

EFFECTS OF AGE AND CALORIE RESTRICTION
ON TUMOR NECROSIS FACTOR-ALPHA SIGNALING IN SKELETAL MUSCLE

By

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This work is dedicated to my dad, Norman Phillips, 1940-2001. Love always.

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EFFECTS OF AGE AND CALORIE RESTRICTION
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The stark reduction in muscle mass and function noted with age, termed sarcopenia, is a normal yet debilitating feature of aging. Stemming from the lack of clarity surrounding the role of TNF- α in age-associated muscle loss, we investigated the involvement of this cytokine in promoting reductions in muscle mass, and the cellular signaling pathways through which these effects were executed. We studied the inflammatory and apoptotic pathways emanating from TNF- α stimulation in the muscle cells, and also compared the responses to TNF- α in two different muscles, the soleus and the superficial vastus lateralis. These muscles were selected as they contained different types of muscle fibers known to exhibit the effects of aging (i.e., muscle loss) to differing degrees. We found that aging was paralleled with increased TNF- α , and that the inflammatory and apoptotic signaling capability of TNF- α was dependent on the muscle being examined. In the soleus with age, we report a greater capacity to cultivate inflammatory signaling through the transcription factor, NF- κ B, compared to that

detected in the superficial vastus lateralis. Alternatively, in the superficial vastus lateralis, TNF- α stimulated apoptotic signaling with age to a much higher extent than was observed in the soleus. Moreover, a reduction in muscle cell area in the superficial vastus lateralis coincided with this age-linked elevation in apoptosis.

Application of the life-extending intervention, calorie restriction, was also relied upon to provide further elucidation of the contribution of TNF- α to skeletal muscle loss with age. Calorie restriction is the only robust intervention shown to repeatedly evade the physiological declines associated with aging, and in agreement with this ability, TNF- α stimulation of both inflammatory and apoptotic pathways were abrogated when calorie restriction was applied. Our results suggest that specific fiber types may play a regulatory role in determining the nature of the TNF- α signal transmitted at the cellular level, with the decision of selecting life or death signals intimately tied to the extent of fiber loss experienced in the muscle; such a potential may constitute a major proponent in the pathogenesis of sarcopenia.

CHAPTER 1 INTRODUCTION

Significance

Aging, identified by a progressive and irreversible decline in cellular structure and function, is exhibited in most multicellular organisms, with the molecular basis for such depreciation yet undefined. In mammals, the only intervention repeatedly shown to retard the onset of age-associated cellular deficits is calorie restriction (CR) in the absence of malnourishment (1). Most CR studies have involved laboratory rodents which, when subjected to 25 to 50% reduction in caloric intake displayed a delay in the onset of age-associated pathological and physiological changes and an extension of median and maximum life span (2-5). Simply put, calorie restriction imparts aging resistant qualities to the organism (that serve to stall cellular defects attributable to age, lessen the severity and prevalence of age-related pathologies, and permit a lengthier median and maximum life span). Attempting to establish the mechanisms behind CR's anti-aging effects has and continues to be fervently, yet elusively, investigated. However, the effects of CR provide a base from which several theories have emerged to explain the aging process.

Of the multiple hypotheses proposed (6), the oxidative-stress hypothesis of aging (7) (which arguably provides the most comprehensive elucidation of the aging process and other age-related phenomena) points toward a reduction/oxidation (redox) imbalance as founding the characteristic changes witnessed with aging (7-9). This redox imbalance describes a disparity between oxidative stress and antioxidant buffering capabilities

culminating in damage to cellular structures (e.g., protein, lipid and DNA) (10-12). In terms of aging, the frequency and presence of cell structural damage is thought to accumulate. One of calorie restrictions' anti-aging mechanisms is believed to be targeted against the redox inequality, manifest as an improved redox-balancing capability exhibited by the organism, and a reduction in oxidative stress-induced damage (1).

An additional area closely implicated with redox flux explored in conjunction with aging is inflammation. Specifically, key mediators of inflammatory pathways, (i.e., tumor necrosis factor-alpha (TNF- α) and nuclear factor-kappa B (NF- κ B)) are under investigation with regard to a possible role in aging (13-15). Inflammatory reactions are known to be an extremely complex series of physiological reactionary events prompted to minimize cellular trauma and promote repair. Critically, TNF- α and NF- κ B, responsive to multiple stimuli including oxidative stress, have the ability to rapidly direct cellular remodeling. However, persistent, unwarranted immunological episodes implicating these and other pivotal inflammatory agents (e.g., inhibitor of κ B kinase (IKK) complex and inhibitor of κ B (I κ B) proteins) have been observed with increased frequency in aged tissues (13, 15-17). Given that many of the inflammatory cell types are sensitive to reactive oxygen species (ROS) and regulate their function based on ROS presence (18, 19), the possibility exists of a pro-inflammatory state becoming more chronic concurrent with advancing age. What's more, few studies have examined the anti-aging effects of CR from the perspective of the inflammatory response in post-mitotic tissue.

In the case of tissue-specific responses to aging processes, skeletal muscle, (which accounts for approximately 40% of the total body mass and 75% of the body's cell mass) (20), provides a substantial target for age-associated degeneration. Furthermore, the

different skeletal muscle fiber types have been reported to experience the effects of aging to varying degrees, with the fastest fibers (i.e., type II) argued to suffer the most deleterious effects (21-27). The stark reduction in muscle mass and function noted with age (sarcopenia) has been well characterized (24, 28). However, the impact of sarcopenia is not clearly established because of the lack of suitable approaches for estimating its prevalence and incidence in elderly populations (29). From a functional perspective, weakness of the lower extremities has been implicated with difficulty in balance problems and falls (30) and is thus recognized as holding great public health significance (31) both economically and socially. Furthermore, other age-related diseases with an inflammatory flavor (e.g., congestive heart failure) often impair skeletal muscle. The loss of muscle mass and function contribute substantially to morbidity and mortality in sufferers of such conditions (29, 32).

In summary, given the proposed involvement of TNF- α and NF- κ B in aging and in age-linked disorders, and given the paucity of studies examining this area, we intended to investigate the TNF- α / NF- κ B signaling pathway concurrently with aging in skeletal muscle. Additionally, in view of the ability of CR to retard the debilitating changes seen with aging, we proposed to apply this intervention to evaluate whether CR exerted its anti-aging effect by counteracting inflammatory mediators involved in TNF- α / NF- κ B signaling. Accordingly, the following questions were devised.

Questions and Hypotheses

Question 1. How do age and calorie restriction affect the inflammatory cytokine TNF- α and its receptor TNF-R1 in skeletal muscle?

Hypothesis 1. Skeletal muscle will display an increased presence of TNF- α and TNF-R1 with age, and calorie restriction will attenuate this elevation.

An increase in TNF- α , particularly in skeletal muscle, could play a role in the processes contributing to aging, and to many age-linked disorders with inflammatory and catabolic elements (e.g., sarcopenia, rheumatoid arthritis, and cachexia). Protein and mRNA of TNF- α have been detected in biopsy samples from human skeletal muscle (33) and adherence to an exercise regimen was found to reduce TNF- α in elderly humans (14). However, no studies have explored the effects of age and calorie restriction on TNF- α in skeletal muscle.

Question 2. What effects do age and calorie restriction have on the transcription factor NF- κ B in skeletal muscle?

Hypothesis 2. We anticipate an age-associated increase in NF- κ B and an attenuation of this elevation by calorie restriction.

Aging was found to induce an increase in NF- κ B nuclear binding activities in cardiac muscle and liver (16, 34). Others have reported a decline in NF- κ B activity when CR was used as an intervention in a variety of models (35-37). To our knowledge, no studies have assessed the combined effect of age and calorie restriction on NF- κ B in skeletal muscle.

Question 3. How are the IKK and I κ B regulatory proteins altered by age and calorie restriction in skeletal muscle?

Hypothesis 3. Age will result in an heightened stimulation of IKK and I κ B regulatory proteins, and calorie restriction will reduce this stimulus.

Several studies examining mitotic tissue (i.e., liver) (13, 15) reported an increase with age and a downregulation by calorie restriction of the NF- κ B regulatory proteins (inhibitor of κ B kinase (IKK) and inhibitor of κ B (I κ B) proteins). Whether this same phenomenon occurred in post-mitotic tissue (such as skeletal muscle) was addressed in these proposed experiments.

Question 4. Does oxidative stress act as an endogenous mediator of the TNF- α / NF- κ B signaling pathway in skeletal muscle, and what is the effect of age and calorie restriction?

Hypothesis 4. Oxidative stress will participate in mediating the TNF- α / NF- κ B signaling pathway in skeletal muscle; this involvement will be heightened with age and reduced by calorie restriction.

Reactive oxygen-mediated NF- κ B activation in response to TNF- α has been reported in skeletal-muscle myocytes (38). This association was confirmed by adding an antioxidant enzyme (catalase) that served to inhibit NF- κ B activation, and also adding a known oxidant (hydrogen peroxide) that increased activation.

We endeavored to investigate oxidative-stress involvement in TNF- α induced NF- κ B activation in aged skeletal muscle, and assessed the affects of age and CR on antioxidative factors.

Question 5. Will type I and type II skeletal muscle fibers display differences in the TNF- α / NF- κ B signaling mediators (TNF- α , TNF-R1, IKK, I κ B, and NF- κ B), and how will age and calorie restriction affect the fiber-type responses?

Hypothesis 5. Type II fibers will display a greater presence of TNF- α , TNF-R1, IKK, I κ B and NF- κ B compared to type I fibers, and this will be accentuated with age and alleviated by calorie restriction.

Type II (fast-glycolytic) fibers have been argued to be more susceptible than type I (slow-oxidative) fibers to age-related fiber atrophy and fiber loss (21-24, 26, 27, 39, 40). Several explanations (including a preferential loss of larger motoneurons, recruitment of motor units, and integrity of oxidative metabolic pathways) reflect but a few of the ideas put forth to explain this occurrence (5, 23-26). We set out to determine whether a difference in TNF- α / NF- κ B signaling was connected to this fiber type-specific aging response, and how it may have been affected by age and calorie restriction.

CHAPTER 2 REVIEW OF LITERATURE

Aging is characterized by a progressive decline in physiological function in the absence of disease and malnutrition. The underlying causes for the functional deterioration of aging have been extensively but inconclusively investigated. Accordingly, our focus was the role of inflammation in aging and age-associated conditions, and the means by which calorie restriction affords considerable resistance to the physiological declines of aging.

Aging and Inflammation: TNF- α / NF- κ B Signaling Pathway

The inflammatory response performs an essential role in detecting and preventing prolonged cellular insult caused by different stressors including physical agents (UV and gamma radiation); chemical agents (components of the body and products of metabolism such as free radicals); and biological agents (viruses, bacteria). A complex circuitry of inflammatory mediators works in concert to sense, apprehend, and rectify the ensuing cellular damage. Such capabilities are critical for survival. Yet with age, the very responses needed to ward off stressors are thought to become the players contributing, in part, to the functional declines noted with aging (41-44). Among the candidates argued to instigate an inflammatory aspect of aging are tumor necrosis factor- α (TNF- α) and nuclear factor κ B (NF- κ B). Thus, the TNF- α / NF- κ B signaling pathway in association with aging and CR is considered in the following paragraphs.

Overview of Tumor Necrosis Factor-Alpha (TNF- α)

The cytokine, tumor necrosis factor-alpha (TNF- α), originally named cachectin due to its catabolic action (45), is a homotrimer of 157 amino-acid subunits that promotes antitumor and immune responses (46). The catabolic activity of TNF- α has afforded the cytokine an association with muscle pathology (47), but has also been implicated in a wide variety of functions in many other cell types (48). Thought to contribute to age-related skeletal-muscle degeneration (13), TNF- α is primarily produced by activated macrophages and has been evidenced in patients with heart failure (49), sepsis (50), and other inflammatory diseases that result in secondary muscle weakness (51-53). Further, elevated TNF- α levels have been reported with age in heart, liver, kidney and brain tissues (16, 17).

TNF- α signaling pathways. Cellular responses to TNF- α are varied and dependent on cell type. A post-receptor, intricate cascade of signaling events holds the key to a host of responses attributed to this pleiotropic cytokine (46). Three major pathways that transduce the TNF- α signal have been identified (54, 55). These include a proapoptotic pathway regulated by interaction of the TNF- α -receptor complex with the Fas-associated death domain. A second pathway activates the transcription factor activator protein-1 via Jun-NH2-terminal kinases. The third means of signaling activates NF- κ B. This last pathway, known to occur in skeletal muscle (19, 38, 47), represents a major mechanism of transcriptional control by TNF- α particularly in terms of promoting the inflammatory response.

Signaling through TNF-R1. The biological and signaling activity of TNF- α is exerted through binding to two types of receptors: TNF-R1 and TNF-R2. These

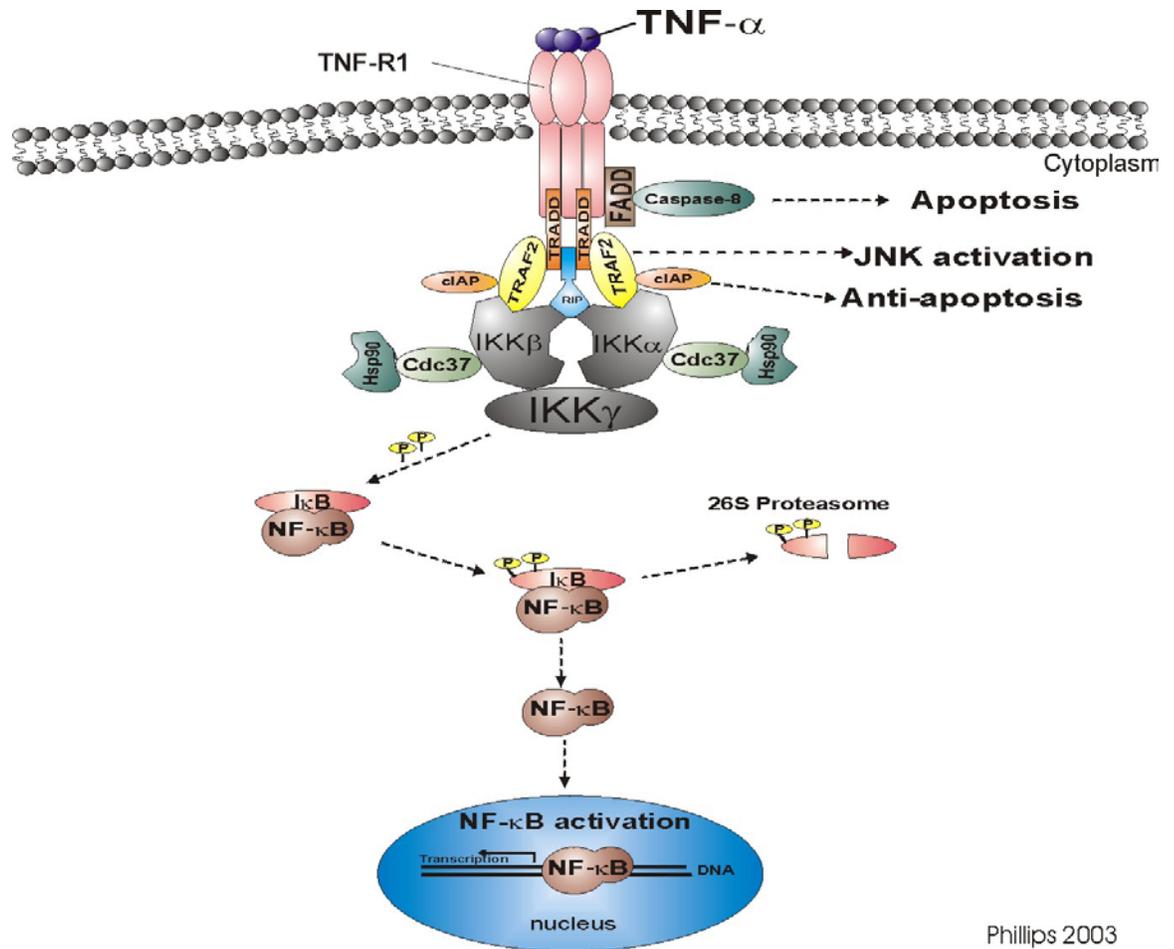


Figure 1. The TNF- α signal transduction pathway.

receptors belong to a family of type I transmembrane receptors, with one to five cysteine-rich repeats in their extracellular domain, and a common death domain in their cytoplasmic tail (56). TNF-R1 contains the death domain, whereas TNF-R2 does not. Consequently, TNF-R1 has the ability to direct both cell-survival and cell-death signals, whereas TNF-R2 primarily conveys cell-survival signals (56). Despite the presence of two receptors, multiple studies show that TNF-R1 initiates most of TNF- α 's biological activities (57) (Fig 1). In an analysis of lymphocyte responses to aging, concurrent with elevated serum TNF- α levels, increased expression of TNF-R1 was implicated with T-cell dysfunction and lymphopenia in aging populations (58).

