al., 2002b). In aged rat (24 month-olds) tibialis anterior muscle, atrogen-1 mRNA concentration is 2.5X the amount found in young rats (6 month-olds). tibialis anterior is a fast twitch muscle (Clavel et al., 2006). MAFbx/atrogen-1 mRNA is up-regulated 1.96X in aged soleus muscle. When compared to the larger up-regulation caused by sepsis (16X) it seems that the slower up-regulation seen in aged soleus, is a more true picture of the atrophy that occurs during sarcopenia (Pattison et al., 2003).

Unlike MURF-1, MAFbx/atrogen-1 transcription is not elevated in atrophy induced by NF- κ B activation. It is therefore thought that MAFbx/atrogen-1 and MURF-1 act in separate, but parallel pathways to induce proteolysis (Cai et al., 2004a). MAFbx/atrogen-1's role in muscle atrophy was first demonstrated in C2C12 myotubes when their diameters were reduced after being infected with the MAFbx/atrogen-1 gene. In an experiment which induced disuse and denervation atrophy to mice tibialis anterior and gastrocnemius muscles, mice with the MAFbx/atrogen-1 gene "knocked out" had 56% less muscle wasting than their wild type counterparts. This reduction in muscle atrophy caused by the MAFbx/atrogen-1 knockout was more significant than with the MURF-1 knockout (Bodine et al., 2001). Because the "knocking out" of the MAFbx/atrogen-1 gene causes such significant reduction in muscle wasting, MAFbx/atrogen-1, and its inducers, have been examined as a pharmacological target to reduce muscle wasting. This has promoted much research to identify pathway(s) by which MAFbx/atrogen-1 is transcribed, up-regulated, and acts.

FOXO and MAFbx/atrogen-1

Forkhead box O (FOXO) is a family of transcription factors which directly up-regulates transcription of MAFbx/atrogen-1. FOXO₁, FOXO₃ and FOXO₄ are members of the FOXO family found in mammals. FOXO₁ is an “atrogene” and is up-regulated during muscle atrophy, during fasting, diabetes, uremia, and in cancer (Lecker et al., 2006). During normal muscle metabolism, FOXO₁ becomes phosphorylated through the P13K/AKT pathway (IGF-1/P13K/AKT pathway). In this pathway, high levels of insulin and/or insulin like growth factor-1 (IGF-1) are major anabolic stimuli. When IGF-1 or insulin are present, P13K phosphorylates Akt, creating Akt-P, and then Akt-P phosphorylates FOXO. Once phosphorylated, FOXO₁ is unable to translocate to the nucleus, and therefore unable to induce gene transcription of MAFbx/atrogen-1. During conditions where IGF-1 and/or insulin levels are not sufficient to activate P13K, the subsequent phosphorylations of Akt and FOXO₁ do not occur (Lecker et al., 2006). IGF-1 and/or insulin levels may be low or inactive during diabetes, fasting, cachexia, uremia, and inactivity (Lecker et al., 2006). IGF-1 levels may also be decreased in aging (National Institute on Aging, 2007). Catabolic states (disease, aging?) may correlate, or even cause insulin resistance, further hindering the activation of the Akt protein and preventing FOXO from being phosphorylated (Lee et al. 2004). In its un-phosphorylated state, FOXO translocates to the nucleus and induces transcription of MAFbx/atrogen-1, which increases muscle atrophy via lack of stimulation of the PI3K/AKT pathway (Sandri et al., 2004). During induced insulin deficiency, PI3K activity is suppressed. This leads to increased activation of FOXO, 20-fold increases of MAFbx/atrogen-1 mRNA, gastrocnemius muscle loss, and reduction in body wt. Circumstances that might

mimic induced insulin deficiency include insulin resistance, and insulin insensitivity (Lee et al., 2004). These circumstances occur more frequently in aged subjects (Short et al., 2003). **(Figure 3)**

Figure 3: P13K/Akt Pathway of Proteolysis. Age, cachexia, or other catabolic states result in decreased insulin and IGF signaling. This reduction in signaling causes a cascade effect of inadequate signaling resulting in non-phosphorylated (active state) FOXO. FOXO acts to increase Atrogin 1/MAFbx 1, MURF1, and Atrogenes – all of which contribute to increased proteolysis via the ubiquitin dependent proteasome pathway. Figure and explanation adapted from: Cao et al., 2005; Glass, 2005; Lecker et al., 2006; Lee et al. 2004; Sandri et al., 2004.

The 26S Proteasome

All of the E3 ligases work to attach multiple Ubs to the substrate. Proteins with a polyubiquitin tail are “recognized” by the 26S proteasome and subsequently degraded. The 26S proteasome consists of a 19S regulatory particle, and a 20S core particle. The 19S regulatory particle is an 18 subunit entity made up of a “lid” and a “base”. It serves three main functions in the proteasome. It acts to recognize ubiquitinated proteins, it opens a channel in the 20S chamber which allows substrate entry, and it facilitates substrate entry into the 20S by unfolding proteins. The 19S is adenosine tri-phosphate (ATP) dependent, and 6 of its 18 subunits contain ATPases. The 20S core particle is the primary site for proteolysis (Glickman & Ciechanover, 2002). (**Figure 4**)

Figure 4: The 26 S Proteasome. The 26 S proteasome is composed of two 19S particles and a 20S proteasome. The 19S particles are ATP dependent, and function to recognize and target misfolded proteins for degradation. The 20S proteasome is composed of two α and two β subunits. β subunits contain proteolytic sites and orchestrate protein breakdown. Diagram and explanation adapted from Glickman & Ciechanover, 2002; Tisdale, 2005.

The 20S core is made up of 4 cylindrical stacked rings. The outer two rings are made up of 7 α -subunits, and the inner two rings are made up of 7- β subunits each (Tisdale, 2005) (**Figure 4**). Normally, inner β -rings of the 20S contain the proteolytic sites β 1, β 2, and β 5 (Tisdale, 2005). Proteasome's displaying β 1, β 2, and β 5 subunits are referred to as constitutive proteasomes. The proteolytic subunits β 1, β 2, and β 5 may be replaced by cytokine-induced subunits LMP2, MECL, and LMP7 as a result of inflammatory stimuli (such as from TNF- α). If the LMP2, MECL, and LMP7 subunits are present, the proteasome is referred to as the immunoproteasome. Normally immunoproteasomes represent 5% of all proteasomes. In aging, however, it has been reported that, while levels of the constitutive proteasome stayed the same, the immunoproteasome levels were increased 4.2-4.4-fold (Husom et al., 2004).

The immunoproteasome displays different proteasome activity than the constitutive proteasome. It has lower trypsin, chymotrypsin, and hydrolase activity; it has elevated generation of major histocompatibility complex (MHC) class 1 antigen presentation, and is up to 19-fold more efficient at protein degradation (Reviewed in Husom et al., 2004). In the constitutive proteasome, β 1, β 2, and β 5 sites exhibit 3 types of proteolytic activity: trypsin like, chymotrypsin like, and caspase like (Tisdale, 2005). In aged (22 month old) rats in the postabsorptive state, trypsin, chymotrypsin and hydrolase activity is 41-45% less than in young rats (8 month olds). This indicates that proteasome activity is reduced during the postabsorptive state in aged rats, despite changes in total ubiquitination (Combaret et al., 2005). In vitro muscle studies indicate decreases in trypsin, chymotrypsin, and hydrolase specific activity up to 60% in aged rats. This number was expressed as enzyme activity per proteasome content. Proteasome content,

as measured through α -subunit concentration on the catalytic core, was as much as 3-fold higher in aged rats. The content of the proteasome was higher in old rat muscle preparations; however the activity of the proteasome did not proportionally “keep up” with the increased proteasome expression. Since both immunoproteasome and constitutive proteasomes have α -subunits, the increase in proteasome content and decrease in relative proteasome activity may be due to a higher proportion of immunoproteasomes in aged muscle isolates, rather than errors in the proteolytic process. (Husom et al., 2004).

Other studies of the proteasome have found no differences in enzymatic activity with aging. When comparing tibialis anterior muscle from old (24 months) vs. young (9 month) rats, the ability of the proteasome to hydrolyze proteins via cymotrypsin-like, trypsin-like, or peptidyl glutamyl peptide hydrolase (PGPH) enzyme activity was unchanged by aging (Clavel et al., 2006). Clearly, the role aging plays in proteasome activity needs further investigation.

Substrate Release from the Proteasome

After a substrate is degraded by multiple proteases it is released by the 26S proteasome. Peptides released from the proteasome range from 3-22 residues long. Once peptides are released they are further broken down by proteases and aminopeptidases to yield amino acids, or they are used for antigen presentation (Figure 2). Proteins which are used in antigen presentation are 8-9 residues long and travel through the endoplasmic reticulum (ER) to the immune system, where they are presented by MHC class 1 molecules (Kisselev et al., 1999).

