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Changes in IL-15 expression and death-receptor apoptotic signaling in rat gastrocnemius muscle with aging and life-long calorie restriction

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Abstract

TNF-α-mediated apoptosis is enhanced in aged rodent muscles, suggesting that this pathway may be involved in sarcopenia. Interleukin-15 (IL-15), a muscle-derived anabolic cytokine, mitigates muscle wasting and apoptosis in cachectic rats. This effect is thought to occur through inhibition of TNF-α-triggered apoptosis. We investigated IL-15 signaling and the TNF-α-mediated pathway of apoptosis in the gastrocnemius muscle of Fischer344×Brown Norway rats across the ages of 8, 18, 29 and 37 months, in relation to life-long calorie restriction (CR, 40% calorie intake reduction). Aging caused loss of muscle mass and increased apoptotic DNA fragmentation, which were mitigated by CR. Protein levels of IL-15 and mRNA abundance of IL-15 receptor α-chain decreased in senescent ad libitum (AL) fed rats, but were maintained in CR rodents. Elevations of TNF-α, TNF-receptor 1, cleaved caspase-8 and -3 were observed at advanced age in AL rats. These changes were prevented or mitigated by CR. Our results indicate that aging is associated with decreased IL-15 signaling in rat gastrocnemius muscle, which may contribute to sarcopenia partly through enhanced TNF-α-mediated apoptosis. Preservation of IL-15 signaling by CR may therefore represent a further mechanism contributing to the anti-aging effect of this dietary intervention in skeletal muscle.

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Keywords

sarcopenia; interleukin-15; tumor necrosis factor-α; calorie restriction; apoptosis

Introduction

Sarcopenia, the loss of muscle mass and function, is a common feature of aging and is promoted by multiple etiological factors (e.g., denervation, altered hormonal status, impaired muscle regeneration, altered protein turnover, increased levels of pro-inflammatory cytokines, oxidative damage) [reviewed in (Rolland et al. 2008)]. However, the relative importance of the various contributing factors has not been established. In recent years, evidence has accumulated indicating that an age-dependent acceleration of apoptosis in skeletal muscle may be involved in the pathogenesis of sarcopenia (Strasser et al. 1999;Malmgren et al. 2001;Dirks and Leeuwenburgh 2002, 2004;Phillips and Leeuwenburgh 2005;Whitman et al. 2005;Siu et al. 2005a;Pistilli et al. 2006a;Marzetti et al. 2008a,b). Several apoptotic pathways may contribute to age-related muscle atrophy [reviewed in (Marzetti and Leeuwenburgh 2006)]. In particular, the death receptor-mediated pathway of apoptosis, triggered by TNF-α, is activated in skeletal muscle of old rodents (Phillips and Leeuwenburgh 2005;Pistilli et al. 2006a;Marzetti et al. 2008a), suggesting a role in age-related muscle loss. The relevance of TNF-α signaling to muscle atrophy is further highlighted by its ability to promote protein breakdown in skeletal myocytes (Li et al. 1998;Li and Reid 2000), mainly via activation of the ubiquitin-proteasome pathway (Llovera et al. 1997). The temporal relation between the initiation of apoptosis and the induction of proteolysis as well as the relative magnitude of the two processes during muscle wasting is still unclear. However, it has recently been suggested that apoptotic signaling is required for and precedes protein degradation during muscle atrophy (Argiles et al. 2008).

The death receptor pathway of apoptosis, also referred to as the extrinsic pathway, is triggered by the interaction of TNF-α with TNF-receptor 1 (TNF-R1) and subsequent recruitment of adaptor proteins such as Fas-associated death domain (FADD), TNF-receptor-associated death domain (TRADD) and TNF-receptor-associated factors (TRAFs). The resulting death-inducing signaling complex (DISC) engages and activates procaspase-8 (Danial and Korsmeyer 2004). Cleaved caspase-8 then works downstream to activate caspase-3, which carries out the actual proteolytic events and DNA fragmentation (via caspase-activated DNase, CAD) that result in cellular breakdown.

In the presence of strong pro-apoptotic pressure, such as aging or muscle unloading, skeletal myocytes may produce anti-apoptotic factors as an attempt to limit muscle loss (Dirks and Leeuwenburgh 2004;Siu et al. 2005a,b). In this regard, it was recently reported that expression of interleukin-15 (IL-15) mRNA, a muscle anabolic cytokine, was increased with unloading and aging in rat soleus and plantaris muscles (Pistilli et al. 2007). This finding was consistent with a previous microarray study in rodent soleus muscle (Pattison et al. 2003).