TNF-R1 adaptor proteins. The absence of intrinsic enzymatic activity in the receptor death domains requires multiple adaptor proteins to be recruited for signal conductance to occur. A highly coordinated amalgamation of signaling intermediaries then follows. The extracellular binding of the TNF- α trimer to TNF-R1 releases the inhibitory protein, silencer of death domains (SODD), from TNF-R1's intracellular domain. When exposed, the TNF-R1 intracellular domain is identified by the adaptor protein TNF receptor-associated death domain (TRADD), which then recruits additional adaptor proteins: receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2), and Fas-associated death domain (FADD) (57). These latter adaptor proteins recruit key enzymes to TNF-R1 that, are responsible for initiating the various signaling pathways.

TNF- α signaling to NF- κ B. Knowledge of the signaling intermediaries that facilitate TNF- α induced activation of NF- κ B has progressed substantially in recent times. The crux of this activation relies on phosphorylation-dependent ubiquitination and degradation of the inhibitor of κ B (I κ B) proteins, which usually retain NF- κ B within the cytoplasm of unstimulated cells (59). The phosphorylating agent that assists in the release of this inhibition arises from a multiprotein I κ B kinase (IKK) complex (60). Two catalytic subunits, IKK α and IKK β , and a regulatory subunit, NF- κ B essential modulator (NEMO or IKK γ) comprise the core of the IKK complex. Further, the IKK complex contains a specific chaperone consisting of Cdc37 and Hsp90 that serves to shuttle the complex from cytoplasm to membrane.

Upon TNF- α binding to TNF-R1, the IKK complex is recruited in sync with the adaptor proteins. Binding is dependent on RIP, and once IKK interacts with RIP or an

RIP-dependent kinase, the complex becomes activated. Investigators have shown that phosphorylation of two sites at the activation loop of IKK β , but not IKK α , are essential for the activation of IKK by TNF- α and other proinflammatory cytokines (61). Also, the existence of an autophosphorylation capability of IKK β is postulated to endow a negative regulatory constraint on IKK activation (which contributes to the transient nature of its activation). This holds importance as prolonged activation of the NF- κ B signaling pathway carries with it the potential for toxicity and pathophysiology (62); thus a rapid termination to IKK activation is critical for attainment of an appropriately weighed NF- κ B response.

The noncatalytic component of the IKK complex (IKK γ) has also been keenly investigated with efforts geared toward delineating its role. Early studies noted the essential requisite of the IKK γ subunit in order for NF- κ B activity to be achieved by a variety of stimuli (63, 64). More recent work substantiated these findings and further presented the notion that the oligomeric organization of IKK γ enforces a spatial positioning of the two kinases, IKK α and IKK β , in the IKK complex serving to facilitate transautophosphorylation, activation, and subsequent NF- κ B activation (65).

The specific role of IKK α remains a subject of debate; experiments using IKK α -knockout mice point toward an involvement of IKK α in keratinocyte differentiation in the epidermis independent of its kinase activity and independent of NF- κ B (66). Studies using gene ablation have also ascertained that IKK β and IKK γ are required for NF- κ B activation by proinflammatory stimuli, while IKK α is essential for morphogenic signals (67-69). Once activated, IKK proceeds to phosphorylate the I κ Bs

(which in the case of I κ B α occurs at two conserved serines, S32 and S36) in the N-terminal regulatory region (70), targeting them for ubiquitination and proteasomal degradation; and liberating NF- κ B for nuclear translocation (65).

Although most of the involved parties in this cascade have been validated by both biochemical and genetic means (57), several unresolved interactions and functions linger: for example, the possibility of there being an intermediate factor or kinase operating between RIP and IKK. Such unknowns should not be overlooked when considering the signaling pathway.

Nuclear Factor- κ B (NF- κ B): An Overview

The transcription factor NF- κ B is a central regulator of the immune response whose actions are rapidly induced by proinflammatory stimuli such as TNF- α (Pahl (71) lists known inducers). Further, NF- κ B acts to promote cellular growth, staving off programmed cell death by pro-apoptotic stimuli such as TNF- α (54, 72). The NF- κ B exists as either heterodimers or homodimers of the Rel family of proteins. The predominant form of NF- κ B is a heterodimer consisting of p50/p105 and p65 (RelA). Other forms contain RelB, c-Rel and p52/p100 subunits (73). These dimers bind to a set of ten base pair DNA sites (recognized as the κ B sites) to regulate gene expression. In latent cells, the NF- κ B dimers are retained in the cytoplasm bound to the I κ B proteins. When stimulated, however, the I κ B proteins undergo proteasomal degradation, which allows liberated NF- κ B to translocate to the nuclear binding site and facilitate gene expression (71).

NF- κ B regulated gene expression. The active NF- κ B transcription factor promotes the expression of over 150 target genes (71). Among these are members of the

Rel / NF- κ B / I κ B family. As a result, NF- κ B limits its own activation by prompting the new synthesis of I κ B. Newly-synthesized I κ B can enter the nucleus and dislodge active NF- κ B from its DNA binding site; thus, in most cell types, NF- κ B activation is fleeting (74, 75). In addition, a substantial number of the proteins encoded by the NF- κ B target genes participate in host immune response, giving rise to the designation of NF- κ B as a central mediator of immune response. However, NF- κ B is also involved in transcriptional control of many genes whose functions reach beyond an immediate immune response and are more associated with stress responses. Accordingly, rather than being labeled a central mediator of the immune response, it has been proposed that NF- κ B is more global, and represents a regulator of stress responses (71).

Using knockout mice models, the importance of NF- κ B has been shown for normal physiological function. Knockout mice created for p65/RelA, I κ B α , p50, c-Rel, and RelB have been examined (76-78). Deficiencies of p50, c-Rel, or RelB were reported to result in developmentally normal mice; and a RelA deficiency resulted in embryonic lethality from liver apoptosis (77). Furthermore, I κ B α -deficient mice appeared normal at birth; but in the postnatal period, their growth ceased and death occurred within 7 to 10 days of age (77, 78).

Summary

The inflammatory response is governed and executed by a multifaceted circuitry of inflammatory cell types. While essential for survival against cellular insult, inflammation paradoxically may be implicated in aging and age-related functional declines (47, 79). The TNF- α and NF- κ B influential inflammatory mediators, have been considered in terms of contributing to aging processes.

TNF- α has been found to signal through three major pathways (including the NF- κ B pathway responsible for transcriptional activation of many genes linked with the inflammatory response). Despite the prevalence of two TNF- α receptors, most of TNF- α 's activities are initiated through TNF-R1 (57). The TNF- α / NF- κ B signaling pathway comprises a finely integrated cascade of events (encompassing initial ligand-receptor interaction, adaptor protein recruitment, and inhibitory protein ubiquitination-proteasomal degradation), permitting translocation of NF- κ B from cytoplasm to nucleus. Although a complete understanding of all the involved proteins and the extent of their contributions to signal transduction remains equivocal, key intermediaries have been identified. With respect to proinflammatory (e.g., TNF- α) induced stimulation of NF- κ B, IKK β and IKK γ (kinase and regulatory components) in the IKK complex, respectively, have been shown to be essential for NF- κ B activation (61, 63-65).

Once liberated, NF- κ B has the ability to translocate to the nucleus where it can promote the expression of over 150 target genes (71). The breadth and diversity of proteins whose genes contain NF- κ B binding sites provides some illustration of the global responses (e.g., immune and stress-related) that are enacted through NF- κ B transcriptional control, and exemplifies the wide-reaching impact of this transcription factor.

Aging, Calorie Restriction and TNF- α / NF- κ B Signaling

Immunosenescence, has been used to describe the proposition that a global reduction in the capacity to cope with various stressors and a concurrent progressive increase in proinflammatory status are major characteristics of the aging process (41). What determines the propensity of an individual to be more susceptible to inflammatory

dysregulation is argued to stem from environmental and genetic factors. The genetic component of this postulate identifies the absence of robust gene variants or the presence of frail gene variants (or some combination thereof) as fuelling the likelihood of age-related diseases occurring that contain an inflammatory element. These include Alzheimer's dementia, atherosclerosis, and maturity-onset diabetes mellitus (80, 81).

The environmental contribution to this status describes the lifelong series of inflammatory stimuli that persist and inflammatory reactions that accumulate over time, forming the inflammatory background. Susceptibility to disease occurrence is deemed to be dependent on the nature of the inflammatory background, which is fashioned by individual responses to stressors at the cellular level. To offer further delineation, the responses of influential inflammatory pathways (e.g., TNF- α / NF- κ B signaling) to oxidative stress have been examined because of the intimate link thought to exist between oxidant production and aging processes (7, 8).

Oxidative stress and the molecular inflammatory theory of aging. Free radicals; molecules (typically oxygen or nitrogen derivatives) that contain one or more unpaired electrons in their outer orbit (9), and also nonradical oxidants (e.g., hydrogen peroxide and peroxynitrite) have the propensity to act as signaling agents and modulators of cellular function that can equate to cellular damage. Buffering systems persist that act to protect cellular structures from excessive radical assault and maintain a redox equilibrium. If a disparity exists between the amount of oxidative stress prevalent and the antioxidative force buffering capability, then the net result equates to oxidant stress. Further, studies have reported the sensitivity of NF- κ B to oxidative stress and the presence of NF- κ B binding sites in the promoter regions of antioxidant genes (40, 82-84).

On a related track, a molecular inflammatory theory of aging has been proposed implicating reactive oxygen and nitrogen species and proinflammatory molecules as key players in the aging process (13). In an examination of rat kidney from young and old animals, signaling molecules of the NF- κ B family were shown to be redox sensitive, a condition that was amplified with age (15). Specifically, an increase in the nuclear binding activity of NF- κ B was found to occur with advancing age. This was accompanied by a decrease of I κ B α and I κ B β , the regulatory proteins that prevent nuclear translocation of NF- κ B and subsequent transcription-factor activities. Further, the IKK complex responded in agreement with the increased NF- κ B activity indicated by elevated phosphorylation of this complex with age.

Calorie restriction and inflammation. In the aforementioned study, conclusions drawn from the age-associated elevation of inflammatory mediators were made even more robust by including a calorie restriction intervention (15). The CR animals showed reduced IKK activation, downregulating the NF- κ B activation reportedly through increased bound I κ B α and I κ B β proteins in the cytoplasm (15). These reductions with CR fell concomitantly with lowered reactive oxygen species levels compared to *ad libitum* fed groups at all ages studied, supporting the notion of oxidant stress having some involvement in promoting the inflammatory state found with age. The responsiveness of NF- κ B to oxidative stress has been well documented (18, 85); however, the idea of aging being molded by an interplay between oxidative stress and inflammatory mediators has received much less attention.

Aging and Inflammation: Significance in Skeletal Muscle

Age-associated reductions in skeletal muscle size, mass, and function have been found to persist in various mammalian species (86). Further, the observation of specific skeletal muscles in humans undergoing ~40% decline in muscle mass between the ages of 20 and 80 years (25) imparts significant concern for public health and also impinges on the issue of independence in the elderly (87).

The atrophy and dysfunction of skeletal muscle associated with aging has been investigated from several avenues to determine the underlying cause(s) (29). Use of calorie restriction to further unveil contributing mechanisms has unearthed a preventive intervention against age-related muscle degeneration (88, 89). Work that analyzed the involvement of mitochondrial DNA deletions in muscle fiber loss reported a significantly blunted progression of age-dependent fiber and mitochondrial abnormalities present in CR compared to *ad libitum* fed animals (5). Aspnes et al., hypothesized that CR's ability to attenuate accumulation of oxidative damage in skeletal muscle was responsible for this outcome. Whether a similar effect of CR occurs on age-dependent elevated inflammatory pathways (TNF- α / NF- κ B signaling) while, in turn, maintaining muscle integrity remains to be determined, but this possibility has been reported in other tissues (15).

Alternatively, the influence of inflammatory cytokines such as TNF- α on muscle biology remains interesting given the broad range of biological events this pleiotropic factor can direct, many of which remain to be fully understood. Accordingly, elucidating skeletal muscle responses to TNF- α in the backdrop of normal, progressive aging may help to shed light on age-dependent alterations in signaling patterns that compromise

muscle homeostasis, and ultimately hinder function. In turn, signaling patterns gone awry may hold important clues to the more severe destructive effects of this cytokine on muscle biology reported to accompany inflammatory disorders including cancer (90, 91), chronic obstructive pulmonary disease (92), congestive heart failure (32), and sarcopenia (29).