Although the proteasome usually releases inactive protein products, this is not always the case. For example, when inhibitor complex I κ B α of the NF- κ B/I κ B α complex is phosphorylated, NF- κ B/I κ B α enters the proteasome. The proteasome only degrades I κ B α , NF- κ B is released and it translocates across the nucleus where it affects gene transcription (Chen et al., 1995). NF- κ B may increase transcription of the E3 ligase MURF-1, leading to increased proteolysis (Cai et al., 2004a).

Other Functions of the Ubiquitin Proteasome Pathway

Besides playing a role in gene transcription, the UBPP has other functions. It is involved in the irreversible degradation of proteins, involved in quality control, and it produces amino acids as protein is degraded. The UBPP acts as a quality control regulator by selectively targeting damaged, mutated, or abnormally folded proteins for degradation. Damaged proteins may result from heat exposure, oxidative damage, or genetic errors. In this sense, the UBPP serves a protective function (Lecker et al., 2006). In muscle cells the UBPP targets both short and long lived proteins, as well as proteins residing in the nucleus and cytosol (Solomon et al., 1996).

NF- κ B

Nuclear Factor- κ B (NF- κ B) is a eukaryotic transcription factor which promotes the expression of hundreds of different genes, in a variety of tissues, and acts as the central regulator of stress and to a lesser extent, immune response (Paul, 1999). Normal regulation of NF- κ B has important physiological roles in immune function, specifically B and T cell development and proliferation. Animal studies in which members of the NF- κ B family were “knocked out” produced animals with abnormal B and T functioning, decreased immune response, and poor macrophage production among other problems (Kumar et al., 2004). Abnormal NF- κ B functioning contributes to many diseases. The diseases of which NF- κ B may be a direct effector include, but are not limited to: heart failure, cardiac hypertrophy, atherosclerosis, muscular dystrophy, Alzheimer’s disease, inflammatory bowel disease, renal diseases, sleep apnea, viral infections, acquired immune deficiency syndrome (AIDS), lupus, diabetes (type 1&2), asthma, arthritis, cancer, and skeletal muscle loss during cachexia, disuse, and aging (Kumar et al., 2004). Elevated NF- κ B levels are associated with muscular dystrophy, dermatomyositis, polymyositis (Monici et al., 2003), cachexia (Wyke & Tisdale, 2005), and sarcopenia (Bar-Shai et al., 2005). This discussion will focus on NF- κ B’s role in skeletal muscle loss.

The NF- κ B family

The NF- κ B family consists of 5 transcription factors (p50, p52, p65 (Rel A), cRel, and Rel B). These family members form homo or heterodimers with different activation activities, binding affinities, and deoxyribonucleic acid (DNA) binding

activity. Each NF- κ B family member contains a Rel homology domain (RHD). The RHD plays a role in DNA binding, nuclear translocation, binding to I κ B inhibitors, and dimerization (Siebenlist et al., 1994). RelA (p65), RelB, and c-Rel contain a C-terminal domain. This C-terminal domain allows them to be potent transcriptional activators. P50, and p52 do not have C-terminal domains and cannot act as transcriptional activators (May & Ghosh, 1997). The most common active form of NF- κ B implicated in muscle loss is a heterodimer of p50/p65 (Kumar et al., 2004). Some studies have indicated p65 is regulated differently between tissues during aging. For example, levels of p65 were shown to be up-regulated in aged soleus muscle, but not in superficial vastus lateralis muscle. It is possible that NF- κ B forms tissue specific dimerizations, and that Rel proteins other than p65 are reserved for type-2 muscle fibers (Phillips & Leeuwenburgh, 2005), and this may explain these observations.

Activation pathways of NF- κ B

In its inactivated state, NF- κ B resides in the cytosol, bound to an inhibitory protein called inhibitor of kappa-B (I κ B). I κ B molecules bind to the nuclear localization site (NLS) on NF- κ B and prevent it from translocating to the nucleus. There are 7 types of I κ B molecules: I κ B- α , I κ B-b, I κ B- γ , I κ B- ϵ , Bcl-3, p100, and p105 (May & Ghosh, 1998). I κ B- α , I κ B-b are capable of dissociating NF- κ B bound to enhancer and promoter regions of DNA, thus halting NF- κ B mediated gene expression (Zabel & Baeuerle, 1990). Levels of I κ B- α are up-regulated in aged soleus muscle, but not in superficial vastus lateralis muscle. This could indicate that NF- κ B is up-regulated in aging because increased activation of NF- κ B prompts synthesis of I κ B (Phillips & Leeuwenburgh, 2005).

In the classical (canonic) pathway, NF- κ B becomes activated when it translocates from the cytosol to the nucleus. This is made possible through the action of I κ B kinases (IKKs). IKKs contain one IKK α subunit, one IKK β subunit, and two IKK γ subunits (Senftleben & Karin, 2002). IKK α plays a role in epidermal development, IKK β is responsible for NF- κ B activation by inflammatory stimuli, and IKK γ connects IKK with upstream activators and is essential to begin the NF- κ B activation cascade (Karin & Delhase, 2000). Levels of IKK γ are elevated ~2-fold in aged soleus muscle (Phillips & Leeuwenburgh, 2005). IKKs are activated by many signals including TNF- α , interleukin-1(IL-1), phorbol esters (DiDonato et al., 1996), oxidative stress (Schreck et al., 1992), reactive oxygen species (ROS) (Li, 1998), other inflammatory cytokines, bacterial and viral products, mitogens, and growth factors (Kumar et al., 2004, May & Ghosh, 1997). IKK activation is the first step in NF- κ B activation. When IKKs are activated they cause the phosphorylation of serine 32, and serine 36 on I κ B- α and I κ B- β respectively. This targets I κ B- α and/or I κ B- β for rapid degradation by the 26 S proteasome. Once I κ B is degraded, NF- κ B translocates from the cytosol to the nucleus and affects gene transcription (DiDonato et al., 1996) (**Figure 5**).

In disuse atrophy, NF- κ B may be activated through a non-classical/non-canonical pathway. In the non-canonical pathway, cytokines such as TNF- α do not play a role in activating NF- κ B. Short term disuse atrophy is a good model for the non-canonical pathway, because during the first week of atrophy, cytokine levels are not elevated. During disuse atrophy, levels of p50, c-Rel, and Bcl-3 are up-regulated, but levels of p65, p52, and Rel B are not. In this pathway, the p50/c-Rel heterodimer is predominant, instead of the p65/p50 heterodimer (Hunter et al., 2002). If disuse atrophy continues for

3-4 weeks, the p65/p50 heterodimer becomes predominant, and TNF- α levels become elevated. In old rats, p65 levels increase earlier, and are elevated ~2-fold by the second week of disuse. Aging therefore may contribute to a stronger, earlier, and longer lasting inflammatory response, leading to more profound and accelerated muscle wasting (Bar-Shai et al., 2005).

The non-canonical model of NF- κ B is also evident during conditions of inflammatory response such as ischemia/reperfusion, infection, trauma, and shock. Under these conditions NF- κ B is initially activated by a canonical stimulus (such as TNF- α). Then a “second hit” occurs and results in persistent overstimulation of NF- κ B and its involvement results in a positive feedback loop. This positive feedback loop involves NF- κ B up-regulating transcription of inflammatory cytokines such as TNF- α and IL-1. Then, increased levels of inflammatory cytokines induce more NF- κ B activation. This cycle ends with the apoptosis of tissue and possibly the beginning of multi-system organ failure (Senftleben & Karin, 2002).

While disuse atrophy increases NF- κ B levels, exercise can also increase NF- κ B levels, as investigated in vastus lateralis, and gastrocnemius muscle. Beginning at 2 hours post exercise (treadmill run), rats had increased levels of NF- κ B binding activity and p50 levels in the nucleus (Ji et al., 2004). In gastrocnemius muscle, NF- κ B binding activity increased 50% and remained elevated until 5 hours post exercise (Ho et al., 2005). Immediately after exercise, levels of phosphorylated I κ B α were increased. Increased NF- κ B activity was likely do to increased oxidative conditions, as treatment with pyrrolidine dithiocarbamate (PDTC) (a potent antioxidant) normalized p50 and phosphorylated I κ B α levels (Ji et al., 2004). During fatiguing exercise however (wt.

lifting to the pt. of exhaustion), NF- κ B binding activity is decreased in skeletal muscle nuclei (Durham et al., 2004).

Age also increases NF- κ B/DNA binding activity. In cardiac muscle NF- κ B/DNA binding activity was 123% in 24-month old rats compared to 4 month old rats. This increase in binding activity occurred without significant increases in nuclear or sarcoplasmic p52, p50, or p65 proteins. Elevated binding activities in cardiac muscle may be due to increased exposure to oxidative damage in aged muscles. Binding activity was also increased in mouse brain, liver and kidney (Helenius et al., 1996).