IL-15 belongs to the four-α-helix bundle cytokine family and acts as a growth factor for T and B lymphocytes, and for natural killer cells (Grabstein et al. 1994;Burton et al. 1994;Carson et al. 1994;Tagaya et al. 1996). Actions of IL-15 are transduced by a heterotrimeric receptor with two subunits shared with IL-2 [i.e., common γ-chain (γ_c) and IL-2 receptor β-chain (IL-2Rβ)] (Giri et al. 1994). The third subunit, IL-15 receptor α-chain (IL-15Rα), is responsible for IL-15 specificity and high affinity binding (Giri et al. 1995). In vitro studies showed that IL-15 promoted myosin heavy chain accumulation in differentiated myotubes independent of IGF-1 (Quinn et al. 1995). Quinn et al. (2002) also demonstrated that overexpression of IL-15 in cultured myotubes stimulated protein synthesis and inhibited proteolysis. Interestingly, IL-15

administration attenuated muscle wasting and apoptotic DNA fragmentation in a rat model of cancer cachexia (Figueras et al. 2004).

The anti-apoptotic effect of IL-15 in skeletal muscle may be mediated by inhibition of the TNF-α-mediated pathway. In fact, attenuation of apoptosis severity in muscle of cachectic rats supplemented with IL-15 coincided with decreased expression of TNF-R1 and R2 (Figueras et al. 2004). Furthermore, binding of IL-15 to its receptor enables IL-15Rα to compete with TNF-R1 for adaptor proteins of the DISC in cultured fibrosarcoma cells (Bulfone-Paus et al. 1999), thus preventing activation of downstream caspases. It was also demonstrated that interaction between IL-15Rα and TNF-R1-associated TRAF-2 resulted in activation of NF-κB (Bulfone-Paus et al. 1999). Notably, NF-κB may promote the expression of anti-apoptotic genes such as cellular inhibitor of apoptosis protein-1 and 2 (cIAP-1, cIAP-2), and FLICE-like inhibitory protein long form ($FLIP_L$) (Wang et al. 1998;Micheau et al. 2001).

Based on these premises, we investigated the effects of aging and life-long calorie restriction (CR), an intervention shown to mitigate apoptosis in skeletal muscle of old rodents (Dirks and Leeuwenburgh 2004;Phillips and Leeuwenburgh 2005), on IL-15 expression and the apoptotic pathway activated by TNF-α in rat gastrocnemius muscle. We hypothesized that advanced age would be associated with decreased expression of IL-15 and IL-15Rα, concomitant with increased signaling through the TNF-α-mediated pathway of apoptosis. We further hypothesized that CR would attenuate apoptosis via downregulation of the TNF-α pathway of cell death, accompanied by upregulation of IL-15 and IL-15Rα expression.

Methods

Animals

Seventy-two 8, 18, 29 and 37-month-old male Fischer344×Brown Norway (F344×BNF1) hybrid rats were purchased from the NIA colony at Harlan Industries (Indianapolis, IN). The ages were chosen to reflect a young age (8-month), adulthood (18-month), advanced age (29-month) and senescence (37-month) (Turturro et al. 1999). In each age group, 9 animals were fed ad libitum (AL) and 9 were 40% calorie restricted (CR). CR was initiated at 14 weeks of age at 10% restriction, increased to 25% at 15 weeks, and to 40% at 16 weeks. AL rats had free access to NIH-31 average nutrient composition pellets, whereas CR animals received NIH-31/NIA fortified pellets, once daily, approximately 1 hour before the onset of the dark period. All rats had free access to tap water. Rats were individually housed and maintained on a 12-hour light/dark cycle, at constant temperature and humidity, in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Health status, body weight and food intake were monitored daily. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH and reviewed and approved by the University of Florida's animal care and use committee.

Preparation of subcellular fractions and whole cell homogenates

Rats were sacrificed by rapid decapitation, hind limb muscles removed, trimmed of adipose tissue and tendons, and weighed. Immediately afterward, muscles were snap-frozen in liquid nitrogen and subsequently stored at -80°C. No major pathologies were noted at the time of tissue harvesting. Experiments were performed on the left medial gastrocnemius, predominantly comprised of type II fibers, which undergoes substantial atrophy during aging (Siu et al. 2006;Marzetti et al. 2008b). Subcellular fractionation and preparation of whole cell homogenates were performed as previously described (Marzetti et al. 2008b).