Summary

Inflammatory response as a potential contributor to aging processes in skeletal muscle may help explain the dramatic degeneration observed in this tissue with age. The inflammatory cytokine, TNF- α , attracts particular interest as it has the ability to affect a multitude of cellular responses and drive gene expression through activation of the transcription factor NF- κ B. Further, TNF- α has been linked to many pathologies (some of which are age-related) where muscle degeneration is apparent (29, 32, 91-93), and has also been shown to be elevated with age (16, 17). Moreover, applying the intervention of calorie restriction may help to curb age-dependent deterioration of skeletal muscle induced by inflammation, as proposed by others regarding nonmuscle tissue (13), and supply additional information regarding the mechanisms at play.

Undoubtedly, many other factors must come under scrutiny before we can substantiate the involvement of inflammation in aging. However, the TNF- α / NF- κ B signaling pathway provides an attractive avenue for exploring this potential, and ultimately may assist in further unraveling the mechanisms behind aging and age-related pathologies.

CHAPTER 3 METHODS

Animals

Eight 6-month-old *ad libitum* fed (6AL), eight 26-month-old *ad libitum* fed (26AL) and eight 26-month-old calorie restricted (26CR) male Fischer 344 rats (National Institutes of Aging Colony, Harlan Sprague Dawley, Indianapolis, IN) were used. The 26CR animals had been subjected to calorie restriction starting at 3.5 months of age (10% restriction), increased to 25% restriction at 3.75 months, and maintained at 40% restriction from 4 months throughout the animal's life (until 26-months of age). Fischer 344 rats were used as there was extensive background data available on these animals and they were the species we have used in previous projects. Males were used to avoid possible skeletal muscle protective effects of estrogen which may have functioned as an antioxidant (94). The rats were individually housed in a temperature (18-22°C) and light-controlled environment with a 12-hour light/dark cycle. After one week of acclimation the animals were randomly sacrificed (5 per day) on consecutive days. All treatment of animals throughout this study conformed fully with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society (95), and, received University of Florida institutional animal care and use committee approval.

Tissue Harvesting

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, Chicago, IL) (5 mg/100 g). The superficial vastus lateralis (SVL) and all hind limb muscles from both legs were removed. Soleus (type I)

and SVL (type IIb) (40) from the left leg were weighed, frozen in liquid nitrogen-cooled isopentane and stored at -80°C for use in histochemical and immunohistochemical analyses. The right leg soleus and SVL were used for isolation of the nuclear and cytosolic fractions. Lastly, the chest was opened and blood removed by cardiac puncture drawn directly into Vacutainer® tubes containing ethylenediaminetetraacetic acid (K3EDTA; 8.4mg/ Vacutainer®) for plasma acquisition. The blood aliquots were centrifuged at 4°C at $1500 \times g$ for ten minutes and plasma stored at -80°C until use.

Biochemical Assays

Cellular fractionation

Isolation of cytosolic fraction. Soleus and SVL muscles were homogenized in isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.2 % BSA, 1mM EDTA, pH 7.4) with a dilution of 1:10 using a Potter-Elvehjem glass homogenizer. Homogenate was centrifuged at $1,000 \times g$ for 10 minutes. The supernatant was centrifuged at $14,000 \times g$ for 10 minutes and subsequently used for Western blotting and antioxidant enzyme analyses in the cytosolic fraction.

Isolation of nuclear extracts. Nuclear extracts were isolated from the soleus and SVL muscle using the protocol described by Blough et al (96). Briefly, 50 mg of tissue was homogenized in 35 ml of Buffer 1 (10 mM HEPES, pH 7.5, 10mM MgCl_2 , 5mM KCL, 0.1 mM EDTA, pH 8.0, 0.1% Triton X-100, 1 mM dithiothreitol, 0.1 MM phenylmethyl sulfonyl fluoride, 2 ug/ml aprotinin, and 2 ug/ml leupeptin) and centrifuged for 5 minutes at $3000 \times g$ at 4°C . The pellet was then resuspended in 500 mL of Buffer 2 (20 mM HEPES, pH 7.9, 25% glycerol, 500 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, pH 8.0, 0.5 mM dithiothreitol, 0.2 mM phenylmethyl sulfonyl fluoride, 2 mg/mL aprotinin, and 2 mg/mL leupeptin). Next, the sample was centrifuged for 5 minutes at

3000 x g at 4°C. The supernatant was transferred to a 5000 nominal molecular weight limit (NMWL), 4-ml Ultrafree Filter Unit (Millipore, Bedford, Ma) and centrifuged for 30 minutes at 4500 x g at 4°C. This last step concentrated the sample, which was then used for the measurement of NF-κB activity.

Western blotting

Proteins were separated using 4-20% precast SDS-polyacrylamide gels (BMA, Rockland) under denaturing conditions and electrotransferred onto nitrocellulose for 1 hr at 0.28 amps. For TNF-R1, IKKβ, IKKγ, IκBα, p65, FADD and caspase-8, 30μg of protein was added to each lane. After transfer, membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween 20 (PBS-T/5% milk) overnight at 4°C. Membranes were then incubated with the following primary antibodies and concentrations in PBS-T/5% milk for 1.5 hrs at room temperature: TNF-R1, monoclonal antibody, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); IKKβ, monoclonal antibody, 1:100 (US Biological, Swampscott, MA); IKKγ, monoclonal antibody, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); IκBα, monoclonal antibody, 1:100 (Rockland, Gilbertsville, PA); p65, monoclonal antibody, 1:100 (Rockland, Gilbertsville, PA); FADD, polyclonal antibody, 1:1000 (Stressgen, British Columbia, Canada), and caspase-8, polyclonal antibody, 1:100 (Stressgen, British Columbia, Canada). After washing (2 x 20 mins with PBS), membranes were incubated for 1.5 hrs at room temperature with secondary antibody (anti-rabbit or anti-mouse IgG, horseradish linked whole antibody; Amersham Life Science, United Kingdom) with an appropriate dilution in PBS-T/5% milk. Blots were developed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, United Kingdom) and protein bands analyzed using

Kodak 1D Image analysis software (Eastman Kodak Company, Rochester, NY).

Arbitrary OD units were calculated by multiplying the area of each band by its optical density. Groups were equally represented on each gel, and loading of equal amounts of protein was controlled for by using Ponceau staining (Pierce Biochemicals, Rockford, IL) of the nitrocellulose membrane.

Histochemistries

Hematoxylin & Eosin stain. In an effort to characterize aging effects on the skeletal muscles, muscle mass and representative muscle cross-sectional areas were determined. Whole muscle samples, previously frozen in isopentane-cooled liquid nitrogen and stored at -80°C, were brought to the cryostat temperature (-20°C) before sectioning and placed in OCT mounting media (Miles, Elkhart, IN). Serial cross sections (10 µm thick) were cut from the mid-section of each muscle using a cryostat microtome (Reichert-Jung) maintained at -20°C and mounted on a microscope slide. Sections were placed in 100% then 70% alcohol for 1 minute, respectively; hydrated in dH₂O for 2 minutes then transferred to hematoxylin solution for 2 minutes. After washing in dH₂O, sections were placed in Scott's solution for 15 seconds, rinsed in dH₂O and transferred to 70% alcohol. The sections were then stained in eosin solution for 2 minutes, dehydrated and mounted. Sections were visualized using an Axiovert 200 light microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) and computerized imaging processing software (Scanalytics, Inc., Fairfax, VA). Two representative, random areas of interest were chosen to determine muscle fiber cross-sectional area. Briefly, muscle fiber membranes were traced and all areas within the membranes added to determine the cross-

section. Values were averaged for each sample and the results reported as cellular area per mm².

Immunohistochemistry of skeletal muscle TNF- α . To expose TNF- α production stemming from myocytes, consecutive sections obtained in the same manner (see previous) were subjected to immunohistochemical analysis. In brief, sections were incubated for 2hrs at 37°C with the primary antibody for TNF- α (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The sections were then washed in PBS (1.6 mM NaH₂PO₄; 8.4 mM Na₂HPO₄; and 8.75g NaCl in 1L of dH₂O, pH 7.4) and incubated with biotinylated anti-goat IgG (1:200, Vector Laboratories, Burlington, CA) for 30 minutes at room temperature. After incubation with the secondary antibody, sections were washed with PBS and incubated with ABC peroxidase reagent (Vector Elite ABC Kit, Vector Laboratories, Burlington, CA) for 1 hr at room temperature. Following incubation, the sections were washed 3 times in PBS and the enzyme substrate added (Vector NovaRed substrate). Once the reaction proceeded for approximately 2-10 minutes, the section was washed 3 times in dH₂O.

Sections were viewed using an Axiovert 200 light microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) and computerized imaging processing software (Scanalytics, Inc., Fairfax, VA). For each sample, the number of TNF- α positive cells in two representative regions of interest containing ~100-200 fibers were counted manually, and the mean used for statistical analysis. TNF- α labeled cells was reported as TNF- α expression per number of fibers per mm².

Determination of TNF- α by ELISA. TNF- α was measured in the plasma using a commercially available ELISA kit (R & D Systems, Minneapolis, MN) that employs the

quantitative sandwich enzyme immunoassay technique. Sample absorbances were read at 450 nm off the standard curve and expressed in pg/mL.

NF- κ B Activation. Skeletal muscle NF- κ B activation was measured using an ELISA kit (Active Motif, Carlsbad, CA). Briefly, sample nuclear extracts (96) were treated according to manufacturers instructions, and loaded onto a microplate coated with oligonucleotide containing the NF- κ B consensus site. The primary antibodies used to detect NF- κ B recognized an epitope on p65 that is accessible only when NF- κ B is activated and bound to its target DNA. Following an incubation period, an HRP-conjugated secondary antibody was added to provide a sensitive, colorimetric signal from which absorbance at 450 nm was detected through use of a spectrophotometer. Results were expressed as NF- κ B activation optical density values per mg of protein at 450 nm.

DNA fragmentation

DNA ladder assay. To enable detection of nucleosomal ladders in apoptotic cells the DNA ladder assay was performed. Soleus and superficial vastus lateralis muscle tissues (40-50 mg) were homogenized using a Teflon homogenizer in 1 mL DNAzol (Molecular Research Center Inc., Cincinnati, OH). Proteinase K (Qiagen, Valencia, CA) was added to the homogenates which, after a 3 hour incubation period, were centrifuged (10 000 x g for 10 minutes at 4°C) and the supernatants precipitated and washed with 100% and 75% ethanol, respectively. The isolated DNA was used in the PCR Kit for DNA Ladder Assay (Maxim Biotech, Inc., San Francisco, CA) performed as directed by the manufacturer. Lastly, samples were electrophoresed through 1.8% agarose gels

containing 0.5 µg/mL ethidium bromide at 100 mA for 1 hour, and examined under UV light for the presence of DNA ladders.

Antioxidant enzyme activity

Superoxide dismutase. Superoxide dismutase (SOD) activity was assayed according to Oyanagui (1984) with slight modification (97) in muscle cytosolic fraction. One unit (U) of SOD activity was defined as the concentration of enzyme that inhibited nitrite formation from hydroxylamine in the presence of xanthine oxidase by 50%.

Protein concentration

Cytosolic and nuclear protein concentrations were determined using the method developed by Bradford (1976) (98). Samples and standard were pipetted in triplicate onto a microplate and 200 µl of Coomassie Plus Protein Assay Reagent (Pierce) was added. Optical density was determined using a microplate reader set at 595 nm.

Statistical analysis

A two-way analysis of variance was performed with age and fiber type, and calorie restriction and fiber type as the independent variables using a statistical package from Minitab Inc. (State College, PA). When appropriate, Tukey's post hoc analysis was performed. A P-value of < 0.05 was considered significant.

CHAPTER 4 RESULTS

Morphological Measurements

Animal Body Weights

Compared to the 6-month-old *ad libitum* (6AL) rats (363.4 ± 4.0 g; mean \pm SEM), the 26-month-old *ad libitum* (26AL) animals' body weights (413.6 ± 5.4 g; mean \pm SEM; $P < 0.001$) increased $\sim 13\%$. Further, the 26AL animals' body weights were $\sim 35\%$ greater than those of the 26-month-old calorie restricted (26CR) group (307.1 ± 2.3 g; mean \pm SEM; $P < 0.001$) (Table 1).

Muscle Mass

Soleus muscle mass. The soleus muscle mass showed no difference in wet weight between the 6AL (0.147 ± 0.004 g; mean \pm SEM) and 26AL (0.146 ± 0.005 g; mean \pm SEM) groups with age (Table 1). Expression of soleus wet weight in conjunction with body weight, however, indicated that the 6-month-old *ad libitum* animals had a significantly greater soleus wet weight compared to the 26-month-old *ad libitum* group (0.399 ± 0.006 vs. 0.345 ± 0.011 g; mean \pm SEM; $P = 0.02$) (Fig 2). Alternatively, the 26CR animals displayed an $\sim 33\%$ reduction (0.109 ± 0.003 g; mean \pm SEM; $P < 0.001$) in soleus wet weight versus their *ad libitum* fed counterparts (Table 1); however, no statistical differences were noted when body weight was brought into the equation (0.355 ± 0.011 vs 0.345 ± 0.011 g; mean \pm SEM) (Fig 2).

Table 1. Body mass and muscle mass of 6-month- and 26-month-old *ad libitum* and 26-month-old calorie restricted male Fischer 344 rats.

	6AL (n = 8)	26AL (n = 8)	% change 26AL vs. 6AL	26CR (n = 8)	% change 26CR vs. 26AL
Body mass (g)	363.4 ± 4.0	413.6 ± 5.4*	↑13%	307.1 ± 2.3	↓35%
Soleus muscle mass (g)	0.147 ± 0.004	0.146 ± 0.005**	no change	0.109 ± 0.003	↓33%
Superficial vastus lateralis muscle mass (g)	0.419 ± 0.003	0.364 ± 0.005*	↓15%	0.332 ± 0.006	↓9%

Body mass and muscle mass of 6AL (6-month-old *ad libitum*), 26AL (26-month-old *ad libitum*) and 26CR (26-month-old calorie restricted) male F344 rats. Data were expressed as mean ± SEM. *P < 0.001 26AL vs 6AL and 26CR; **P < 0.001, 26AL vs. 26CR.

Superficial vastus lateralis muscle mass. The predominantly type IIa and IIb superficial vastus lateralis (SVL) (24, 25) demonstrated a significant reduction in wet weight in the 26AL (0.364 ± 0.005 g; mean ± SEM) compared to the younger animals (0.419 ± 0.003 g; mean ± SEM; P < 0.0001). In addition, calorie restriction also imparted a reduced muscle wet weight (0.332 ± 0.006 g; mean ± SEM; P = 0.0007) versus the 26AL group (Table 1). However, when expressed as a percentage of body weight, SVL wet weight increased by 28% in the 26-month-old calorie restricted animals compared with the 26-month-old *ad libitum* animals (1.089 ± 0.022 g vs. 0.850 ± 0.013 g; mean ± SEM; P < 0.0001). Further, when age was considered in the expression of SVL as a percentage of body weight the 26AL group again exhibited a reduced wet weight

compared to the 6AL animals (0.850 ± 0.013 vs. 1.126 ± 0.021 g; mean \pm SEM; $P < 0.0001$) (Fig 2).