NF- κ B and muscle loss

The pathway through which NF- κ B acts to promote muscle loss is mediated by many regulatory components. One of the main components in NF- κ B regulation is TNF- α . TNF- α is a potent inflammatory marker which is capable of directly stimulating protein loss, and decreasing myosin heavy chain (MCH) levels in skeletal muscle. MHC levels are reduced due to increased proteolysis, not deficiency in synthesis. TNF- α stimulates ubiquitin gene expression and ubiquitin conjugation to muscle proteins within 30-60 minutes of exposure. It does this through NF- κ B activation (Li et al., 1998). The TNF- α / NF- κ B pathway is regulated by reactive oxygen species (Li et al., 1998, Schreck et al., 1992) along with other mechanisms. For example hydrogen peroxide, a potent pro-oxidant is capable of inducing NF- κ B activation through I κ B α degradation (Li et al., 1998).

TNF- α regulates NF- κ B in a biphasic manner. This biphasic regulation is similar (and possibly the same as) the non-canonical pathway. During the first phase of biphasic regulation, NF- κ B/DNA binding and nuclear p65 levels peak during the first half hour. In

the second phase, NF- κ B/DNA binding and nuclear p65 levels remain at high levels for 24-36 hours. TNF- α is the only known cytokine to regulate biphasically. The second, long lasting and persistent phase of NF- κ B up-regulation may be responsible for muscle loss during conditions where TNF- α remains elevated such as cachexia (Ladner et al., 2003), and aging (Dirks & Leeuwenhurch, 2006).

Inhibitors of NF- κ B

Many inhibitors of the NF- κ B pathway exist. These inhibitors are of therapeutic interest. Downregulating the NF- κ B pathway could be beneficial to help reduce muscle loss, inflammation, and many other detrimental processes. Some inhibitors include: antioxidants (vitamin E, vitamin C, melatonin), proteasome and protease inhibitors (cyclosporin A, ubiquitin ligase inhibitors), I κ B α phosphorylation and/or degradation inhibitors (ibuprofen, aspirin, nitric oxide), others (glucocorticoids, calcitriol), and inhibitors of NF- κ B activating signals (anything that inhibits TNF- α , IL-1, Phorbol esters, or lipopolysaccharides). Unfortunately, most of these inhibitors have broad action, are not cell specific, potentially interfere with other cellular pathways, and may not be physiologically practical for in vivo situations (Epinat & Gilmore, 1999). Therefore, the search is still on to identify inhibitors that will act specifically, at the molecular and cellular levels, and at concentrations acceptable for in vivo applications.

Leucine

Leucine is a branched chain amino acid that is essential to our diets. It is the most common amino acid in proteins and is found in whole grains, milk, eggs, green leafy vegetables, soy, beef, chicken, and pork. Leucine is a proteogenic amino acid, and only the L-stereoisomer can be used for metabolic purposes (New World Encyclopedia, 2008). Structurally, leucine is an important building block for proteins. Leucine plays a role in regulating gene transcription and translation during protein synthesis, and may have a similar role in preventing protein catabolism (Anthony et al., 1999).

Leucine is capable of regulating muscle protein translation through a rapamycin-sensitive pathway, specifically the pathway involving mTOR. Mammalian target of rapamycin acts to phosphorylate eukaryotic initiation factor 4E binding protein 1 (eIF4E BP1), and p70 S6 kinase 1 and 2 (S6K1/2), which both lead to increases of mRNA synthesis. Stimulatory action of S6K1/2 affects S6 which directly leads to increased protein synthesis. To note, insulin also stimulates mRNA synthesis through mTOR. Insulin acts on protein kinase B/Akt (PKB/Akt) which inhibits tuberous sclerosis complexes 1 and 2 (TSC1/2) from inhibiting mTOR (Nair & Short, 2005) (**Figure 5**). Closely linked to its role in stimulating muscle synthesis is leucine's role in downregulating protein degradation.

Leucine is involved in downregulating protein degradation through the ubiquitin-dependent proteasome pathway, and through mTOR (Combaret et al., 2005). Leucine activates mTOR which inhibits the E3 ligases MAFbx/atrogin-1, potentially resulting in reduced activity of the proteasome pathway and reduced protein degradation.

Leucine activates phosphatidylinositol-3kinase (P13K) which activates PKB/Akt. PKB/Akt acts to inhibit the FOXO family of regulators. Usually FOXO upregulates MAFbx/atrogen-1 and MuRF1, however in the presence of leucine, this does not occur (Glass, 2005) (**Figure 5**).

Figure 5: Leucine's role in muscle metabolism. In catabolism, leucine acts on PKB/Akt which inhibits the FOXO family from stimulating MAFbx/atrogen-1 and MuRF1. MAFbx/atrogen-1 and MuRF1 normally increase transcription of the ubiquitin ligases. Decreased transcription of ligases leads to suppression of the ubiquitin-dependent-proteasome pathway and decreased protein degradation. In anabolism, leucine's action on mTOR causes phosphorylation of p70 S6 kinase 1 and 2 (S6K1/2), and eukaryotic initiation factor 4E binding protein 1 (eIF4E BP-1), which increase mRNA translation and protein synthesis (adapted from Glass, 2005; and Nair & Short, 2005).

The potential mechanisms of Leucine's ability to decrease negative protein balance are clear, however more studies have been done on young adults than the elderly. As a dietary supplement, Leucine could play a significant role in preventing or reversing muscle loss. Many clinical studies of leucine's role in protein metabolism have used animal models, and yielded positive results. Leucine was able to suppress myofibrillar proteolysis in chick muscle during in-vivo studies. During this study, 14 day old chicks were placed on diets containing 250mg/g body weight of leucine, isoleucine, or valine. This level of amino acids represented the amount of leucine consumed by same aged chicks during 24 hours of ad libitum feeding. The control group was food deprived and chicks were provided with saline for hydration. Suppression of myofibrillar proteolysis was due to downregulation of the ubiquitin-proteasome pathway. Specifically, leucine activated P13K and PKC, resulting in decreased levels of ubiquitin and C2 proteasome subunit mRNA. Leucine downregulated proteolysis in a dose-dependent manner, as was confirmed by quantitating output of N-Methylhistidine. N-Methylhistidine is an amino acid used as an indicator of proteolysis because it is formed by post translational methylation of histidine on actin and myosin, and it cannot be reutilized for protein synthesis (Nakashima, 2005). Leucine supplementation may also suppress muscle loss in humans. Adult women (aged 40-56) on a low calorie diet who received 10g/day leucine maintained more muscle, and lost more fat than those who received only 5g/day leucine. It is possible that leucine supplementation has a protein sparing effect on lean body mass (Layman, 2003).

More research has been done on leucine's ability to promote muscle synthesis than its ability to decrease degradation. The ability to increase synthesis is complicated

with aging, and insulin resistance. In vivo studies on rats have indicated that old rats need twice the amount of leucine as adult rats to stimulate protein synthesis (Rieu et al., 2002). In vitro studies are similar. Concentrations of 260 μ mol/L were needed to increase muscle synthesis in old rat tissue (24 months), vs. only 110 μ mol/L for adult rat tissue (6-8 months) (Dardevet et al., 2000). In another study, two groups of rats were run on a treadmill for 2 hours, rates of protein synthesis were measured, and then one group was given a “leucine drink”. Protein synthesis was reduced 18% in the both groups of exercised rats, however when given the “leucine drink” (0.27g leucine), protein synthesis was returned to normal in the experimental group (Anthony et al., 1999).

Human studies using leucine supplements to improve protein synthesis have had mixed results. These results are potentially due to flaws in supplementation design, and differences in protein metabolism which are unique to the elderly. It appears that aged human skeletal muscle may have a blunted response to anabolic stimuli. At concentrations which are considered physiological, anabolic stimuli such as amino acids – leucine specifically, do not stimulate muscle synthesis to the extent that they do in younger adults. Both young (mean age = 28) and elderly (mean age = 70) exhibit dose dependent increases in sarcoplasmic fractional synthetic rates when provided a drink containing increasing amounts of leucine (drink also contained physiologic amounts of isoleucine, phenylalanine, threonine, tryptophan, methionine, and valine). However, in the elderly dose dependent response was depressed, and the highest supplementation dose provided to the elderly (40g total protein) produced smaller synthesis increases than the lowest doses (10g total protein) in the young. Sarcoplasmic fractional synthetic rates were measured using a radioactively labeled leucine tracer. Reduction in synthesis was

believed to be due to decreases in mTOR phosphorylation and subsequent activation by leucine, and potentially reduced nutrient signal transduction in the elderly (Cuthbertson et al., 2005).

In another study, fractional synthetic rates were measured in young (mean age = 30) and elderly (mean age = 72) provided a glucose/amino acid supplement after an overnight fast. The supplement contained 40g glucose and 40g amino acids. Amino acid concentrations were based on levels typical of beef protein, with leucine having the highest concentration. Again, the elderly had lower fractional synthetic rates than the young subjects, and while the addition of glucose appeared to increase protein synthesis in the young, it had no benefit to the elderly, and was possibly detrimental (Volpi et al., 2000). It appears that when given in combination with other dietary components, leucine's ability to promote muscle synthesis appears more blunted than when given alone. Unfortunately, most human studies have involved mixed feeding or supplementation where leucine is given as part of a meal, or with other nutrients. This method would be appropriate in young or adult populations, but in the elderly it is ineffective for two potential reasons.