Western blot analysis

For quantification of TNF-R1 expression, 60 μg of protein were applied to pre-cast Tris·HCl gels (Bio-Rad, Hercules, Ca), whereas 120 μg were loaded to quantify cleaved caspase-8 and -3. Tissue levels of cIAP-1/2 and $FLIP_L$ as well as nuclear content of NF-κB p65 were determined by applying 100 μg of protein. Separated proteins were transferred to PVDF membranes (Immobilon P, Millipore, Bedford, MA, USA) using a semidry blotter (Bio-Rad). Transfer efficiency was verified by staining the gels with GelCode Blue Stain Reagent (Pierce Biotechnology, Rockford, IL) and the membranes with Ponceau S (Sigma-Aldrich, St. Louis, MO). Ponceau S staining was also used as a loading control (Moore and Viselli 2000). For TNF-R1 and cIAP-1/2 experiments, membranes were blocked in StartingBlock Tris-Buffered Saline Blocking Buffer with 0.05% Tween-20 (Pierce Biotechnology), and incubated in rabbit polyclonal anti-TNF-R1 (Abcam, Cambridge, MA), 1:1,000, and goat polyclonal anti-cIAP-1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:200, respectively. Membranes were subsequently incubated with alkaline phosphatase-conjugated secondary anti-rabbit (1:30,000; Sigma-Aldrich) and anti-goat (1:5,000; Santa Cruz Biotechnology) antibodies, respectively. For the analysis of cleaved caspase-8 and 3, NF-κB p65 and $FLIP_L$, the Vectastain ABC-AmP immunodetection kit (Vector Laboratories) was used, according to the manufacturer's instructions. The following primary antibodies and relative dilutions were used: rabbit polyclonal anti-cleaved caspase-8 (Abcam), 1:1,000; rabbit monoclonal anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), 1:1,000; mouse monoclonal anti-NF-κB p65 (Santa Cruz Biotechnology), 1:200; rabbit polyclonal anti-$FLIP_L$ (Santa Cruz Biotechnology), 1:200. Generation of the chemiluminescent signal, digital acquisition and densitometry analysis were performed as previously described (Marzetti et al. 2008b). Spot density of the target bands was normalized to the amount of protein loaded in each lane, as determined by densitometric analysis of the corresponding Ponceau S-stained membranes.

Multiplexed immunoassay for cytokine quantification

Gastrocnemius protein levels of IL-15 and TNF-α were determined via a multiplexed, bead-based immunoassay (Bio-Rad). This assay allows for the simultaneous quantification of multiple analytes by a dual-laser fluorescent microsphere detection system (Bio-Rad). Briefly, ~50 mg of frozen tissue were pulverized using a dry ice-chilled BioPulverizer (BioSpec Products, Bartlesville, OK). The powder was suspended in 500 μL Bio-Plex Lysis solution with protease inhibitors (Bio-Rad), frozen at -80°C, thawed on ice and sonicated 20 times (duty cycle=40, output=4). Debris and lipid were removed by centrifugation at 20,800×g for 30 min at 4°C. The resulting supernatant was collected and stored at -80°C. Protein concentration was determined by the BCA Protein Assay (Pierce Biotechnology), and protein content normalized to 3 mg/ml. Concentration of TNF-α and IL-15 was determined by using rat TNF-α and mouse IL-15 assay kits (Bio-Rad), respectively. The use of the mouse IL-15 assay for detection of rat IL-15 had been previously validated with recombinant rat IL-15 (Cell Sciences, Canton, MA), yielding R^2=0.9985 (data not shown). Standard curves for each analyte were run concurrently for all the assays, and all samples were run in triplicate. Intra- and inter-assay coefficients of variation were less than 20% and 30%, respectively. Cross-reactivity between the cytokines was negligible, as indicated by the absence of fluorescence above the background when using the BioRad Mouse Cytokine II standard dilutions (which includes recombinant mouse IL-15) with the rat TNF-α system (BioRad antibody-conjugated beads and detection antibody). Additionally, no fluorescence above the background was detected with the BioRad Rat Cytokine standard dilutions (which includes recombinant rat TNF-α) when used with the mouse IL-15 system (BioRad antibody-conjugated beads and detection antibody). Concentration of both analytes is expressed as pg/mg protein.