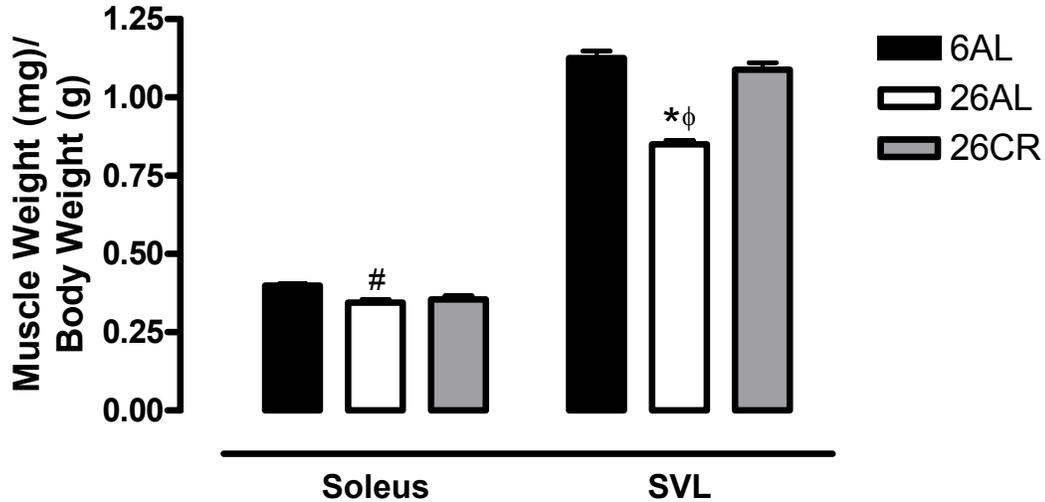


Figure 2. Soleus and SVL (superficial vastus lateralis) muscle wet weights (mg) of 6AL (6-month-old *ad libitum*), 26AL (26-month-old *ad libitum*), and 26CR (26-month-old calorie restricted) F344 rats expressed per gram of body weight. #Different from 6AL, $P = 0.0205$; *Different from 6AL, $P < 0.0001$; ϕ Different from 26CR, $P < 0.0001$.

Muscle Cross Sectional Area

Histochemistry. Hematoxylin and eosin staining was conducted in an effort to characterize aging on the soleus and superficial vastus lateralis muscle cross-sectional areas. From each sample, two random areas of interest were chosen to determine muscle fiber cellular area per mm^2 as a representative measure of muscle cross sectional area (Table 2). In soleus, the 6AL animals demonstrated a greater percentage of cellular area per mm^2 compared to the 26AL group (77.5 ± 3.7 vs. 54.5 ± 1.5 %; mean \pm SEM; $P = 0.002$) (Fig 3A, B). We did not detect a statistical difference in percentage of cellular area per mm^2 between the 26AL and 26CR animals (54.5 ± 1.5 vs. 61.9 ± 6.0 %; mean \pm SEM) (Fig 3B,C).

Table 2. Soleus and Superficial vastus lateralis cross sectional areas of 6-month- and 26-month-old *ad libitum* and 26-month-old calorie restricted male F344 rats.

	6AL	26AL	% difference 26AL vs. 6AL	26CR	% difference 26CR vs. 26AL
Soleus (% Cell area / mm ²)	77.5 ± 3.7	54.5 ± 1.5*	↓42%	61.9 ± 6.0	↑13%
Superficial vastus lateralis (% Cell area / mm ²)	77.2 ± 7.1	68.1 ± 3.9**	↓13%	78.0 ± 2.6	↑15%

Representative cross sectional areas (expressed as % cell area / mm²) of 6AL (6-month-old *ad libitum*), 26AL (26-month-old *ad libitum*) and 26CR (26-month-old calorie restricted) male F344 rats obtained via H&E staining. Data were expressed as mean ± SEM. *P < 0.01, 26AL vs. 6AL; **P < 0.05, 26AL vs. 6AL and 26CR.

In contrast, both age and diet effects were evident in the superficial vastus lateralis (Fig 3D, E, F). Compared to the 6AL rats, the 26AL animals had ~13% lower cell area per mm² (77.2 ± 7.1 vs. 68.1 ± 3.9 %; mean ± SEM; P = 0.0364) (Fig 3D, E). Also, when compared to the 26CR animals, a similar scenario was found with the 26AL animals demonstrating ~15% reduction in cell area per mm² (78.0 ± 2.6 vs 68.1 ± 3.9 %; mean ± SEM; P = 0.0406) (Fig 3E, F).

In agreement with previous findings (99) this data , offered support to the idea that a decline in muscle fiber size occurred in parallel with age. What's more, there appeared to be a fiber type specific response to aging-associated muscle loss, highlighted by the ability of the dietary intervention to impart a greater resistance in the SVL against muscle fiber loss and cell area reduction compared to that which was displayed in the soleus.

Immunohistochemistry. Immunohistochemical analysis to expose TNF-α expression stemming from myocytes was performed in an attempt to discern whether

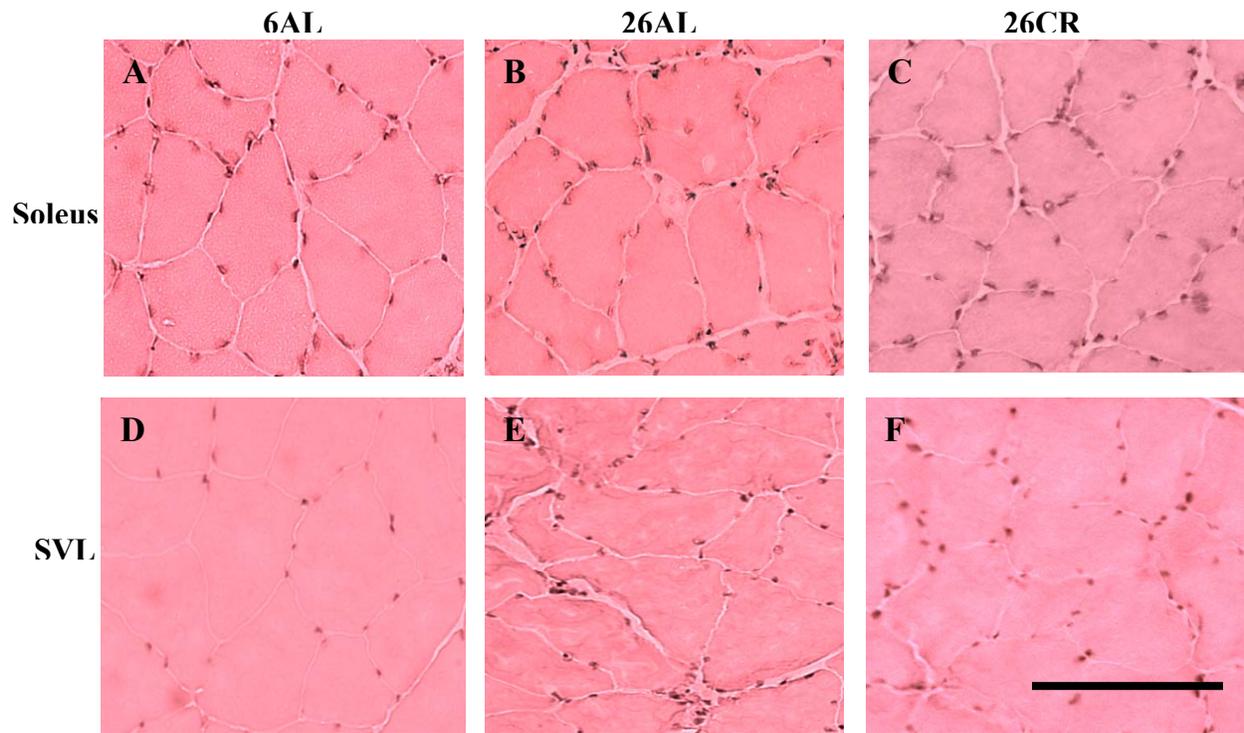


Figure 3. H&E staining in soleus (A-C) and SVL (superficial vastus lateralis) (D-F) sections from 6AL (A,D), 26AL (B,E) and 26CR (C,F) Fischer-344 rats. Bar 100 μ m.

muscle fiber type exerted an influence on the cytokines' expression. For each sample, the number of TNF- α positive cells in two representative regions of interest containing ~100-200 fibers were counted manually, and the mean used for statistical analysis. In soleus, we found no detectable differences between the 6AL and 26AL animals (0.03 ± 0.005 vs. 0.043 ± 0.003 TNF- α expression / fiber number / mm^2 ; mean \pm SEM) nor did we reveal differences when comparing 26AL with 26CR (0.043 ± 0.003 vs. 0.03 ± 0.01 TNF- α expression / fiber number / mm^2 ; mean \pm SEM) (Fig 4). There were, however, far different results found in the superficial vastus lateralis muscle. The 26-month-old *ad libitum* animals exhibited significantly greater TNF- α expression per fiber number per mm^2 compared to the 6-month-old *ad libitum* animals (0.069 ± 0.02 vs. 0.039 ± 0.003

TNF- α expression / fiber number / mm²; mean \pm SEM; $P < 0.05$) and also compared to the 26-month-old calorie restricted animals (0.069 ± 0.02 vs. 0.033 ± 0.008 TNF- α expression / fiber number / mm²; mean \pm SEM; $P < 0.05$) (Figs 4 and 5).

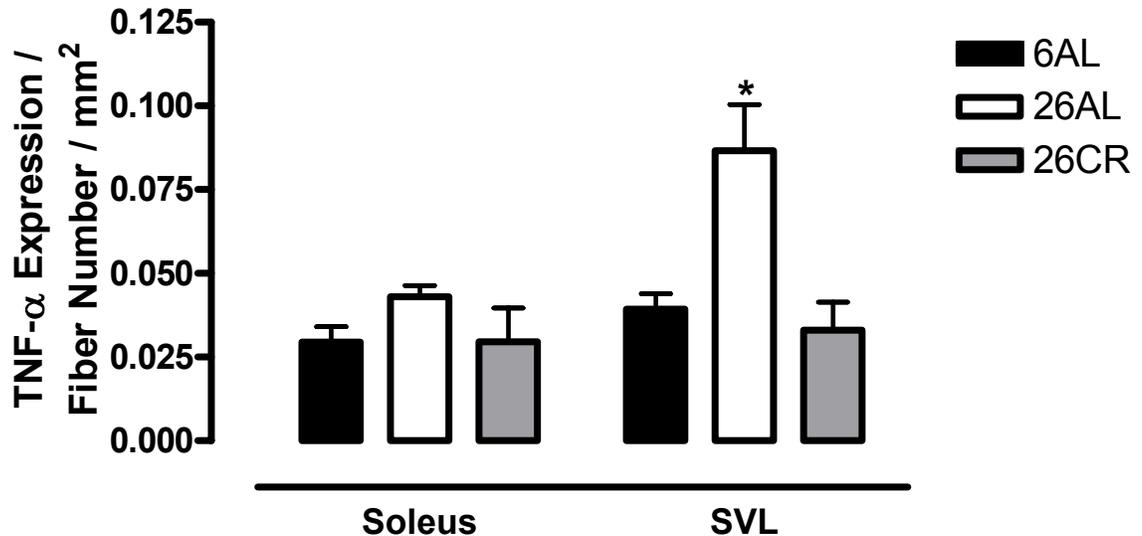


Figure 4. TNF- α expression from skeletal muscle myocytes. The number of TNF- α positive cells in two representative regions containing ~ 100 - 200 fibers were counted manually, and the means used for statistical analysis. No statistical differences were detected between groups in soleus. In SVL, 26AL was significantly different from 6AL and 26CR ($*P < 0.05$).

Elevated TNF- α levels have been reported in frail elderly individuals in myocytes acquired from vastus lateralis muscle (14), a finding which the present data falls in agreement with. However, to our knowledge few studies have reported the occurrence of a fiber type-specific expression of this catabolic cytokine and a possible tendency for type II over type I fibers to emanate greater expression.

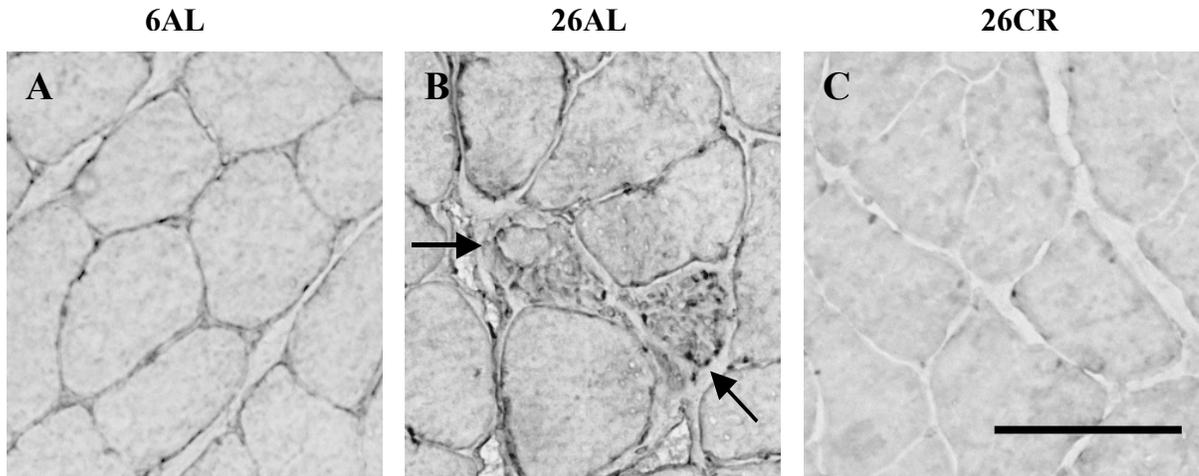


Figure 5. TNF- α immunohistochemical staining in superficial vastus lateralis sections from 6AL (A), 26AL (B) and 26CR (C) Fischer-344 male rats. Arrows indicate muscle fibers positive for TNF- α . Bar 100 μ m.

Biochemical Assays

Antioxidant Enzyme Activity

Superoxide dismutase activity. As an index of superoxide production, activity level of the antioxidant enzyme superoxide dismutase (SOD) was assayed in the cytosol fractionated from the soleus and SVL of the 6-month and 26-month-old *ad libitum* and 26-month-old calorie restricted animals (Table 3).

Table 3. Antioxidant enzyme activity in cytosol from 6-month- and 26-month-old *ad libitum* and 26-month-old calorie restricted male F344 rats.