The first reason is decreased dietary intake that results from aging. When given a supplement which has caloric value, elderly adjust their eating so that the supplement becomes a caloric exchange for something that is left on their plate. For the elderly, supplements become isocaloric diet substitutions, instead of additions, and there is no net increase in energy or nutrients (Morley, 1997).

The second reason that leucine supplementation has had mixed results may be due to carbohydrate present in most meals. In young adults carbohydrates stimulate insulin

production, and insulin is an anabolic stimulus to muscle synthesis. In elderly, there is an age-associated insulin resistance of muscle, regardless of whole body insulin tolerance. Carbohydrate does not have anabolic action and, may actually be detrimental to muscle synthesis in elderly, by preventing amino acid action (Volpi et al., 2000). Future studies on leucine may be successful by using supplements containing only leucine, and higher concentrations than those known to increase protein synthesis in the young.

Currently, Recommended Dietary Allowances (RDA's) for protein are the same for adult and elderly populations. This issue has been controversial, and many researchers suggest that RDA's be increased for elderly populations. These suggestions postulate that increased protein may help preserve muscle mass in elderly populations. Some evidence is based on studies comparing nitrogen balance in the young to the elderly. To maintain nitrogen balance elderly subjects required 0.89-1.0g/kg protein, whereas young subjects required 0.75g/kg protein. Observations of food intake in the elderly suggest that usual intakes are often suboptimal when compared to recommended amounts. While observations of suboptimal intakes alone are not reason to change the RDAs, they are an important consideration when looking at the overall picture, and should not be ignored (Walrand & Boirie, 2005). Since leucine is likely the most potent amino acid contributor to muscle protein synthesis (Anthony et al., 2000 & Blomstrand et al., 2006), and may reduce proteolysis, it seems that increased intake of leucine would be a good idea for the elderly. Also, since aged subjects (animal and human) require twice as much leucine as adults (Layman, 2003; Rieu et al., 2002; Dardevet et al., 2000), perhaps the definitions of "physiological concentrations" for leucine should be redefined for the elderly and new intervention trials should be conducted.

Conclusion

Longevity has long been celebrated as a blessing. In order to prevent longevity from becoming problematic, attention needs to be paid to quality of life concerns that affect the aging population. The maintenance of muscle tissue, and avoidance of sarcopenia, is of key importance to successful aging. The best strategies to prevent muscle loss and simultaneously promote muscle gain have yet to be perfected. It is only through our understanding of the pathways – both anabolic, and catabolic, that a chance to improve protein metabolism exists. This chance could involve a supplement, a lifestyle change, a targeted gene or protein, a hormone or medical intervention, or a combination of the above. As a starting point, manipulation of NF- κ B could potentially involve both anabolic and catabolic pathways to result in more favorable protein metabolism in aging. Additionally, supplementation of the amino acid leucine could not only provide substrate for protein metabolism, but may facilitate decreased proteolysis and increased protein synthesis. It seems that sarcopenic muscle loss can be prevented, and even possibly reversed. Well-designed interventions may promote positive protein balance in the elderly, and should be further explored.

The overall objectives of this research are three-fold. Objectives include establishing evidence of proteolysis induced by age, determining mechanisms involved in proteolysis induced by aging and suboptimal leucine levels, and determining a level of leucine that reduces proteolysis in adult and old rats. Specifically, this study will examine the effects of aging on proteolysis in rats fed a diet containing suboptimal

leucine levels. Hypotheses include that proteolysis and NF- κ B levels are elevated in aging, and that NF- κ B elevation contributes to age induced muscle loss.

Materials and Methods

Animals

All rats used during these studies were male Sprague-Dawley rats, purchased from Harlan (Indianapolis, Indiana).

Initially three, 3 month old rats were purchased. These rats (group A) were used to establish techniques used during sacrifice, dissection, tissue homogenization, nuclear extraction, and ELISA tests. Data from this group is not included in this study but may be further analyzed during future research.

In an attempt to investigate the effect of varying leucine levels on proteolysis in same aged rats, a second group of ten, 6 month old rats was purchased (group B). The ten rats were randomly divided into two groups fed either a “low” or “high” level of leucine diet. Rats in the “low leucine” group received a diet containing 0.9g/kg pellet leucine. Rats in the “high leucine” group received a diet containing 10.7g/kg pellet leucine. Two different types of ELISA tests were performed on group B rats. Experimentation with group B rats contributed to the development of techniques used on group C and D rats. None of the data collected from group B rats will be used in this study, but may be used in future investigations.

The core of this study revolved around two groups of 7 rats. Rats were grouped according to age, either 6 month olds (group C = Adult), or 21 month olds (group D = Old). Both group C and D rats were fed a diet containing 1.8g/kg pellet leucine. ELISA and western blot analysis was performed on group C and D rats.

All rats were housed individually in polypropylene wire top cages with 12 hour light/dark cycles. Diet treatment ranged from 10-17 days. During this time, rats were

allowed to consume pelleted diet ad libitum, and had free access to water. Body weight of each rat was measured on arrival and daily thereafter. Food intake was measured daily.

Diets

Diets were purchased from Dyets, Inc (Bethlehem, PA). Diets for group A, C and D rats were formulated to provide leucine at lower levels than suggested for “maintenance”. The level of L-glutamic acid was increased to compensate for the L-leucine reduction and provide an optimal level of nitrogen. Low leucine rats in group B received a diet containing 0.9g/kg pellet leucine, with L-glutamic acid increases to provide an optimal level of nitrogen. High leucine rats in group B received “L-Amino Acid Defined AIN-93M Diet”, containing 10.7g leucine/kg pellet. Diet ingredients for diet containing 1.8g/kg leucine are listed in **Table 1**. Rats were allowed to eat ad libitum up until time of death. Control diets were not used during this study.

Table 1. Diet Composition

Ingredient	Grams/Kilogram
L-Arginine (free base)	4.4
L-Histidine (free base)	3.2
L-Lysine HCl	11.3
L-Tyrosine	6.4
L-Tryptophan	1.5
L-Phenylalanine	6.1
L-Methionine	3.2
L-Cystine	2.4
L-Threonine	4.6
L-Leucine	1.8
L-Isoleucine	5.9
L-Valine	6.9
Glycine	2.2
L-Proline	14.3
L-Glutamic Acid	34.2
L-Alanine	3.2
L-Aspartic Acid	7.9
L-Serine	6.6
Cornstarch	451.192
Dyetrose	150
Sucrose	130
Cellulose (microcrystalline)	50
Soybean Oil	40
TBHQ	0.008
Salt Mix # 210053	35
Sodium Bicarbonate	5.2
Vitamin Mix # 310025	10
Choline Bitartrate	2.5

Tissue Collection

Rats were sacrificed on days 10-17 of the diet. Grouped pairs were sacrificed on the same day to eliminate discrepancy between young and old rats. For example, rats 7C and 7D were killed on the same day. Rats were anesthetized using carbon dioxide gas in a closed chamber. Blood samples were collected via cardiac puncture into a syringe containing ethylene-diamine-tetra-acetic acid (EDTA), and were centrifuged at 4°C for 10 minutes. The plasma portion was separated and frozen in liquid nitrogen for storage at -80°C. Whole dissections of muscle and organ tissues were extracted from each rat and weights were recorded (results section). Smaller fractions were then prepared and frozen in liquid nitrogen for storage in cryovials at -80°C (**Table 2**).

Table 2. Muscles and Organs Collected From Adult and Old Rats

Muscles and Organs Collected From Adult and Old Rats
Gastrocnemius
Tibialis Anterior
Soleus
Quadriceps
Heart
Liver
Kidney
Lungs

Lysate Preparation for Group A

Group A rats were used in methods development for nuclear extraction and NF- κ B quantification using enzyme linked immunosorbent assay (ELISA). Kidney, liver, heart, gastrocnemius (gastro), quadriceps, and tibialis anterior (tib) from group A rats were processed for nuclear extraction using the Imgenex (San Diego, CA) NF- κ B/p65 ActivELISA Kit®. All samples were thawed on ice, weighed into 1g portions, homogenized using a polytron homogenizer and processed using Imgenex recommendations for “preparation of lysates from cells” into whole cell, cytosolic and nuclear fractions. Because extracted soleus muscle weighed less than 1 gram, it was not used during this procedure.