Cell death ELISA for quantification of DNA fragmentation

The extent of apoptotic DNA fragmentation was quantified in the gastrocnemius muscle by measuring the amount of cytosolic mono- and oligonucleosomes (180 base pair nucleotides or multiples) using an ELISA kit (Roche Diagnostics, Mannheim, Germany). This assay relies on the quantification of histone-complexed fragmented DNA. Although the kit does not allow for the discrimination between apoptotic vs. necrotic cell death in cytosolic extracts, occurrence of significant necrosis in skeletal muscle during normal aging has not been reported. Therefore, the impact of necrotic cell death in our system may be considered negligible. Absorbance was determined at 405 nm using a Synergy HT Multi-Detection microplate reader (BioTek, Winooski, VT) and reported as arbitrary OD units/mg cytosolic protein (apoptotic index).

Quantitative Polymerase Chain Reaction (Q-PCR)

To determine the relative gene expression of TNF-α, IL-15, γ_c, IL-2Rβ and IL-15Rα, Q-PCR analysis was performed. Total RNA was isolated and DNase-treated as previously described (Marzetti et al. 2008a). First strand cDNA synthesis was achieved from 2 μg of RNA using the MasterScript kit (5 Prime, Hamburg, Germany). Q-PCR was performed using an Applied Biosystems 7300 Real-Time PCR System (ABI, Foster City, CA). Amplification of cDNA was achieved using predesigned primers and probes (ABI, Table 1), employing ABI universal cycling conditions. All samples were examined in triplicate, with the young AL group as a calibrator. For all genes, negative controls (i.e., no template and no reverse transcriptase) were also included. Differences in the expression of the target genes were determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) with β-actin as housekeeping gene, the expression of which did not change among the experimental groups (data not shown).

Statistical analysis

Statistical analysis was performed using GraphPrism 4.0.3 software (GraphPad Software, San Diego, CA). The experiments were fully crossed, two-factor designs, with four levels of age (8, 18, 29 and 37 months) and 2 levels of diet (AL and CR). The two-way analysis of variance (ANOVA) allowed for the distinction between age and diet effects as well as the determination of a possible interaction between the two factors. In cases where no interaction effect was observed, we reported whether there was a main age effect (independent of diet) and/or main diet effect (independent of age). When applicable, Bonferroni's post-hoc test was performed. Linear regression analyses were performed to explore correlations between variables. All tests were two-sided, with significance set at $p<0.05$. All data are reported as mean ± SEM.

Results

Morphological characteristics

Body weight—Within each age group, CR rats weighed less than their AL counterparts (diet-effect: $p<0.0001$; Figure 1a). However, the trajectory of changes in body weight (BW) over time was different between diet groups (age×diet interaction: $p<0.0001$). In AL rats, BW increased from 8 to 29 months and declined thereafter (age-effect: $p<0.0001$). In contrast, in the CR group, BW remained relatively stable, with the exception of a small increase between 18 and 29 months.

Muscle weight—Gastrocnemius muscle absolute wet weight (MW) declined at advanced age, indicative of sarcopenia (age-effect: $p<0.0001$; Figure 1b). However, changes in MW with age were different in the two diet groups (age×diet interaction: $p<0.0001$). In AL rats, MW increased between 8 and 18 months, and declined thereafter. In contrast, in the CR group, MW remained unchanged until senescence, when a marked decrease was observed. Overall, CR animals displayed a lower MW in comparison to their AL counterparts (diet-effect: $p<0.0001$),

except for the oldest animals, where no differences were detected. However, when MW was normalized to BW (MW/BW ratio), CR rats displayed a higher ratio relative to their AL controls in all age groups (diet-effect: $p<0.0001$; Figure 1c). MW/BW decreased over the course of aging in both AL and CR rats (age-effect: $p<0.0001$). However, AL animals displayed a steeper decline than their CR counterparts (age×diet interaction: $p<0.0001$). Overall, these data confirm and extend previous findings in this rodent model of aging demonstrating that CR attenuates sarcopenia (Mayhew et al. 1998;You et al. 2007).

Expression of IL-15 and IL-15 receptor subunits

Gastrocnemius protein and gene expression levels of IL-15 were analyzed to determine age and diet effects. Additionally, gene expression of the trimeric receptor subunits of IL-15 was determined.

IL-15—mRNA levels of IL-15 did not change among groups (Table 2). However, protein levels of IL-15 declined with age in AL rats (age-effect: $p<0.001$; Figure 2a), but were not significantly changed in CR animals.

IL-15 receptor subunits—Gastrocnemius γ_c and IL-2Rβ mRNA expression was unchanged among the experimental groups (Table 2). However, an age×diet interaction was detected for IL-15Rα mRNA expression ($p=0.02$; Figure 2b). Specifically, we observed a progressive age-dependent decrease in IL-15Rα mRNA levels in the AL group that was significant at 37 months (age-effect: $p=0.01$). This decline was prevented by CR (diet-effect: $p=0.04$).