	6AL	26AL	26CR
Superoxide dismutase			
Soleus	31.6 \pm 0.9	38.4 \pm 1.9*	33.7 \pm 0.9
SVL	33.0 \pm 1.3	35.6 \pm 1.6**	30.1 \pm 0.5

Superoxide dismutase (SOD) activity is expressed as units / mg protein and was measured according to Oyanagui (97). * Different from 6AL, $P < 0.05$; ** Different from 26CR, $P < 0.05$

In soleus, SOD was found to be elevated in the 26AL animals compared to the 6AL group (38.4 ± 1.9 vs. 31.6 ± 0.9 units / mg protein; mean \pm SEM; $P < 0.05$) but no differences were observed between 26AL and 26CR groups. In the SVL, age effects were not evident but there were differences accompanying diet with 26AL demonstrating greater SOD activity compared to the 26CR animals (35.6 ± 1.6 vs. 30.1 ± 0.5 units / mg protein; mean \pm SEM; $P < 0.05$).

Determination of Plasma TNF- α

Significant age and dietary effects on TNF- α levels. Greiwe et al. (14) showed in human plasma and skeletal muscle that TNF- α levels elevated with age were attenuated by resistance exercise. We investigated the efficacy of calorie restriction in being able to exert similar effects. In agreement with others (100, 101), plasma TNF- α levels were increased in 26-month- compared to 6-month-old *ad libitum* animals (21.8 ± 0.7 vs. 7.2 ± 1.4 pg / mL; mean \pm SEM; $P = 0.0001$) (Fig 6).

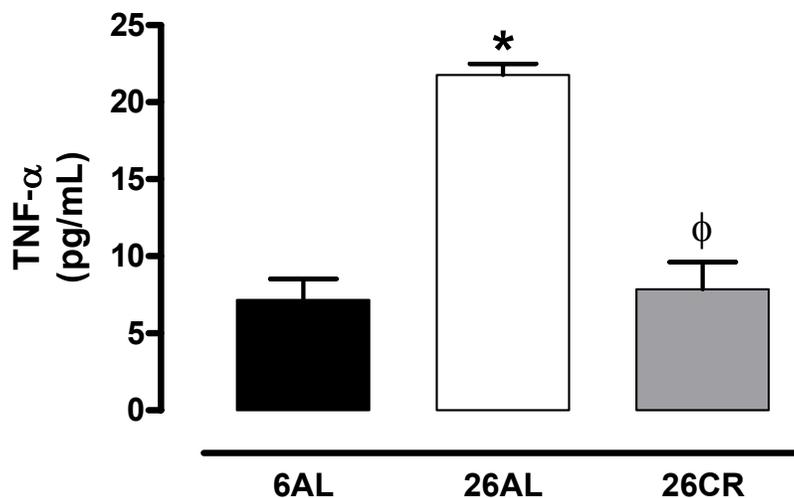


Figure 6. Plasma TNF- α levels in 6AL (6-month-old *ad libitum*), 26AL (26-month-old *ad libitum*) and 26CR (26-month-old calorie restricted) F344 rats. * Different from 6AL, $P = 0.0001$; ϕ Different from 26AL, $P = 0.0001$

Further, calorie restriction invoked a near 3-fold reduction in plasma TNF- α levels as found when the 26AL animals were compared to the 26CR group (21.8 ± 0.7 vs. 7.9 ± 1.8 pg / mL; mean \pm SEM; $P = 0.0001$) (Fig 6).

Western Blotting Detection of TNF- α / NF- κ B Signaling Proteins

TNF-R1 protein content in muscle of 6AL, 26AL and 26CR rats. Despite the presence of two receptors for TNF- α , multiple experimental approaches have revealed that TNF-R1 initiates the majority of TNF- α 's biological activities and has the ability to direct both cell survival and cell death signals (56, 57). After homogenization of the muscle samples, the homogenate was centrifuged at 1,000 x g for 10 minutes. The supernatant was then centrifuged at 14,000 x g for 10 minutes and the cytosolic fraction used for TNF-R1 protein determination (Fig 7).

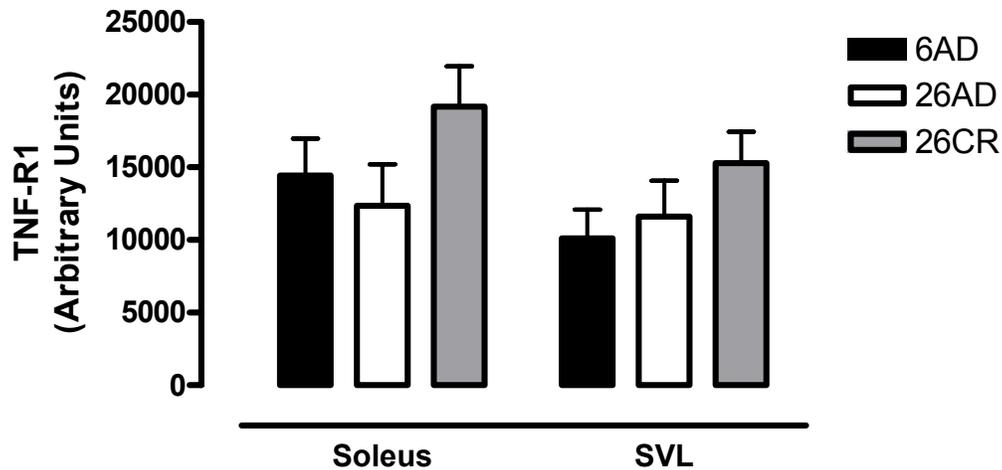


Figure 7. TNF-R1 in soleus and SVL (superficial vstus lateralis) muscle. Homogenates were centrifuged at 1,000 x g for 10 minutes. The supernatant was then centrifuged at 14,000 x g for 10 minutes and the cytosolic fraction assayed for TNF-R1 content via Western blotting. There was no difference statistically in TNF-R1 protein content between 6AL and 26AL, nor 26AL and 26CR in either muscle.

We found no statistical differences in the protein content of TNF-R1 in the soleus cytosol with age (14444 ± 2530 vs. 12355 ± 2850 , 6AL vs. 26AL, respectively; mean \pm SEM of arbitrary OD units / mg protein) nor when examined for effects of diet (12355 ± 2850 vs. 19178 ± 2773 , 26AL vs. 26CR, respectively; mean \pm SEM of arbitrary OD units / mg protein) (Fig 7). Furthermore, we did not detect differences in TNF-R1 expression in the SVL muscle with age (10112 ± 1987 vs. 11599 ± 2484 , 6AL vs. 26AL, respectively; mean \pm SEM of arbitrary OD units / mg protein) or dietary intervention (11599 ± 2484 vs. 15289 ± 2148 , 26AL vs. 26CR, respectively; mean \pm SEM of arbitrary OD units / mg protein).

IKK complex subunits, IKK β and IKK γ , presence in soleus and superficial vastus lateralis. In the IKK complex, IKK β and IKK γ , kinase and regulatory subunits, respectively, have been shown to be required for proinflammatory (e.g., TNF- α) induced stimulation of NF- κ B (61, 63-65). Accordingly, we examined these two subunits of the IKK complex to determine how they would respond to the effects of age and calorie restriction. In the soleus, we found that IKK β content was reduced with age ($P = 0.0073$ vs. 6AL) and was unaffected by calorie restriction (Fig 8). Conversely, calorie restriction appeared to evoke a decrease in IKK β content in the SVL compared to 26AL; however, no age effect was noted in this muscle (Fig 8).

The regulatory noncatalytic component of the IKK complex, IKK γ , has been singled out as an essential requisite in order for NF- κ B activation to be achieved by a variety of stimuli (63, 64). We chose to investigate the response of this protein first, to age and dietary intervention, and second, in muscles comprised of different fiber types.

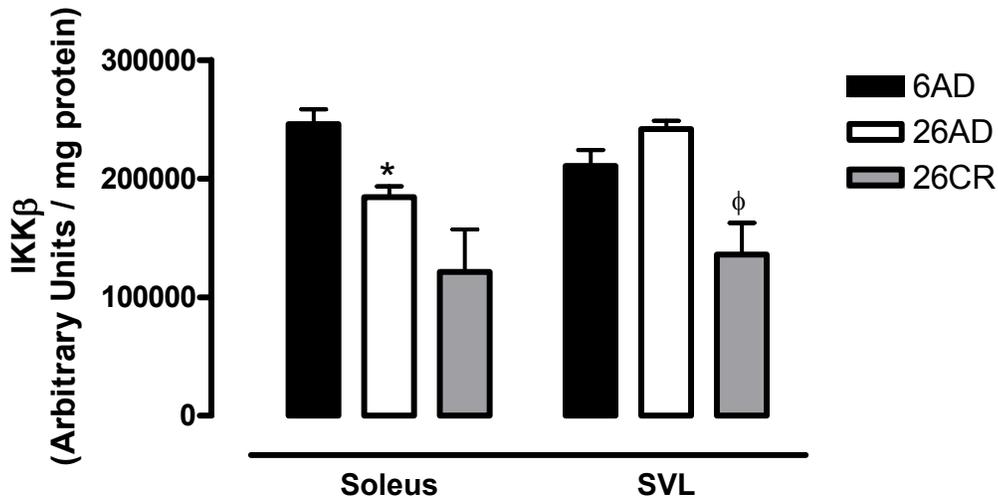


Figure 8. Western blot analysis of IKK β content in soleus and SVL (superficial vastus lateralis) muscle from 6AL (6-month-old *ad libitum*) (n=8), 26AL (26-month-old *ad libitum*) (n=8) and 26CR (26-month-old calorie restricted) (n=8) male F344 rats. Soleus IKK β content decreased with age (246106 ± 12408 vs. 184415 ± 9170 arbitrary units / mg protein; mean \pm SEM; *P = 0.0073) with no dietary effect detected. In the SVL, however, a dietary effect was found and IKK β appeared reduced in the 26CR group compared to the 26AL animals (135844 ± 26675 vs. 241832 ± 6855 arbitrary units / mg protein; mean \pm SEM; ^φP = 0.0377). Age effects were not significant in the SVL.

In the SVL we did not detect any change in content of IKK γ between the 6AL and 26AL animals (25987 ± 5259 vs. 28644 ± 3705 arbitrary units / mg protein, respectively; mean \pm SEM) nor did we see an influence of diet when comparing the 26AL to the 26CR group (28644 ± 3705 vs. 20168 ± 3322 arbitrary units / mg protein, respectively; mean \pm SEM) (Fig 9). Alternatively, when the effects of age and diet were evaluated in the soleus significant differences were displayed. In terms of age, the 6AL animals had substantially reduced protein content of IKK γ compared to the 26AL group (43984 ± 7150 vs. 92587 ± 9794 arbitrary units / mg protein, respectively; mean \pm SEM; P = 0.003). Calorie restriction imparted similar effects, with the 26CR rats demonstrating a

reduced IKK γ content compared to the 26AL animals (29447 ± 8410 vs. 92587 ± 9794 arbitrary units / mg protein, respectively; mean \pm SEM; $P < 0.0001$) (Fig 9).

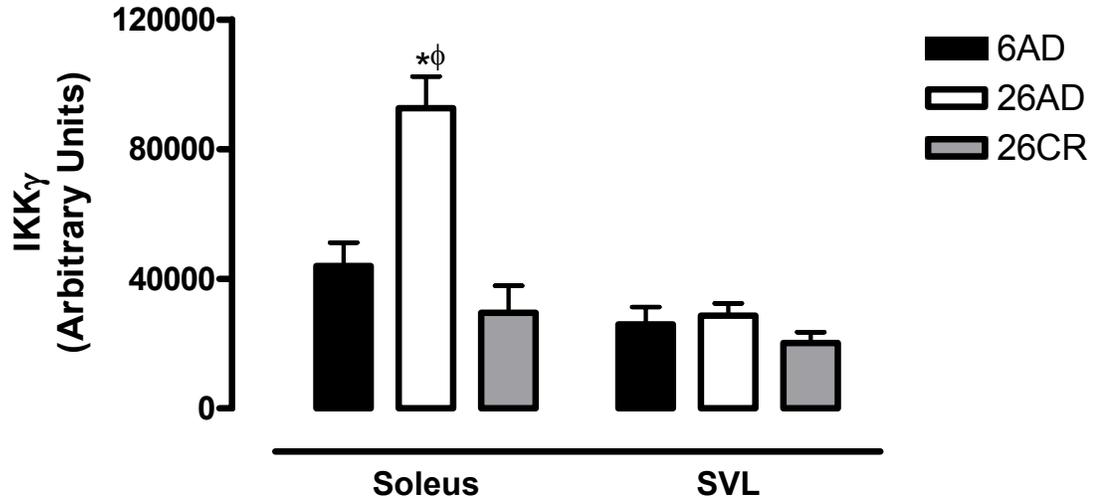


Figure 9. IKK γ content in soleus and SVL muscle from 6AL, 26AL and 26CR male F344 rats. There was no significant difference with age or diet in the SVL. In soleus, age and diet evoked significant differences. 26AL was greater in IKK γ content compared to 6AL ($*P = 0.003$) and 26AL was also greater compared to 26CR ($\phi P < 0.0001$). IKK γ content is reported as arbitrary units / mg protein.

The NF- κ B inhibitor protein, I κ B α , is significantly affected by age and diet in soleus muscle. In quiescent cells, NF- κ B is maintained in an inactive state through binding to inhibitor of κ B proteins, which cover the nuclear localization sequence of NF- κ B and prevent translocation of the transcription factor to the nucleus. We measured the protein content of I κ B α to determine how it would respond to age and calorie restriction in different fiber types. Beginning with the soleus, we found that age and diet effects existed. The 26-month-old *ad libitum* animals possessed a much greater content of I κ B α protein compared to the 6-month-old *ad libitum* animals (12069 ± 1399 vs. 6758 ± 1032 arbitrary units / mg protein, respectively; mean \pm SEM; $P = 0.0103$) (Fig 10).

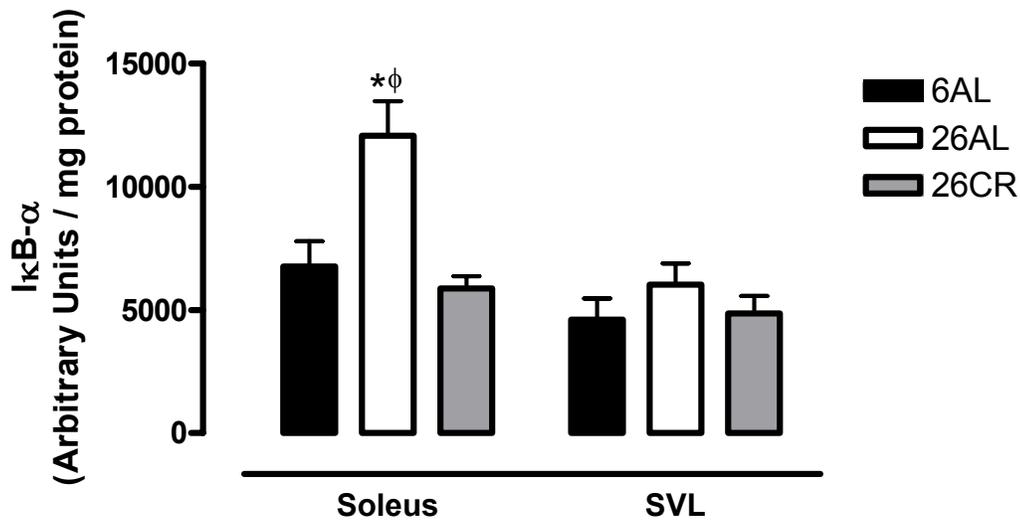


Figure 10. Western blot analysis of I κ B α in soleus and SVL (superficial vastus lateralis) muscle of 6-month-old- (6AL), 26-month-old *ad libitum* (26AL) and 26-month-old calorie restriction (26CR) F344 rats. I κ B α content is reported as arbitrary units / mg protein. In soleus, significant differences were found between 26AL and 6AL groups (12069 \pm 1399 vs. 6758 \pm 1032 arbitrary units / mg protein, respectively; mean \pm SEM; *P = 0.0103). Also, calorie restriction affected I κ B α content in the same direction as age, with greater levels found in 26AL compared to the 26CR animals (12069 \pm 1399 vs. 5866 \pm 507 arbitrary units / mg protein, respectively; mean \pm SEM; ϕ P = 0.0005).