Nuclear and Cytosolic Protein Extraction for Group B

Group B rats were processed for nuclear extraction using the Active Motif (Carlsbad, CA) TransAM Nuclear Extraction Kit®. During rat dissection, 1 g samples of gastrocnemius, and tibialis anterior, and 0.5 g of soleus were excised from rats and used for lysate preparation (instead of being frozen in liquid nitrogen). Gastrocnemius, soleus, and tib samples were cut into smaller pieces and manually homogenized on ice, following extraction protocols per Active Motif recommendations. Cytosolic and nuclear fractions were obtained, frozen in liquid nitrogen, and stored at -80°C.

Nuclear and Cytosolic Protein Extraction for Groups C and D

Gastrocnemius and tibialis anterior muscles from groups C and D rats were immediately processed for nuclear extraction using the Active Motif (Carlsbad, CA) TransAM Nuclear Extraction Kit®. A hypotonic buffer solution was prepared using 6ml of 1x hypotonic buffer/gram tissue + 6 μ l 1M DTT/g tissue + 6 μ l detergent/g tissue, and

was incubated on ice for 15 minutes. Next, 0.5 gram samples of gastro and tib were manually homogenized on ice in the buffer solution, until the mixture was a smooth consistency and the pestle could be easily manipulated. The homogenate was centrifuged for 10 minutes, 850x g at 4°C, and the supernatant was transferred into prechilled microcentrifuge tubes. The supernatant was considered the “cytosolic fraction”. The homogenate pellet was suspended with 500µl 1x hypotonic buffer and incubated on ice for 15 minutes. Fifty µl detergent solution was added and the mixture was vortexed for 10 seconds at max speed. It was then centrifuged for 30 seconds at 14000xg in a prechilled microcentrifuge tube at 4°C. Following centrifugation, the supernatant was pooled with the “cytosolic fraction” from above and equally aliquoted among 5 centrifuge tubes. Aliquot amounts were recorded and the “cytosolic fraction” was frozen in liquid nitrogen and stored at -80°C.

The pellet was resuspended in 200µl “Complete Lysis Buffer” (178µl Lysis Buffer AM1+ 2µl protease inhibitor cocktail (PIC) + 20µl 10mM DTT), vortexed for 10 seconds, and incubated on ice on a rocking platform (150rpm), for 30 minutes. After incubation the product was vortexed for 30 seconds, and centrifuged for 10 minutes at 14000xg at 4°C. The resulting supernatant was aliquoted equally between 10 prechilled centrifuge tubes, was frozen in liquid nitrogen and stored at -80°C. This product was considered the “nuclear fraction”.

Protein Quantification

Nuclear and cytosolic protein concentrations from all rat groups were quantified using PIERCE® (Rockford, IL) bovine serum albumin (BSA) protein assay kit (Product # 23225): test tube method. The albumin standard (BSA) was diluted in DI water to

prepare standards ranging in BSA concentration from 0-2000µg/ml BSA. Working reagent was prepared using a ratio of 50 parts Reagent A to 1 part Reagent B per kit protocol. Nuclear and cytosolic extracts were prepared in a 1:5 dilution with DI water. nuclear lysis buffer was used as a “blank” (125µl DTT + 2350µl nuclear extract buffer (from TransAM kit), + 25µl PIC 100x) and prepared in 1:5 dilutions with DI water. Rat nuclear and cytosolic extracts, and standard samples were prepared in triplicate. Fifty µl of each standard/sample was added to 1ml working reagent, vortexed, and incubated at 37°C for 30 minutes. Tubes were allowed to cool and samples were transferred into a cuvette, where absorbances were read at 562nm. DI water was used to blank the spectrophotometer.

Absorbances were recorded on Excel®. A standard curve was created using the average absorbances from tubes with 0-1000µg/ml BSA concentration, and concentrations above 1000µg/ml were not used for the standard curve due to decreased linearity. All absorbances were standardized for the “blank”. From the standard curve an equation was generated, allowing calculation of protein concentration from the samples. Outliers in triplicate samples were identified and were excluded from calculations of the standard curve and sample protein concentrations.

ELISA Testing of Group A

ELISA testing on group A rats was performed using Imgenex NF-κB/p65 ActivELISA Kit® manufacturer recommendations. A standard curve was created using concentrations of 0-100ng/well recombinant p65. One hundred microliters of a 0.5µg/µl solution was added to individual wells representing nuclear extractions from kidney, liver, heart, gastro, quad, and tib. Nuclear extractions from quadriceps muscle were

plated in a dilution series from $\sim 5\mu\text{g}/\mu\text{l}$ to $0.0625\mu\text{g}/\mu\text{l}$ in attempts to optimize absorbance readouts. All nuclear extraction and standard samples were loaded in duplicate. The ELISA reaction was visualized at 405nm.

ELISA Testing of Group B

The Active Motif TransAM ELISA Kit® was used for group B rats. Twenty microliters of a $0.1\mu\text{g}/\mu\text{l}$ solution from nuclear fractions of gastro, tib, and soleus were loaded into wells producing sample concentrations of $\sim 2\mu\text{g}/\text{well}$. Tibialis samples from both nuclear and cytosolic fractions were used in solutions up to $1\mu\text{g}/\mu\text{l}$ ($\sim 20\mu\text{g}/\text{well}$) to optimize ELISAs. Jurkat samples were used as a positive control for p65 and complete lysis buffer was used as a negative control. Wild type and mutant oligonucleotides were used with tibialis cytosolic and nuclear samples and with Jurkat controls to confirm specificity of the ELISA. Primary and secondary antibodies specificity was confirmed by exclusion of each individually. The ELISA reaction was visualized at 450nm.

ELISA Testing of Group C and D

ELISA testing on group C and D rats was performed using the Active Motif TransAM ELISA Kit®. Due to results obtained from group B ELISA testing, variations were made from previous procedures using the TransAM ELISA Kit®. Manual homogenization on ice was performed as suggested however only 0.5g of each muscle sample was homogenized, and the amount of buffer used during the homogenization was doubled from $\sim 3\text{ml}$ to $\sim 6\text{ml}$ (as reviewed in nuclear and cytosolic protein extraction of group C and D). Only gastrocnemius samples were used for ELISA. Gastrocnemius nuclear extract was added to ELISA wells in a concentration $\sim 1\mu\text{g}/\text{well}$, and gastro cytosolic extract was added in concentrations of $\sim 1\mu\text{g}/\text{well}$ and $\sim 10\mu\text{g}/\text{well}$. Jurkat cells

were used as a positive control for p65. Wild type and mutant oligonucleotides were added to both gastro nuclear and cytosolic extract and jurkat cells as controls for specificity. The ELISA reaction was visualized at 450nm.

Western Blotting

After extensive yet unsuccessful trials with ELISA testing, western blots were attempted. Western blots were advantageous to ELISA techniques because of their ability to detect denatured p65, and because non specific binding could be visually identified and excluded during quantification of the blots.

Electrophoresis:

PIERCE Precise Protein Gels (4-20%) Tris-HEPES-sodium dodecyl sulfate (SDS) precast polyacrylamide mini gels were used for electrophoresis. Cytosolic and nuclear extractions from gastro and tib were prepared in microcentrifuge tubes by adding 5x load buffer with SDS (Boston BioProducts), using phosphate buffered saline (PBS) as a diluent, and adding cytosolic fraction of rat samples, to create a 1µg/µl solution. Fifty µl of this solution was loaded for each rat sample with alternate loading of adult and old rat samples. Ten µl Full Range Rainbow Recombinant Protein Molecular Weight Marker (GE Healthcare, Piscataway, N.J) was added to one lane of each gel to validate protein sizes and transfer. Ten µg of nuclear extract from jurkat cells was used as a positive control for NF-κB (Santa Cruz Biotechnologies, Santa Cruz, CA). Rat muscle protein samples, jurkat, and molecular weight marker were boiled for 4 minutes, and cooled prior to loading. Because each gel had only 10 lanes, we were unable to load all the samples from young and old rats onto the same gel. Muscle samples from rats 1-4 C and D were loaded on one gel, and 5-7 C and D were loaded onto a separate gel. Unfortunately, this

may have created considerable variation which was unavoidable given the resources available for the study. The gel chamber was connected to a BioRad PowerPac 3000 and samples were electrophoresed for 5 minutes at 100V, then at 80V, for a total time of 1 hour, 10 minutes.

Transfer:

After electrophoresis, gels were soaked in BupH Tris-Glycine Buffer (PIERCE) for 20 minutes. Nitrocellulose Transfer Membranes (PIERCE) were cut to size, and soaked in BupH Tris-Glycine Buffer for 15 minutes. Thick filter paper was cut to size briefly submerged in BupH Tris-Glycine Buffer, and placed on a BioRAD Semi-Dry Transfer apparatus. The nitrocellulose membrane was layered on top of the filter paper, followed by the gel, and then a final layer of filter paper. The transfer was run for 45 minutes at 15V constant. Gels were incubated with GelCode Blue Stain Reagent (PIERCE) to visualize protein bands, and stored in air tight containers. Membranes were air dried, and covered in plastic wrap prior to Western Blots.