Expression of markers within the death receptor-mediated apoptosis

In order to investigate the effects of age and diet on the extrinsic pathway of apoptosis, muscle TNF-α mRNA and protein levels were evaluated by Q-PCR and the multiplexed immunoassay, respectively. Western blot analysis was performed to quantify protein expression of TNF-R1, cleaved caspase-8 and cleaved caspase-3.

TNF-α—TNF-α mRNA expression was unaffected by age and diet (Table 2). In contrast, TNF-α protein levels increased with advancing age (age-effect: $p<0.0001$; Figure 3a). However, this increase was attenuated in CR animals (diet-effect: $p=0.02$). No significant age×diet interaction was detected ($p=0.26$), possibly as a consequence of the small number of rats analyzed (5-6 per group).

TNF-R1—An age×diet interaction ($p<0.0001$) was observed. Specifically, in AL rats, expression of TNF-R1 increased at 29 months compared to younger animals, and even further at 37 months (age-effect: $p<0.0001$; Figure 3b). Conversely, expression of TNF-R1 remained unchanged in CR rats.

Cleaved caspase-8—Muscle content of cleaved caspase-8 increased with advancing age (age-effect: $p<0.0001$; Figure 3c). However, there was an age×diet interaction ($p<0.0001$), reflective of a different pattern of changes over time between diet groups. In fact, in AL rats, levels of cleaved caspase-8 increased at 29 months compared to 8- and 18-month-old animals, with a further increase at 37 months. Conversely, in CR animals levels of cleaved caspase-8 were not elevated until senescence.

Cleaved caspase-3—Similar to cleaved caspase-8, cleaved caspase-3 levels displayed a different trajectory over the course of aging in AL and CR rats (age×diet interaction: $p<0.0001$; Figure 3d). Specifically, in the AL group, levels of cleaved caspase-3 increased with advancing age, starting at 29 months (age-effect: $p<0.0001$). Conversely, no changes were evident in CR animals across the age groups.

Activation of NF-κB and tissue levels of cIAP-1/2 and $FLIP_L$

It was recently reported that, upon binding to IL-15, IL-15Rα induced NF-κB activation in cultured fibroblasts (Bulfone-Paus at al. 1999). Activation of NF-κB may result in enhanced transcription of anti-apoptotic genes such as cIAPs and $FLIP_L$ (Wang et al. 1998;Micheau et al. 2001). In order to evaluate the impact of age and CR on NF-κB activation, nuclear levels of p65, a subunit of the NF-κB heterodimer, were determined by Western blot analysis. Furthermore, muscle tissue levels of anti-apoptotic cIAP-1/2 and $FLIP_L$ were quantified.

Nuclear p65—A progressive age-dependent increase in nuclear p65 levels was evident (age-effect: $p<0.0001$; Figure 4a). However, nuclear p65 content was not correlated with either IL-15 protein levels (R^2: 0.04, $p=0.18$; data not shown) or IL-15Rα gene expression (R^2: 0.01, $p=0.52$; data not shown).

cIAP-1/2 and $FLIP_L$—Gastrocnemius cIAP-1/2 content remained unchanged over the course of aging (Figure 4b). However, CR rats displayed higher levels compared to their AL controls (diet-effect: $p=0.0006$). In contrast, content of $FLIP_L$ did not change across age and diet groups (Figure 4c).

Apoptotic index

Internucleosomal DNA fragmentation represents a hallmark of apoptotic cell death. A sandwich ELISA was employed to quantify the amount of cytosolic mono- and oligonucleosomes. Values for the AL rats were previously published (Marzetti et al. 2008b). Data from CR rats, which were collected simultaneously, are presented here. As reported earlier (Marzetti et al. 2008b), apoptotic cell death was elevated at 29 months of age in AL rats compared to younger animals, with a further increase at 37 months (age-effect: $p<0.0001$; Figure 5). However, as a result of an age×diet interaction ($p=0.001$), apoptosis was not enhanced in CR animals until 37 months. The age-associated increase in DNA fragmentation was attenuated by CR (diet-effect: $p<0.0001$).