In a similar fashion, differences were evidenced between the 26-month-old *ad libitum* and 26-month-old calorie restricted groups (12069 \pm 1399 vs. 5866 \pm 507 arbitrary units / mg protein, respectively; mean \pm SEM; P = 0.0005) (Fig 10). Upon examination of I κ B α in the superficial vastus lateralis, however, we did not detect differences amongst groups for age (4599 \pm 868 vs. 6022 \pm 857 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM) nor diet (6022 \pm 857 vs. 4851 \pm 708 arbitrary units / mg protein, 26AL vs. 26CR, respectively; mean \pm SEM).

Content of p65, a member of the family of dimers comprising NF- κ B. The predominant form of NF- κ B is a heterodimer consisting of p50/p105 and p65 (RelA) of the Rel family of proteins. We elected to focus on the responses of p65 to aging and diet

as past studies have lamented on the frequent incidence of p65 as one of the elements of the NF- κ B dimer (73). In soleus, significant effects of age and diet were exhibited with regard to p65 protein content (Fig 11). There were increases in p65 content in the 26AL animals compared to the 6AL group (391925 ± 45310 vs. 199460 ± 19335 arbitrary units / mg protein, respectively; mean \pm SEM; $P = 0.0014$). Also, 26AL demonstrated significantly higher p65 content versus the 26CR animals (391925 ± 45310 vs. 224699 ± 20726 arbitrary units / mg protein, respectively; mean \pm SEM; $P = 0.0423$).

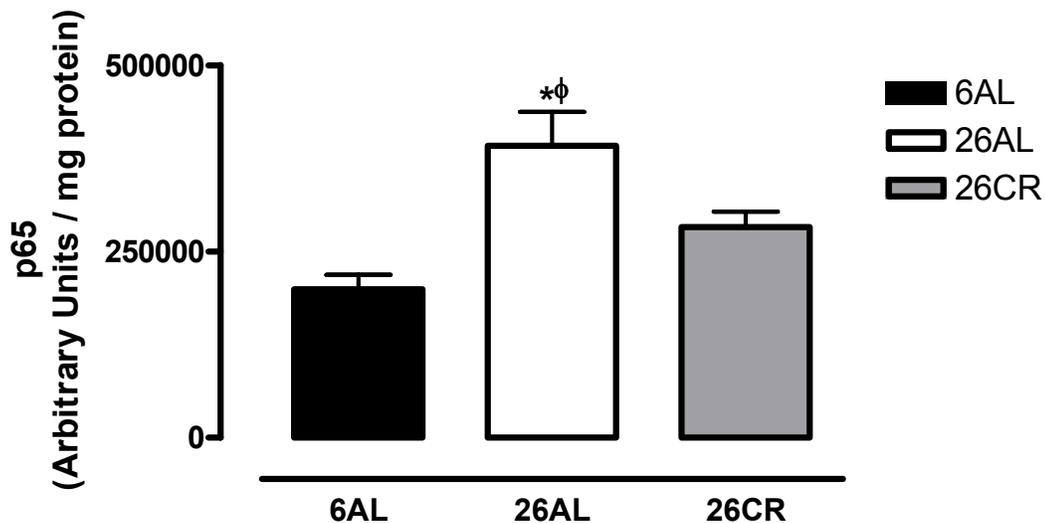


Figure 11. p65 protein content in soleus muscle of 6-month-old *ad libitum* (6AL), 26-month-old *ad libitum* (26AL) and 26-month-old calorie restricted (26CR) male F344 rats. Western blot analysis revealed a significant increase with age for p65 content (199460 ± 19335 vs. 391925 ± 45310 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM; $*P = 0.0014$). Further, calorie restriction reduced p65 content compared to the *ad libitum* fed group (224699 ± 20726 vs. 391925 ± 45310 arbitrary units / mg protein, 26CR vs. 26AL, respectively; mean \pm SEM; $^{\phi}P = 0.0423$). p65 content is reported as arbitrary units / mg protein.

To our surprise, we did not detect evidence of p65 content in the superficial vastus lateralis muscle tissue using western blot analysis. The possibility exists that p65 may not be the dominant protein subunit of NF- κ B expressed in this muscle type. Rather,

other members of the Rel family, such as p50/p105, RelB, c-Rel and p52/p100 (73) may experience a preferential expression in forming the NF- κ B dimer. Accordingly, the factors involved in shaping this intriguing prospect and determining the arrangement of the NF- κ B dimer in this tissue merit further investigation.

Nuclear Binding Activity of NF- κ B

NF- κ B activation is greater in the soleus compared to the superficial vastus lateralis. Soleus and SVL muscle sample nuclear extracts were isolated (96) and NF- κ B activation was measured using an ELISA kit (Active Motif, Carlsbad, CA) (Fig 12). In the soleus, we did not detect significant effects of age or diet on the activity levels of NF- κ B (Fig 12). Alternatively, we did reveal an effect of age in the SVL, which identified greater NF- κ B activity in the young compared to the old animals (1.662 ± 0.134 vs. 1.327 ± 0.137 OD values / mg protein; 6AL vs 26AL, respectively; mean \pm SEM; $P = 0.0332$). Furthermore, when NF- κ B activity was examined between muscle types, soleus demonstrated ~56% greater degree of activation compared to the superficial vastus lateralis (2.151 ± 0.051 vs. 1.378 ± 0.072 OD values / mg protein; soleus vs. SVL, respectively; mean \pm SEM; $P = 0.0001$). This significantly different NF- κ B activation profile existent between the two muscles may be linked to upstream events at the level of the receptor - adaptor protein complex. The decision of which signal will be transmitted, that is, NF- κ B activation or apoptosis, is determined by the type of adaptor proteins drawn to the receptor, TNF-R1. These current data lend support to the idea that there may be a fiber type-specific characteristic directing downstream signaling pathways, specifically, the soleus, a muscle comprised of type I fibers (39), electing to activate

NF- κ B, compared to the SVL, composed predominantly of type IIa and IIb fiber types (25, 39), opting for the apoptotic signaling paths.

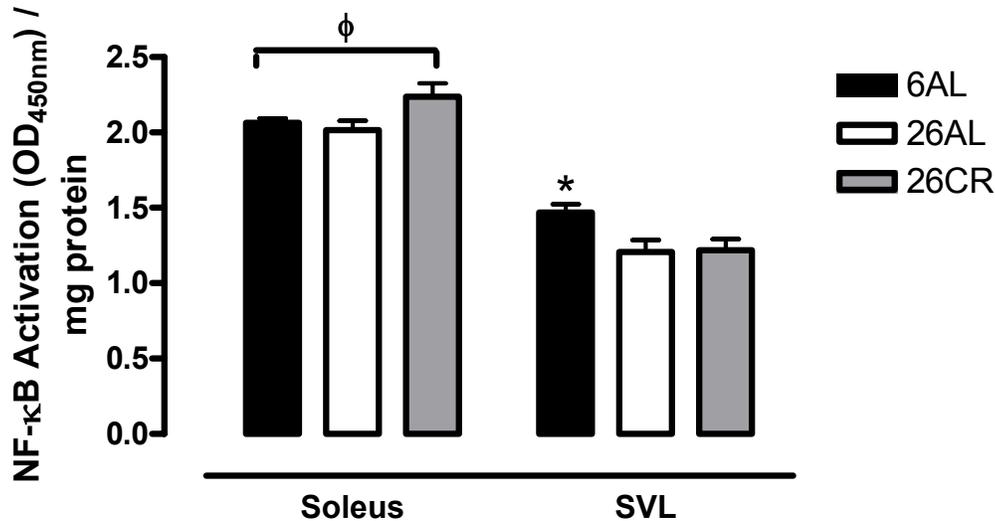


Figure 12. NF- κ B binding activity in soleus and SVL (superficial vastus lateralis) nuclear extracts of 6-month-old *ad libitum* (6AL), 26-month-old *ad libitum* (26AL) and 26-month-old calorie restricted (26CR) male F344 rats. In soleus, we did not detect a significant age effect (2.129 ± 0.07 vs. 2.073 ± 0.077 OD values / mg protein; 6AL vs 26AL, respectively; mean \pm SEM) nor diet effect (2.073 ± 0.077 vs. 2.241 ± 0.111 OD values / mg protein; 26AL vs. 26CR, respectively; mean \pm SEM). SVL NF- κ B binding activity produced an age effect with higher levels observed in the 6AL group (1.662 ± 0.134 vs. 1.327 ± 0.137 OD values / mg protein; 6AL vs 26AL, respectively; mean \pm SEM; * $P = 0.0332$), but no diet effect (1.327 ± 0.137 vs. 1.220 ± 0.137 values / mg protein; 26AL vs 26CR, respectively; mean \pm SEM). Also notable, however, was the difference ($\phi P = 0.0001$) in NF- κ B activity between muscles. NF- κ B binding activity is reported as OD values at 450nm per mg protein.

The TNF- α receptor-mediated pathway of apoptosis

Arising from our data describing the composition of TNF- α / NF- κ B signaling in skeletal muscle and the effects on this pathway of age and calorie restriction, we proceeded to investigate the possibility of muscle fiber types playing a regulatory role in directing the nature of the TNF- α signal conducted. Specifically, electing to recruit adaptor proteins in the course of events that transmitted signals promoting inflammatory

events or apoptotic events. To achieve this end, we examined the occurrence of two of the proteins intrinsic to the TNF- α receptor-mediated pathway of apoptosis, FADD (Fas-associated death domain) and caspase-8 (57).

FADD content elevated with age

When TNF- α binds to TNF-R1, modifications to the TNF-R1 – adaptor protein complex promote the formation of a new complex which dissociates from TNF-R1 and attracts FADD and other proapoptotic intermediates (102). Thus, we chose to assess how age and calorie restriction in different muscle tissues would affect the expression of this proapoptotic protein.

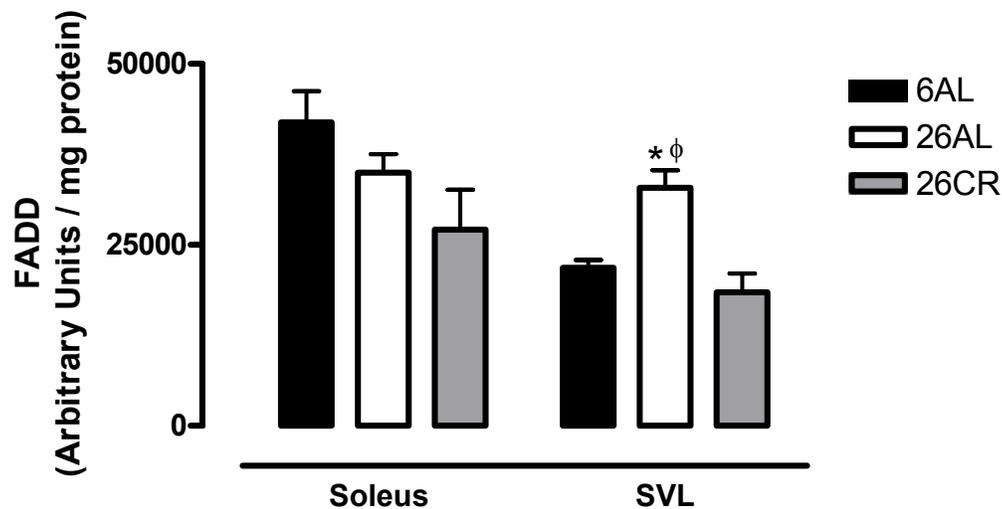


Figure 13. FADD protein content in soleus and SVL (superficial vastus lateralis) muscle of 6AL, 26AL and 26CR male F344 rats. Western blot analysis revealed a significant increase with age in the SVL for FADD content ($P = 0.0281$). Further, in the SVL calorie restriction reduced FADD content compared to the *ad libitum* fed group (18429 ± 2628 vs. 32876 ± 2406 arbitrary units / mg protein, 26CR vs. 26AL, respectively; mean \pm SEM; $\phi P = 0.0477$). We did not observe significant differences in FADD protein levels in the soleus with age or diet. FADD content is reported as arbitrary units / mg protein.

In the soleus, we did not detect a statistical difference in protein levels of FADD with age (41924 ± 4306 vs. 34993 ± 2526 arbitrary units / mg protein, 6AL vs. 26AL,

respectively; mean \pm SEM) nor with diet (34993 ± 2526 vs. 27108 ± 5493 arbitrary units / mg protein, 26AL vs. 26CR, respectively; mean \pm SEM). When we examined FADD content in the SVL (superficial vastus lateralis), however, we did observe a different outcome. The 26AL group displayed a greater level of FADD content compared to the 6-month-old *ad libitum* animals (32876 ± 2406 vs. 21844 ± 1048 arbitrary units / mg protein, respectively; mean \pm SEM; $P = 0.0281$) (Fig 13). Additionally, we found an intervention effect on FADD content, with a reduced level of the protein being detected in the 26CR animals compared to the 26AL group (Fig 13).

Caspase-8: zymogen and cleaved product

Another proponent of the TNF- α stimulated receptor-mediated pathway of apoptosis and affiliated with FADD is caspase-8. Caspase-8, the activated and cleaved form of procaspase-8, belongs to the family of cysteine proteases, and functions to activate other caspases with the potential to generate programmed cell death (103). We chose to determine the effects of age and calorie restriction in the soleus and SVL on the zymogen and cleaved product of caspase-8. When we analyzed procaspase-8 in the soleus, we found no differences between the young and old *ad libitum* fed animals (32261 ± 7532 vs. 29451 ± 3809 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM), nor between the old *ad libitum* fed and calorie restricted groups (29451 ± 3809 vs. 43367 ± 6050 arbitrary units / mg protein, 26AL vs. 26CR, respectively; mean \pm SEM) (Fig 14). Likewise, our results for procaspase-8 content in the SVL muscle indicated no differences with age (32547 ± 4128 vs. 33884 ± 4225 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM) or with calorie

restriction (33884 ± 4225 vs. 35636 ± 5547 arbitrary units / mg protein, 26AL vs. 26CR, respectively; mean \pm SEM) (Fig 14).