Western Blots:

All probing procedures were performed at room temperature on a rocking platform. Membranes were washed 3x for 5 minutes/wash with 20ml Wash Buffer (WB) [10X TBS (Boston Bio Products) + 500ul Tween 20 Surfact-Amps (PIERCE) + 89.5ml DI water]. Blocking was then performed using 20ml StartingBlock™ buffer (PIERCE) for 20 minutes. Membranes were washed 5x with WB for 5minutes/wash. Primary antibodies were aliquoted into 15ml StartingBlock™ buffer (**Table 3**) and membranes were probed for 1.5 hours.

Western blots were probed with primary antibodies for p65, Ub, Actin and histone deacetylase 1 (HDAC1) (**Table 3**). Ubiquitin and p65 antibodies were used to determine potential proteolysis. Actin is a protein expressed in all eukaryotic cells, not known to change with age, which was used as a control for loading differences between adult and old rats. Actin produces a band at 43kDa. HDAC1 is a mammalian protein expressed in the nucleus of cells and was used to determine if the nuclear and cytosolic fractions contained only nuclear or cytosolic components as expected. HDAC1 produces a band at 62kDa and was used as a control for loading differences. All antibody concentrations used were based on manufacturer recommendations.

After incubation with primary antibodies, membranes were washed 5x with WB for 5 minutes/wash, and incubated with secondary antibody (**Table 3**), diluted in 15ml StartingBlock™ buffer for 1 hour. Again, membranes were washed 5x with WB for 5minutes/wash. Membranes were exposed to the working solution [2.5ml Luminol Enhancer (PIERCE) + 2.5ml Stable Peroxide Solution (PIERCE)] for 5 minutes. After exposure to working solution, membranes were wrapped in plastic, and transported to the chemiluminescence imaging instrument.

Table 3. Antibodies used in Western Blotting

Primary Ab	Amount	Secondary Ab	Amount	Protein of Interest
NF-kB p65 (C-20) sc-372 (S.C.B)	37.5ul	Immuno Pure Goat Anti Rabbit IgG, (H+L), Peroxidase Conjugated (T.S.)	0.4ul	NF-kB (p65 subunit)
Ub P4D1 sc-8017 (S.C.B)	75ul	Donkey Anti Mouse IgG (S. C. B.)	10ul	Ubiquitinated Proteins
HDAC1 (S.C.B)	15ul	Immuno Pure Goat Anti Rabbit IgG, (H+L), Peroxidase Conjugated (T.S.)	0.4ul	HDAC1
Actin (H-300) sc-10731 (S.C.B)	75ul	Immuno Pure Goat Anti Rabbit IgG, (H+L), Peroxidase Conjugated (T.S.)	0.4ul	Actin

S.C.B = Santa Cruz Biotechnologies

T.S. = Thermo Scientific

Imaging/Quantitation:

Membranes were imaged using BioRad Chemiluminescence XRS Instrument and the Quantity One program. The “Chemi High Sensitivity” setting was used to capture luminescence, and membranes were exposed for 40 minutes. Lanes were quantitated using “quick volume analysis” and the volume contour tool, with local background subtraction according to the recommendations of BioRad.

Creating a Standard Curve for Western Blots:

A standard curve was created to establish linearity for jurkat cells and cytosolic muscle fractions and to optimize future levels of jurkat and cytosolic sample used. Jurkat samples containing 1.25, 2.5, 5, and 10µg, and cytosolic gastrocnemius samples containing 6.75, 12.5, 25, and 50µg were loaded into a PIERCE Precise Protein Gel (4-20%) Tris-HEPES-SDS precast polyacrylamide mini gel membrane. The membrane was

subsequently electrophoresed, blotted, imaged, and individual lanes were quantified into “adjusted volume” as described previously (**Figure 6**). Graphs of jurkat and cytosolic samples were made by plotting adjusted volume on the y-axis, and concentration ($\mu\text{g}/\text{well}$) on the x-axis (**Figure 7**). A line of best fit was added. Both jurkat cells and cytosolic samples appeared to be linear for all concentrations tested. Jurkat linearity was established between adjusted volumes of 4081.82-82674.45 ($\text{INT} \cdot \text{mm}^2$) Gastrocnemius cytosolic linearity was established between adjusted volumes of 585.37-72918.43 ($\text{INT} \cdot \text{mm}^2$). All quantitated membranes from subsequent experiments were used only if both jurkat and cytosolic samples fell within established ranges of linearity. Jurkat was best visualized at concentrations of $10\mu\text{g}$ and cytosolic samples were best visualized at concentrations of $50\mu\text{g}$. These optimal concentrations were loaded in all subsequent experiments.

Figure 6: Gel used to create standard curve. Jurkat samples containing 1.25, 2.5, 5, and 10 μ g, and cytosolic gastrocnemius samples containing 6.75, 12.5, 25, and 50 μ g were loaded into a PIERCE Precise Protein Gel (4-20%) Tris-HEPES-SDS precast polyacrylamide mini gel membrane. Ten μ l Full Range Rainbow Recombinant Protein Molecular Weight Marker (M.W.M) was loaded to validate protein size and transfer. Lanes were quantitated using the volume contour tool and local background subtraction as suggested by BioRad.

Figure 7: Standard curve of gastrocnemius cytosolic sample. The standard curve was created using Microsoft Excel®. Adjusted volume (INT*mm²) of each lane was plotted on the y axis. Concentration (µg/well) of sample loaded was plotted on the x axis, and a line of best fit was added.

Results

Body and Organ Weights, and Dietary Intake Analysis

Initial and final body weights of adult and old rats were significantly different, with old rats weighing 26% more initially, and 28% more than adult rats at time of death. Percentage body weight loss was not different between the two groups, nor was gross daily food intake. However, when food intake was calculated as a percentage of body weight, old rats ate 39.6 % less than adult rats (**Table 4**). Gross weights of tibialis anterior, heart, kidneys, and lungs were significantly higher in old rats than adult rats. Gross weight of soleus muscle was nearly significantly higher with $p = 0.0632$ (**Table 5**). When expressed per body wt * 10², both soleus and gastrocnemius weights were significantly less in old rats, while all other tissues appeared to adjust proportionally to varying body weights (**Table 6**).

Table 4. Average body weight and food intake of adult and old rats

	Adult ¹	Old ²	P value (2 tailed)
Initial body weight (g)	444.43 ± 18.96	560.29 ± 37	<0.0001
Body weight at death (g)	410.43 ± 23.4	526.29 ± 39.69	0.0002
% Weight loss	7.63 ± 2.61	6.11 ± 2.13	0.1757
Daily food intake (g)	10.19 ± 1.54	9.89 ± 0.95	0.6982
Daily food intake (g) as % body weight	2.4 ± 0.40	1.45 ± 0.29	0.0014

¹ Values represent means ± SD from 7 rats aged 6 months

² Values represent means ± SD from 7 rats aged 21 months

Table 5. Gross average tissue weights (g)

	Adult ¹	Old ²	P value (2 tailed)
Soleus	0.330 ± 0.023	0.372 ± 0.047	0.0632
Tibialis Anterior	1.299 ± 0.266	1.477 ± 0.192	0.0377
Gastrocnemius ³	2.487 ± 0.304	2.448 ± 0.264	0.6435
Heart	1.469 ± 0.115	1.844 ± 0.199	0.0073
Kidney	2.337 ± 0.134	3.189 ± 0.287	0.0003
Lungs	1.912 ± 0.199	2.398 ± 0.341	0.0200

¹ Values represent means ± SD from 7 rats aged 6 months

² Values represent means ± SD from 7 rats aged 21 months

³Represents average weight of only one hindlimb gastrocnemius muscle

Table 6. Average tissue weight per body weight * 10²

	Adult ¹	Old ²	P value (2 tailed)
Soleus	0.0805 ± 0.0042	0.0710 ± 0.0112	0.0471
Tibialis Anterior	0.3181 ± 0.0704	0.2817 ± 0.0417	0.1549
Gastrocnemius ³	0.6067 ± 0.0655	0.4679 ± 0.0685	0.001
Heart	0.3579 ± 0.02	0.3526 ± 0.0535	0.798
Kidney	0.5695 ± 0.0184	0.6064 ± 0.0459	0.1006
Lungs	0.4683 ± 0.0686	0.4589 0.0829	0.8182

¹ Values represent means ± SD from 7 rats aged 6 months

² Values represent means ± SD from 7 rats aged 21 months

³Represents average weight of only one hindlimb gastrocnemius muscle

ELISA Testing

Absorbances from group A nuclear extracts were not different from negative controls used in the Imgenex NF- κ B/p65 ActivELISA Kit®. Appropriate absorbances were produced from provided standards allowing successful creation of the standard curve. The Imgenex kit reacted as expected with provided standards and controls but produced no reaction with our nuclear extracts.