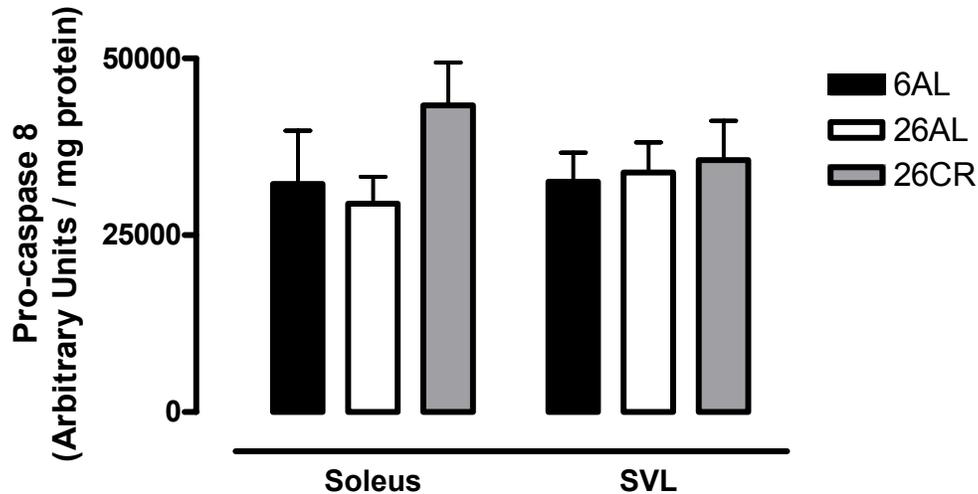


Figure 14. Procaspase-8 protein content in soleus and SVL (superficial vastus lateralis) muscle of 6-month-old *ad libitum* (6AL), 26-month-old *ad libitum* (26AL) and 26-month-old calorie restricted (26CR) male F344 rats. We did not detect differences in protein content of the zymogen in either muscle with age or dietary intervention using Western blot analysis. Procaspase-8 content is reported as arbitrary units / mg protein.

Next we determined whether effects of age and diet would be evidenced in caspase-8 content. In the soleus muscle, there were no differences between the 6AL and the 26AL animals for caspase-8 protein content (14557 ± 5879 vs. 10905 ± 4300 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM); similarly, we did not observe differences when comparing the 26AL rats to the 26CR rats (10905 ± 4300 vs. 15848 ± 800 arbitrary units / mg protein, 26AL vs. 26CR, respectively; mean \pm SEM) (Fig 15). In the superficial vastus lateralis, we found a significant effect with age (7605 ± 1296 vs. 22918 ± 4486 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM; *P = 0.0478) in caspase-8 content.

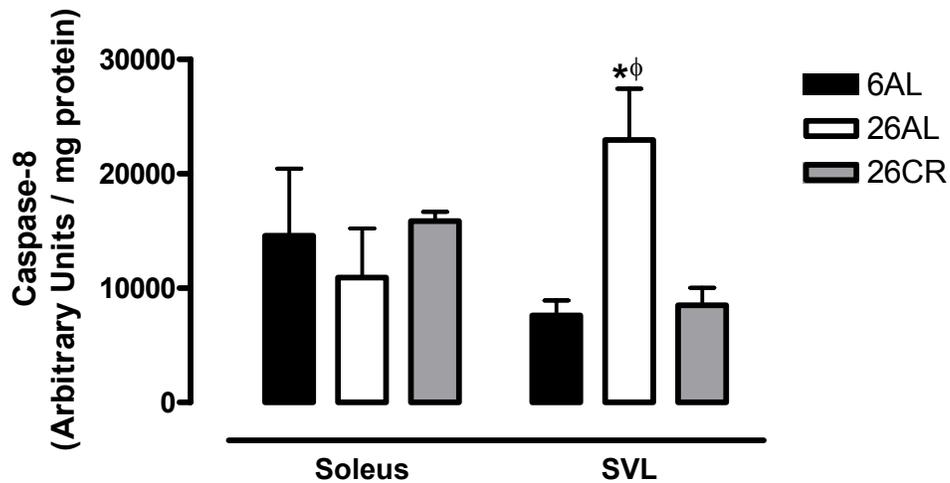


Figure 15. Caspase-8 protein content in soleus and SVL (superficial vastus lateralis) muscle of 6-month-old *ad libitum* (6AL), 26-month-old *ad libitum* (26AL) and 26-month-old calorie restricted (26CR) male F344 rats. Western blot analysis revealed a significant increase with age in the SVL for Caspase-8 content (7605 ± 1296 vs. 22918 ± 4486 arbitrary units / mg protein, 6AL vs. 26AL, respectively; mean \pm SEM; * $P = 0.0478$). Further, in the SVL calorie restriction reduced caspase-8 content compared to the *ad libitum* fed group (8497 ± 1522 vs. 22918 ± 4486 arbitrary units / mg protein, 26CR vs. 26AL, respectively; mean \pm SEM; $^{\phi}P = 0.0142$). We did not observe significant differences in caspase-8 protein levels in the soleus with age or diet. Caspase-8 content is reported as arbitrary units / mg protein.

Further, the dietary intervention reduced caspase-8 levels compared to the age-matched *ad libitum* fed counterparts (8497 ± 1522 vs. 22918 ± 4486 arbitrary units / mg protein, 26CR vs. 26AL, respectively; mean \pm SEM; $^{\phi}P = 0.0142$) (Fig 15).

DNA fragmentation

DNA was isolated from the soleus and superficial vastus lateralis muscles and assessed on agarose gels containing 0.5 $\mu\text{g/mL}$ ethidium bromide for the presence of DNA ladders, a characteristic of apoptosis. The presence of DNA ladders were revealed in the superficial vastus lateralis samples from the 26-month-old *ad libitum* fed animals; however, DNA ladders were absent in both the 6-month-old *ad libitum* and 26-month-old calorie restricted groups (Fig 16A). When we examined the soleus for DNA

fragmentation a different profile was found. In contrast to the superficial vastus lateralis, the only trace of DNA laddering was observed in the 6-month-old *ad libitum* fed animals (Fig 16B). Neither the 26-month-old *ad libitum* group nor the 26-month-old calorie restricted group displayed signs of DNA fragmentation in the soleus.

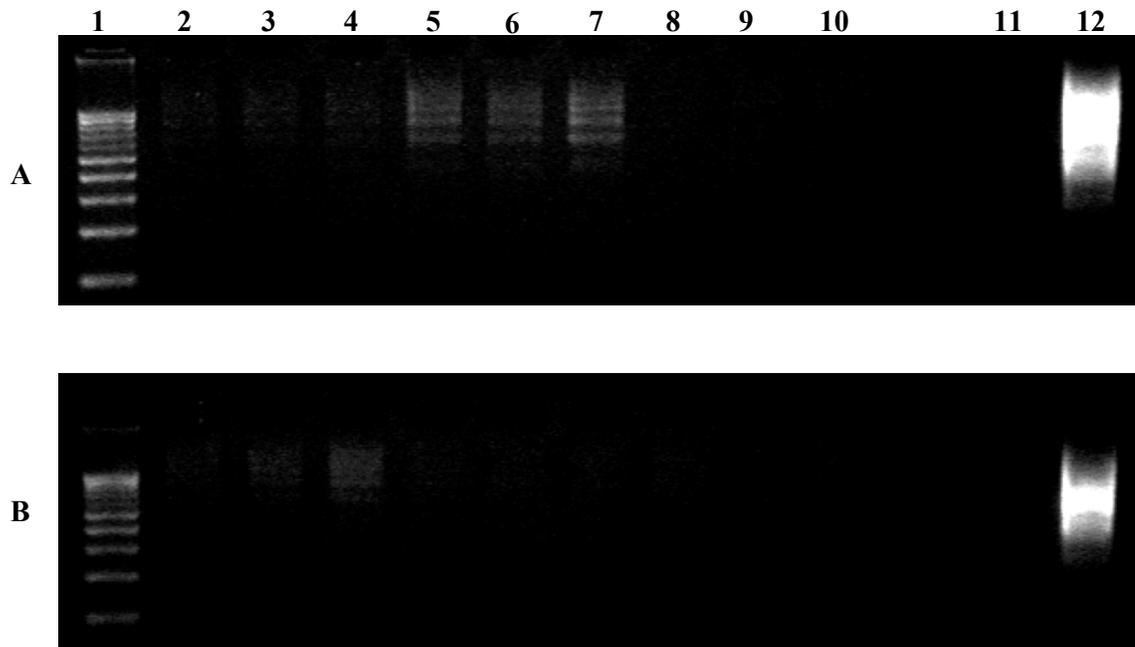


Figure 16. DNA fragmentation in superficial vastus lateralis (panel A) and soleus (panel B). DNA ladders were observed in the superficial vastus lateralis muscles of the 26-month-old *ad libitum* animals (lanes 5 to 7) but were not observed in the 6-month-old *ad libitum* animals (lanes 2 to 4) nor the 26-month-old calorie restricted animals (lanes 8 to 10). In soleus muscles, traces of DNA ladders were observed in the 6-month-old *ad libitum* animals (lanes 2 to 4; panel B) but were not detected in the 26-month-old *ad libitum* or the 26-month-old calorie restricted animals (lanes 5 to 7, and lanes 8 to 10, respectively; panel B). Lane 1 comprised the molecular weight marker; lane 11 the negative control and lane 12 the positive control for both panels A and B. One representative image is shown from two experiments that yielded similar results.

CHAPTER 5 DISCUSSION

Aging and Sarcopenia

Sarcopenia (describing the decline in skeletal muscle mass with age) and an elevated inflammatory milieu are among the physiological changes deemed synonymous with the aging process, and have been cited as significant indicators of mortality in older populations (104). Given the social and economic impact of sarcopenia and subclinical inflammation with age our research has focused on elucidating the mechanisms driving such occurrences. The direct healthcare costs attributable to sarcopenia in the United States alone in 2000 were estimated as \$18.5 billion, representing approximately 1.5% of the total healthcare expenditures for that year (105). Sources responsible for sarcopenia are believed to be multivariate in nature, and include a reduction in hormones promoting growth (106, 107), increased fat mass (108), a decline in central motor system alpha motor neurons (109), elevated apoptosis (110) and increases in catabolic cytokines (104). We sought to investigate sarcopenia from the perspective of involvement of the proinflammatory and catabolic cytokine, tumor necrosis factor- α (TNF- α) as little research has explored a role for this pleiotropic cytokine in the pathology of the sarcopenic condition.

TNF- α , Aging and Skeletal Muscle

We measured plasma TNF- α to establish the presence of an age effect with regard to systemic levels of the cytokine, and in agreement with findings from previous work (104) observed an age-associated increase in plasma TNF- α which the intervention of

calorie restriction attenuated. The impact of aging on skeletal muscle mass was next explored. In an attempt to discern whether TNF- α played an active role in muscle mass reduction with age we evaluated the expression of TNF- α from the soleus and superficial vastus lateralis (SVL) muscles immunohistochemically. These particular muscles were selected as they are composed of vastly different fiber types, with soleus containing primarily type I fibers and SVL type IIa and IIb, representing slow and fast fiber types, respectively (23, 25). Furthermore, the soleus and SVL have been reported to display differing degrees of age-associated atrophy (26). A reduction in the SVL wet weight only was evidenced with age, concurring with previous reports of a greater susceptibility of fast type muscles to yield to age-linked muscle mass loss or atrophy, compared to slow fiber type muscle (26, 27). A recent study by McKiernan et al., (111) substantiated the greater resistance to age-linked atrophy demonstrated by the soleus muscle (composed of type I fibers) than was evident in the vastus lateralis and rectus femoris muscles (predominantly of type II fibers). These researchers in a paralleled study (112) hinted toward the involvement of mitochondrial DNA deletions and associated electron transport system enzymatic abnormalities as providing the trigger for loss of skeletal muscle fibers with age, and for this sequela to be more pronounced in muscles comprised predominantly of type II fibers (99). They reasoned that the lower mitochondrial content in the type II fibers presented a more vulnerable target with respect to the frequency of mitochondrial DNA mutations occurring, thereby increasing the likelihood of these fibers ultimately succumbing to atrophy.

Age Decline in Muscle Cell Area and Increased TNF- α Expression

Skeletal muscle atrophy with age is thought to be manifest by either a reduction in fiber mass, fiber number or some combination thereof (5, 25, 27). Our findings from hematoxylin and eosin stains of muscle fiber sections fell in agreement with these reports. Both soleus and superficial vastus lateralis muscles displayed reductions in the areas measured for myocytes per square millimeter in the aged animals than was found in the young animals. Notable also, was the ability of calorie restriction to elicit a reversal of this age-associated decline in cell area in the superficial vastus lateralis. Concomitant with the decrease in SVL mass and cellular area with age, was an increase in TNF- α expression. The age-associated elevation in TNF- α stemming from myocytes has been acknowledged by earlier work (14). The study by Greiwe and coworkers (14), deployed an exercise intervention which successfully resulted in a reduction of TNF- α expression in skeletal muscle of elderly humans.

In the current study, the dietary intervention of calorie restriction was used to suppress age-associated increases in the cytokine. We found lowered TNF- α expression in the calorie restricted group compared to their age-matched, *ad libitum* fed counterparts. Furthermore, when we performed the same analysis in soleus we found no detectable differences in myocyte-expressed TNF- α levels, presenting the possibility of a fiber type dependent regulation of TNF- α expression. Alternatively, the presence of other cell types that may reside in skeletal muscle, for example adipocytes and macrophages, both of which also have the propensity to produce TNF- α , may also be part contributors to the higher levels of TNF- α observed in the SVL with age. Moreover, the lowered superficial vastus lateralis muscle TNF- α expression accompanying calorie restriction may be

attributable to either the reduced fat mass which this intervention imparts (113) or may be the result of a decline in inflammatory genes expressed, as has been reported in DNA microarray studies profiling gene expression in skeletal muscle subjected to calorie restriction (114).

Fiber Type Variations in NF- κ B Signaling

The actions of TNF- α are transmitted via two receptors and an intricate assortment of signaling proteins. The roles of most of these components have been ascertained; however, certain points of contention remain, particularly with respect to the manner in which adaptor proteins are recruited to the TNF- α receptors. With these considerations in mind, we wanted to determine in skeletal muscle the response to aging and calorie restriction of the signaling intermediaries integral to TNF- α stimulation of NF- κ B. With the majority of TNF- α 's biological actions initiated through TNF-R1 (57) we began our examination of the signaling pathway at this juncture. We did not observe differences in protein content of TNF-R1 with age or calorie restriction in either muscle tissue, an outcome that may have been influenced by the cellular fraction we chose to examine, namely the cytosolic fraction. Although this receptor does persist in a soluble form, a population also prevails that is membrane bound and this population may have responded differently to age and calorie restriction.