Absorbances from group B rat nuclear and cytosolic extracts were not different from negative controls used in the Active Motif TransAM ELISA Kit®. Varying concentrations of nuclear and cytosolic extracts did not affect absorbance readouts. Jurkat cell positive controls produced significant levels of absorbances thereby suggesting detection of p65. Wild type oligonucleotides were able to lower absorbances in Jurkat cells but had no effect on rat nuclear and cytosolic extracts. Mutant oligonucleotides had no effect on Jurkat positive controls or rat extracts. Therefore, specificity of the kit for p65 was confirmed using Jurkat cells, and wild type and mutant oligonucleotides. However, unfortunately the Active Motif kit was unable to detect p65 in either nuclear or cytosolic rat samples.

Despite changes in procedures during nuclear extraction, absorbances from group C and D nuclear and cytosolic extracts were not different from negative controls used in the Active Motif TransAM ELISA Kit®. ELISA testing on group C and D rats produced the same results found in group B rats. Again, the kit appeared to accurately detect p65 in provided controls but not in our samples.

HDAC1, Actin, NF- κ B p65, and Ub levels in Adult and Old rats

HDAC1 was observed in both nuclear and cytosolic extractions from gastrocnemius. This indicated that our cytosolic fraction contained nuclear proteins, and our nuclear extraction did not extract 100% of the nuclei. Because levels of HDAC1 were higher in cytosolic than in nuclear fractions, the majority of the nuclei were believed to be in the cytosolic fraction. Since p65 is activated in the nucleus, and the majority of the nuclei appeared to be in our cytosolic fraction, “cytosolic fractions” were used for all proceeding western blots in an attempt to maximize detection of p65.

We were unable to detect actin in any of our cytosolic or nuclear samples despite extensive troubleshooting, probing with 2 different actin antibodies, and attempts at optimizing antibody concentrations. Consequently, actin was abandoned as a control for loading, and HDAC1 was used instead.

NF- κ B p65 levels were significantly higher in gastrocnemius muscle of old than in adult rats. Levels of p65 also tended to be higher in tibialis anterior, however significant differences did not exist between ages (**Figure 6**). HDAC1 concentrations were not significantly different between ages in either muscle. This confirmed that differences in Ub or p65 concentrations were not due to differences in loading between ages. The p65/HDAC1 ratio was significantly higher in old gastrocnemius muscle, indicating that p65 is elevated in gastrocnemius with age. Quantitating p65/HDAC1 reduced the p value in tibialis anterior muscle, however the differences between ages was not significant. Ub concentrations appeared almost twice as high in gastrocnemius of old vs. adult rats, however this was insignificant, likely due to large degree of variation. Age

did not produce significant differences in Ub isolated from tibialis anterior muscle (**Table 7 & Table 8**)

Table 7. Western blot analysis from gastrocnemius muscle

Gastrocnemius Muscle	Adult ¹	Old ²	P Value (2 tailed)
p65*	24866.8 ± 10181.1	44403.5 ± 18848.8	0.0443
HDAC1*	62837.3 ± 37565.9	64403.4 ± 40542.6	0.904
Ub*	272607.7 ± 281502.4	506686.8 ± 483645.5	0.224
p65/HDAC1	0.5366 ± 0.3144	0.9615 ± 0.6719	0.0482

* Results expressed as Adjusted Volume (INT*mm²)

¹ Values represent means ± SD from 7 rats aged 6 months

² Values represent means ± SD from 7 rats aged 21 months

Table 8. Western blot analysis from tibialis anterior muscle

Tibialis Anterior Muscle	Adult ¹	Old ²	P Value (2 tailed)
p65*	7451.1 ± 4200	12397.64 ± 7547.5	0.1289
HDAC1*	13819.4 ± 10584.3	22280.2 ± 23704.9	0.322
Ub*	399076.4 ± 221754.0	423375.5 ± 216221.26	0.7546
p65/HDAC1	0.50383 ± 0.23333	0.76211 ± 0.35920	0.1705

* Results expressed as Adjusted Volume (INT*mm²)

¹ Values represent means ± SD from 7 rats aged 6 months

² Values represent means ± SD from 7 rats aged 21 months

Figure 8: Graphical Representation of Gastrocnemius and Tibialis Anterior p65
Data from western blot analysis of adult and old rats. Values expressed as Adjusted Volume (INT*mm²). Error bars represent standard deviations, n = 7.

Discussion

All attempts to quantify p65 using ELISA were unsuccessful. Although both Imgenex and ActivMotif insisted that their nuclear extraction procedures and subsequent ELISA tests could detect p65 in skeletal muscle, this study was unable to detect p65 in levels different from negative controls. Western blotting confirmed that p65 was in fact present in samples used for ELISA. During multiple conversations and attempts at troubleshooting with manufacturers, manufacturers could not produce references citing successful use of skeletal muscle for p65 ELISA, nor provide any solutions to improve the assay. Therefore, it is our conclusion that skeletal muscle may be problematic for use in p65 ELISAs due to inability to control for nonspecific binding. All discussion of this study is based on results of western blotting, as ELISA tests were unsuccessful.

Aging is associated with many physiologic changes. Some of the changes were observed in our 21-month old rats. Rats appeared more sedentary, hair was thinner, and older rats were visually more obese than their younger counterparts. During dissection and muscle extraction, fatty tumors were observed in some of the older rats.

Old rats consumed the same gross amount of diet as adult rats, however when assessed as diet intake per body wt., older rats ate only 60% as much as adult rats. Although caloric needs are decreased with aging – and we may expect a decline in total kcal intake, many studies suggest protein needs are elevated with aging. In order to prevent deficiencies, intakes of protein may need to be increased with aging (Walrand & Boirie, 2005). In this study, rats were fed a diet containing suboptimal concentrations of the amino acid L-Leucine in attempts to replicate conditions in which skeletal muscle proteolysis was more likely to occur. It seems logical that when faced with suboptimal

protein levels, rats would consume more in order to meet protein needs. This may have been the case with adult rats, however old rats did not increase their food intakes to provide for their larger body sizes, nor for potentially increased age associated needs. This is possibly due to the decreased desire to eat, decreased sensation of hunger, extended periods of satiety, and slower gastric emptying, as observed in elderly humans and reviewed by Clarkston et al. (1997) & Morley (2001). In human studies, high nutrient/high caloric diet supplementation does not result in any net increase in nutrients of calories consumed by the elderly. If an elderly person does eat/drink some supplement, they eat proportionally less of their meal resulting in isocaloric substitutions of supplement for meal (Morley, 1997) as if they were operating on a calorie “budget”. Perhaps old rats use a similar caloric budget or cutoff point causing them to inadequately compensate for suboptimal nutrient provision. Perhaps age associated intake declines (as observed when consumption diet was expressed per body wt. during this study) are some sort of survival mechanism and in some way beneficial to aged rats. With the potential for reduced gastric motility with age, high intakes may be uncomfortable or even painful for the elderly. Perhaps old rats have less energy to perform the act of eating (stand up on hind legs, chew pellet until it is small enough to fall through holes in wire cage top, eat remaining pellet, repeat). Decreased food intake may also have something to do with leucine’s effect on appetite. Interestingly, L-leucine administered intracerebroventricularly in rats produces increased mTOR signaling, and results in anorexia lasting for 24 hours and subsequent weight loss (Cota et al., 2006).

Likely, multiple reasons contribute to the “anorexia of aging” and decreased pellet consumption/body wt. observed in old rats. Whatever those multiple explanations may

be, the results were overall wt. loss, and significant reduction in soleus and gastrocnemius weight as a percentage of total body weight when compared to adult rats. Of note, aging did not seem to affect the vital organs as it did the skeletal muscle. Heart, lungs, and kidney weights increased proportionally to increased body size in old rats. Percent weight lost was not different between adult and old rats, despite differences in consumption/body weight. Decreased activity and likely lower metabolic rates in old rats may have reduced their caloric needs, and led to similar wt changes between adult and old rats.

Older rats appeared to have more body fat than adult rats, however, body composition was not studied. In addition to observed overall body fatness, organs from older rats were often coated with a fatty layer. This fatty coating of the organs may have contributed to increased weight. In future studies photographs comparing side by side organs from adult and old rats would be beneficial.

Although body composition studies were not performed on the rats, it appears that younger rats may have preserved muscle mass while losing fat, with older rats experiencing the opposite and losing more muscle. This is partially confirmed by the fact that when expressed per body weight, old rats had proportionally smaller hindlimb muscles than adult rats. Soleus and gastrocnemius muscles were both smaller when expressed per body weight. Differences were significant with soleus 0.88x and gastrocnemius 0.77x smaller proportionally in old vs. adult rats. Tibialis anterior muscle appeared smaller, but the difference was not significant. Age related decreases in gastrocnemius weight/total body weight have also been found by other investigators (Kimball et al., 2004). Gastrocnemius and soleus muscle size did not increase

proportionally with increased body mass during aging. This may be due to decreased muscle synthesis, and/or increased muscle loss.