Kim and associates (15) in their study of rat kidney from young and old animals, showed signaling molecules of the NF- κ B family to be amplified with age (15); we wanted to establish whether skeletal muscle acted in a similar fashion. A focal point in the pathway of NF- κ B activation by TNF- α is the IKK complex. The catalytic subunit of the complex, IKK β , was affected by age in the soleus but not in the superficial vastus

lateralis muscle. The lowered IKK β protein content observed in the aged animals falls in contrast to the response of this protein reported in the aged kidney (15). When we proceeded to examine the regulatory unit of the IKK complex, IKK γ or NEMO, a stark increase in this protein was found with age in the soleus but no age effects were found in the superficial vastus lateralis. What's more, in the calorie restricted animals, the levels of IKK γ were significantly reduced compared to those demonstrated in the age-matched *ad libitum* fed counterparts. There is much debate surrounding the extent of each of the subunits' contribution in promoting the downstream events leading to NF- κ B activation; however, several lines of evidence contend that NF- κ B activation is contingent on the presence of IKK γ (63, 64) to a much greater degree than that of the other subunits. Thus, if this relationship holds true for skeletal muscle, then the current data would support the notion that in spite of the reduced protein level of IKK β , the elevated IKK γ level ensured an increased stimulation of NF- κ B with age. Further work is necessitated in order to corroborate this idea.

The target of the IKK complex and the protein inhibiting NF- κ B translocation from cytosol to nucleus, I κ B α , was next subjected to analysis. Others have reported a decline in this inhibitor of κ B with age (15) but in the current study we found the opposite to be true. Reflective of the IKK γ responses observed earlier, we found all of the significant differences in I κ B α content to occur in the soleus and not the superficial vastus lateralis muscle. The young *ad libitum* fed animals and the old calorie restricted animals both exhibited substantially lower levels of I κ B α compared to the old *ad libitum* fed animals. This illustration of increased I κ B α content with age may act as an indirect indicator of

elevated NF- κ B activity. Specifically, contained in the NF- κ B program of genes targeted for transcription (71) are members of the Rel / NF- κ B / I κ B family. When NF- κ B is subject to activation it prompts the new synthesis of I κ B, and limits its own activation (74, 75). Given that the current data demonstrated an increased content of I κ B α with age supports the proposition of an age-associated elevation in NF- κ B activity necessitating an elevated I κ B α transcription to quash NF- κ B's activation for an ill-desired extended period of time.

Fiber Type Variations in NF- κ B Subunits and Binding Activities

Following a very similar pattern to those uncovered for the upstream signaling proteins, was the response of p65 to age and diet. An elevation with age, which calorie restriction was able to attenuate, was exuded in the soleus for this subunit of NF- κ B. Others have reported an analogous relationship in non-muscle tissue for p65 expression obtained from aged and aged calorie restricted rats (15). Unexpectedly, however, we could not find evidence of p65 protein in the superficial vastus lateralis muscle, nor references in the literature that have documented this anomaly. Given that there are five members of the Rel family that are relied upon to form the hetero- or homodimer unit that embodies NF- κ B (73) presents the possibility that in the SVL, the other family member subunits may experience a preferential expression over and above p65 in forming the NF- κ B dimer. In any event, further research is warranted to explore whether or not this is the case and the underlying elements that may regulate this prospect.

Binding of NF- κ B to the DNA nuclear localization site represents the green light for transcriptional activities to be initiated. To gauge the effects of age and calorie restriction on this event, we measured NF- κ B binding activity in nuclear extracts

obtained from the soleus and superficial vastus lateralis muscles. We did not detect age or diet effects on the degree of NF- κ B activity in the soleus, and the only significant difference, symbolized by increased NF- κ B binding, observed in the superficial vastus lateralis fell in the young group, who displayed greater NF- κ B activation compared to the old animals. An intriguing finding did emerge, however, with respect to the NF- κ B binding activities exhibited by the different muscles. The superficial vastus lateralis exuded a notably lower level (~56%) of transcription factor nuclear binding compared to the soleus. This profile of NF- κ B binding may be representative of an intrinsic fiber type-specific capability, or in other words, a fiber type driven preference for signaling to NF- κ B. Support for this proposition is derived from the pattern that emerges in soleus of the inflammatory factors' responses with age. The amplified presence of the inflammatory advocates, i.e., IKK γ , I κ B α , p65 and NF- κ B binding, consistently appearing in the soleus more so than the superficial vastus lateralis, puts forth a strong case for fiber type holding some authority in the decision to promote inflammatory signaling, a decision which appears to be coupled with the aging process. Furthermore, the ability of calorie restriction to attenuate most of the age-associated elevations in the components of inflammation studied, may be representative of the effectiveness of this intervention against chronic, sub clinical levels of inflammation which accompanies aging (104) and which has also been suggested by others (13).

It must also be recognized, however, that when signaling to NF- κ B predominates over the other TNF- α signaling paths, (i.e., proapoptotic activation) that alongside inflammatory signaling, NF- κ B promotes gene transcription responsible for cell survival and repression of apoptosis (54, 76). Therefore, the pattern of signal conductance in the

soleus of old animals may be indicative of signaling efforts in this tissue to promote growth and survival (71). If indeed fiber type does exert a regulatory influence over the cell signal conveyed, then the balance of cell survival or cell death signaling may differ in skeletal muscle with different fiber type profiles. In order to investigate this conjecture, a closer examination of TNF- α signaling apoptotic pathways was undertaken.

Fiber Type Differences in Apoptotic Signaling

An increase in apoptosis has been described as a contributor to age-associated atrophy (110); thus, to address the possibility of TNF- α transmitting signals to recruit apoptotic proteins versus those linked to NF- κ B, we measured the content of two proteins in the programmed cell death cascade, FADD and caspase-8, and sought out evidence of DNA fragmentation, one of the final morphological events in the conserved sequence of apoptosis.

In the old *ad libitum* fed animals, we observed significant increases in the content of both FADD and caspase-8 in the superficial vastus lateralis compared to the young and the old calorie restricted animals, with no differences with age or diet detected in the soleus for either protein. Furthermore, DNA fragmentation (as denoted by DNA laddering) persisted in the superficial vastus lateralis muscles of the 26-month-old *ad libitum* fed animals but was absent in the 6-month-old *ad libitum* and the 26-month-old calorie restricted animals. In the soleus, traces of DNA fragmentation emerged in the young animals, but were not observed in either of the old groups. This may have been a reflection of normal apoptotic activity to maintain cellular homeostasis in the young animals (115).

Scrutiny of the data comparing apoptotic signaling in the type I soleus and the type II superficial vastus lateralis muscle suggests a more pronounced stimulation of cell death signaling in the type II muscle which would concur with the greater loss of muscle mass demonstrated with age in this particular muscle (26, 27). Previous research has reported a tendency for fast (type II) muscles to be more prone to develop apoptosis than slow (type I) muscles (116, 117). These researchers explored the skeletal muscle myopathy linked with muscle bulk loss in a model of congestive heart failure. In the tibialis anterior muscle, composed primarily of type II fibers, the magnitude of apoptosis detected in the tissue mirrored the increase in circulating TNF- α and was accompanied by muscle atrophy (117). At variance, however, was the degree of apoptosis and muscle atrophy detected in the soleus. Despite, the levels of TNF- α being equivalent to those reported in the sister study (117) the investigators did not observe any degree of muscle atrophy in the soleus, and noted the near three fold reduction in myocyte apoptosis compared to that determined in the type II fiber muscle (116). Granted, additional investigation is required to confirm that with aging similar signaling events occur. Nonetheless, on the basis of the current findings, type II muscles possessing a predisposition to develop apoptosis to a greater extent than type I muscles with age, remains a distinct possibility.

Final Conclusions: TNF- α Promoting Skeletal Muscle Cell Survival or Cell Death?

Differences in fiber type responses to age-associated atrophy have been attributed to a collection of intrinsic and systemic factors (26, 27, 104, 106-110). The prospect of a variation in TNF- α signaling representing one such fiber type difference in the regulation of age-linked atrophy, however, is a novel proposition. Age was associated with greater

signaling to NF- κ B in the soleus than was observed in the superficial vastus lateralis. In contrast, a greater presence of TNF- α (the upstream signaling cytokine of NF- κ B) was expressed in the superficial vastus lateralis compared to the soleus, presenting a somewhat paradoxical picture. However, reexamination of the cell signaling capabilities of TNF- α taken in conjunction with the apoptotic signaling pattern exhibited, may offer a potential explanation for these inconsistencies.

TNF- α bound to TNF-R1 may not only elicit intracellular signaling to NF- κ B, but may also signal activation of cellular apoptosis via recruitment of cysteine proteases (57). The current data presents the idea that as we age, the manner of TNF- α signaling in skeletal muscle (promoting cell survival or cell death) may to some degree be fiber type dependent (Fig 17). This postulate would provide an alternative explanation addressing why age coincides with differential atrophy in muscles with different fiber types. What's more, further questions are raised surrounding the specific muscle attributes that may play a role in shaping the cells' decision to opt for TNF- α signaling to NF- κ B versus apoptosis. Recent speculation (102) has been directed toward the fidelity of the stimulation of NF- κ B and subsequent transcriptional activity of NF- κ B as determining the cells' fate. Specifically, cells accommodating defective NF- κ B signals (resulting in low quantities of antiapoptotic proteins) undergo TNF- α -induced apoptotic elimination. This scenario may provide one explanation for the different signaling profiles observed in the soleus and superficial vastus lateralis. The absence of measurable p65 protein in the superficial vastus lateralis may embody one potential flaw (compromising the integrity of NF- κ B signaling fidelity), and in so doing, prompting the tissue to resort to apoptotic signaling pathways. However, given that four alternative subunits of NF- κ B exist,

implies a sense of redundancy and safeguard against any one subunit deciding the success of the NF- κ B signal. Nonetheless, further detailing the role of p65 (particularly in skeletal muscles exhibiting a greater predisposition to age-linked atrophy) may unfold the requisite of this NF- κ B subunit to the success of the NF- κ B signaling pathway.

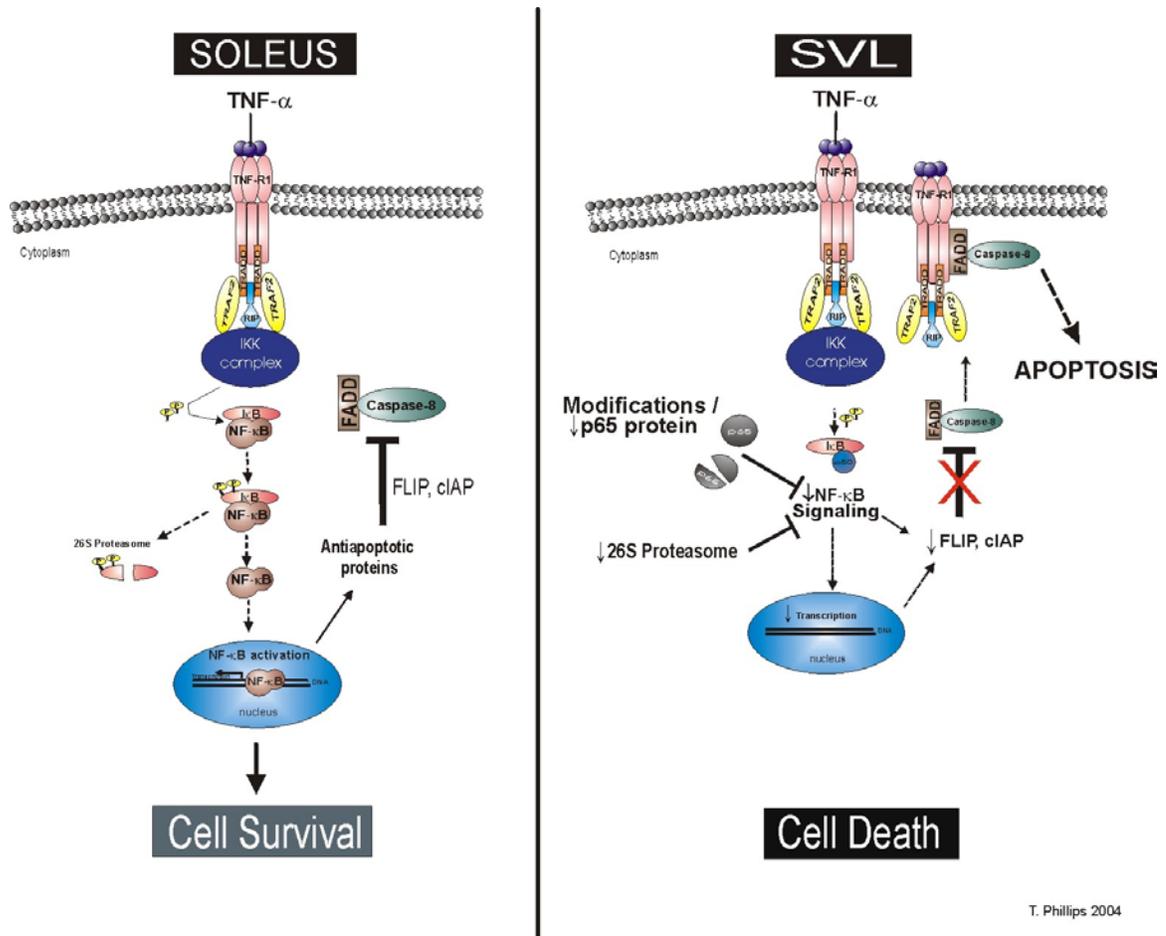


Figure 17. Proposed model of TNF- α signaling with age in soleus and superficial vastus lateralis (SVL) skeletal muscles

Achieving a 10% reduction in sarcopenia prevalence in the United States would result in healthcare savings of \$1.1 billion per year (105). Accordingly, further examination of muscle specific attributes from the perspective of TNF- α signal transmission (in an attempt to make known why sarcopenia affects different muscles disproportionately) presents an avenue worthy of additional investigation. Realizing this

goal may potentially improve or generate new therapeutic interventions geared toward the pathogenesis of sarcopenia, and drastically curb the physical and financial burdens imposed by sarcopenia.

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BIOGRAPHICAL SKETCH

Tracey Phillips was born in Edinburgh, Scotland, growing up in the city suburb of Clermiston. After attending Edinburgh University for one year, she embarked upon a bachelor's degree at Moray House Institute of Education which she completed at Phillips University, in Enid, Oklahoma, in May 1998. She then moved to Charleston, Illinois to undertake a master's degree in cardiac rehabilitation and exercise physiology. In 1999, Tracey began work on her Doctor of Philosophy degree in exercise physiology at the University of Florida. Following graduation, she will begin a degree in medicine at Liverpool University.