Decreased muscle synthesis may be due to suboptimal leucine levels in diet. Leucine has potential to be a strong anabolic stimulus, promoting muscle synthesis through the mTOR pathway (Nair & Short, 2005). In this study, dietary levels of leucine were reduced to a suboptimal state as an attempt to exacerbate age-induced muscle loss. Decreased leucine levels may have led to decreased mTOR signaling, decreased phosphorylation of p70S6K1/2, decreased phosphorylation of eIF4E BP1, and subsequent reduction of mRNA translation and protein synthesis. In addition, multiple studies have suggested that old rats need up to 2.36X more leucine than adult rats to stimulate muscle synthesis (Rieu et al., 2002 & Dardevet et al., 2000). It can be postulated then, that the older rats consumed inadequate leucine levels to promote protein synthesis as they consumed less diet/body weight, and their needs were higher than adult rats. Older rats may also be insensitive to anabolic stimuli as was investigated in other studies (Combaret et al., 2005).

Under normal metabolism, insulin acts anabolically to promote muscle synthesis, and reduces levels of muscle catabolism. Since insulin sensitivity decreases with age (Short et al., 2003), and with obesity (Roubenoff, 2003a) in humans, it is possible that our old rats had impaired insulin sensitivity. In other studies, impaired insulin sensitivity in rats has been associated with decreases of protein synthesis up to 37%, and increases in protein degradation at 141% (Rodriguez et al., 1998). Likely, impaired insulin sensitivity would prevent increases in protein synthesis by inhibiting or blunting activation of PKB/Akt-P and mTOR (**Reviewed in Figure 1**). Insulin insensitivity is also linked to

insufficient utilization of BCAAs (Marchesini et al., 1987), of which leucine would be a primary concern.

Increased muscle degradation in old rats significantly contributed to reduced gastro muscle size/body weight, as evidenced by increased p65 and Ub levels. Multiple conditions and pathways likely contributed to increased degradation. Noteworthy is the possibility of age-induced increases in oxidative stress and TNF- α as confirmed in a study by Clavel et al. (2006) which demonstrated increased levels of oxidative stress and TNF- α in 24-month old rats, but not in 6-month old rats. Elevated TNF- α levels have been identified in vastus lateralis muscles of old rats resulting in a reduction of muscle mass of 15%. TNF- α was not found be elevated in aged soleus muscle (Phillips & Leeuwenburgh, 2005). Further analysis to measure TNF- α levels in our rats would be beneficial, as TNF- α was not examined during this study.

Although this study was unable to confirm increased proteolysis through elevated TNF- α , it was able to confirm age induced increases in NF- κ B. Expression of the p65 subunit of NF- κ B was 1.79x higher in old vs. adult gastrocnemius muscle. This measurement represented whole cell levels of p65. p65 is only able to affect gene transcription in the nucleus of cells. This study assumed that increased whole cell concentrations of p65 likely correlated with increased potential for nuclear translocation, NF- κ B binding, and NF- κ B mediated transcription. NF- κ B mediated transcription of atrogenes and E3 ligases would result in increased protein degradation and explain the decreased muscle weight found in gastrocnemius and soleus muscles.

If, in fact, increased cellular NF- κ B levels in gastrocnemius muscle were causing increased transcription of E3 ligases, we may expect increased activity of the 26S

proteasome, and increased ubiquitination of proteins. Confirming this assumption, levels of ubiquitinated proteins in old rat gastrocnemius appeared nearly 2 fold higher than in adult rats. These results were not significant however, possibly due to large variation between samples loaded onto different gels, and difficulty quantifying the images derived from ubiquitin westerns. Elevations in ubiquitinated proteins would likely correlate with increased protein degradation and at least partially explain reduced gastrocnemius muscle in old rats.

In this study, age associated increases in p65 or ubiquitinated proteins were not seen in tibialis anterior muscle of old rats. The lack of increase in p65 or ubiquitinated proteins couples nicely with the lack of age associated muscle wt decline. With decreased levels of p65 and ubiquitinated proteins (as compared to gastrocnemius muscle), one can propose an overall decreased activity of catabolic pathways including decreased ubiquitination and degradation by the 26S proteasome, and decreased activity of E3 ligases. However, our results conflict with other investigations. Tibialis Anterior of aged rats (24 month-old) were found to have 90% more ubiquitinated proteins than adult rats (6 month-old) (Clavel et al., 2006). Additionally, tibialis anterior muscle from aged rats is known to have 3.3x MURF-1 mRNA, and 2.5x Atrogin-1 mRNA than in adult rats, resulting in decreased muscle fiber diameter and muscle fiber area (Cai et al., 2004a & Clavel et al., 2006). Both MURF-1 and Atrogin-1 are believed to be upregulated by NF- κ B. Therefore we would expect increased MURF-1/Atrogin-1 mRNA to be coupled with increases in NF- κ B activity in tibialis anterior muscle. However, this was not confirmed during this study. Possible explanations may be differences between 21 month-old and 24 month-old rats, dietary differences, and procedural variations. Future studies

examining levels of MURF-1/Atrogin-1 mRNA, and examining muscle fiber area (instead of weight) may be warranted to determine pathophysiology of muscle metabolism in 21 month-old rats, and confirm findings of our study.

During our study, p65 concentrations in soleus muscle were not measured, however increased p65 in aged soleus has been confirmed by other investigators (Phillips and Leeuwenburgh, 2005). Increases in MAFbx/atrogin 1 mRNA also occur in aged soleus muscle (Pattison et al., 2003). When considered with elevated p65, elevations in these E3 ligases would likely cause increased protein degradation, and result in reduced soleus size observed during this study. Interestingly, ubiquitin expression has previously not been found to be elevated in soleus muscles, (Cai et al., 2004b). Soleus muscles are considered type 1 (slow twitch), and respond differently to sepsis than type 2 (fast twitch) muscles. In sepsis, levels of MURF1 and atrogin 1 are not elevated in the soleus as they are in the extensor digitorum longus muscle (Wray et al., 2002b). TNF- α is not elevated in aged soleus muscle (Phillips and Leeuwenburgh, 2005). Soleus muscle is the only type 1 muscle examined by this study, and is somewhat atypical in age-induced responses to TNF- α and Ub. In soleus, it is possible that degradation occurs predominantly through pathways other than through ubiquitin dependent proteolysis. Of note, Cathepsin L expression is elevated in soleus muscle of aged rats (Pattison et al., 2003).

This study was able to confirm age-induced decline in muscle weight of gastrocnemius and soleus, and age-associated increases in the transcription factor NF- κ B in gastrocnemius. Sub-optimal dietary leucine levels used in this study may have been a factor, possibly exaggerating the decline in muscle mass associated with age, and the expression of NF- κ B. Age associated muscle loss is debilitating, but possibly avoidable.

Future studies involving optimal and supra-optimal dietary leucine levels in different aged rats may reveal opportunities to bypass or blunt age induced proteolysis, and promote positive protein balance. Additionally, studies examining downstream targets of NF- κ B may uncover potential targets to reduce protein loss.

This study has confirmed that muscle cell protein balance is a complicated matter. Clearly multiple pathways of both proteolysis and anabolism are involved, and pathophysiology of muscle loss may be age, and muscle specific. Age exhibits a significant effect on muscle homeostasis as confirmed during our study. Elderly rats experienced significant decline in muscle mass/body weight of both gastrocnemius and soleus muscle. Elderly rats also consumed less diet/body weight than adult rats, and did not compensate for suboptimal dietary leucine by increasing their consumption of diet. Our study of elderly rats exposed to suboptimal diet, may mimic actual living conditions of some elderly humans. As we age, access and intakes of an optimal diet may become more challenging due to decreased ability to perform ADLS, economic hardships, depression, physiologic reasons (chewing/swallowing/malabsorption) or many other issues. Providing the elderly with easily accessible and consumable nutrient dense diets, as well as encouraging and assisting intakes, may help reduce age related muscle loss.

There are several potential changes in techniques or strategies that could be used in our future animal studies to improve our understanding of age induced muscle loss. Body composition studies on adult and old rats would allow measurement of changes in lean body mass and adipose tissue with age. Lean body mass could be measured on elderly rats fed diets containing different levels of leucine to confirm that dose dependent relationships exist between leucine intake and muscle metabolism. Pair feeding studies

would prevent potential differences in protein and caloric intakes between groups. Adiposity changes could be examined together with potential changes in insulin sensitivity and inflammatory markers (TNF- α , or IL-1). This may allow determination of the expression levels of inflammation, degree of insulin insensitivity, and percent adiposity at which muscle metabolism is negatively affected. Additionally, downstream targets of NF- κ B such as MURF-1 may be interesting to quantify in adult and old rats fed diets containing different levels of leucine.

In research, it seems that when one question is answered, many new questions are created. This study is no exception, and has only skimmed the surface in investigating the etiologies of age related muscle loss. Hopefully, others can learn from the strengths, weaknesses, failures, and successes of this study, and move forward with future investigations.

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