Regression analysis to explore the relationship among TNF-α, IL-15, apoptosis and gastrocnemius muscle atrophy

Regression analyses were performed to explore the hypothesis that TNF-α promoted apoptosis and muscle atrophy, whereas IL-15 partly mediated CR protection against muscle loss. First, the ratio between protein levels of TNF-α and IL-15 (TNF-α/IL-15) was calculated and its changes among groups evaluated. TNF-α/IL-15 increased at advanced age in AL, but not in CR rats (age-effect: $p<0.0001$; diet-effect: $p=0.004$; age×diet interaction: $p=0.001$; Figure 6). TNF-α/IL-15 was then used as the independent variable in regression analyses. A positive correlation was observed between TNF-α/IL-15 and the apoptotic index (R^2: 0.41, $p<0.0001$; Figure 7a). Additionally, a negative correlation was detected between TNF-α/IL-15 and muscle weight (R^2: 0.44, $p<0.0001$; Figure 7b). For this particular analysis, muscle weight in each dietary regimen was normalized to the average of their respective 8-month-old group, whose muscle weight was therefore considered as 100%. This procedure was adopted to minimize the possible confounding effect of differences in body weight between the two dietary groups. Finally, a negative correlation was evident between the apoptotic index and muscle weight expressed as described above (R^2: 0.51, $p<0.0001$; Figure 7c). To ensure that the regressions were not solely driven by the 37-month-old AL group, the same analyses were run after excluding the senescent AL rats. TNF-α/IL-15 remained significantly correlated with both the apoptotic index (R^2: 0.20, $p=0.004$) and muscle weight (R^2: 0.12, $p<0.05$). Likewise, a significant negative correlation persisted between the apoptotic index and muscle weight (R^2: 0.26, $p<0.0001$).

Discussion

Chronic low-grade systemic inflammation has been indicated as a possible contributing factor to the development of sarcopenia (Roubenoff 2003). Among the pro-inflammatory cytokines, TNF-α has been suggested to be involved in the pathogenesis of age-related muscle atrophy (Visser et al. 2002), owing to its ability to promote muscle protein wasting (Llovera et al. 1993;Garcia-Martinez et al. 1993) and apoptosis (Carbo et al. 2002;Phillips and Leeuwenburgh 2005;Pistilli et al. 2006a;Marzetti et al. 2008a). On the contrary, IL-15, a cytokine highly expressed in skeletal muscle, inhibits muscle protein breakdown in various experimental models (Carbo et al. 2000;Quinn et al. 2002;Busquets et al. 2005) and mitigates apoptotic DNA fragmentation in tumor-bearing rats (Figueras et al. 2004). These actions have been attributed to the ability of IL-15 to counteract TNF-α signaling (Bulfone-Paus et al. 1997, 1999; Hiromatsu et al. 2003;Figueras et al. 2004).

In the present study, advanced age was associated with reduced protein expression of IL-15 and mRNA levels of IL-15Rα in rat gastrocnemius muscle. Enhanced TNF-α apoptotic signaling was also observed in old and senescent AL animals. In contrast, attenuation of sarcopenia and TNF-α apoptotic signaling in CR rats was accompanied by sustained protein levels of IL-15 and mRNA abundance of its receptor through senescence. Our hypothesis that the dynamic interaction between IL-15 and TNF-α might be relevant to apoptosis and muscle atrophy was supported by a positive correlation between the TNF-α to IL-15 ratio (TNF-α/IL-15) and the apoptotic index (R^2: 0.41; $p<0.0001$; Figure 7a). Additionally, a negative and stronger correlation was detected between TNF-α/IL-15 and muscle weight (R^2: 0.44; $p<0.0001$; Figure 7b). This observation highlights the multifaceted interaction between TNF-α and IL-15, which may encompass the regulation of apoptosis as well as muscle protein turnover. Based on these findings, it might be hypothesized that as a result of an age-related imbalance between TNF-α and IL-15, the loss of protein may be accelerated compared to the extent of apoptosis. Indeed, the relative magnitude of these processes in the context of sarcopenia is still unclear, but initiation of apoptosis appears to be required for the execution of muscle proteolysis (Argiles et al. 2008). However, changes in protein synthesis and degradation were not assessed in the present investigation. Additionally, the correlation between the apoptotic index and muscle weight was stronger (R^2: 0.51, $p<0.0001$; Figure 7c) than observed between either TNF-α/IL-15 vs. muscle weight or TNF-α/IL-15 vs. the apoptotic index. This may indicate the existence of multiple apoptotic pathways operating simultaneously in aged skeletal muscle. However, the relative impact of each of these pathways in the pathogenesis of sarcopenia will require further investigation. Furthermore, in 29-month-old AL rats, elevation of the apoptotic index and activation of the death receptor signaling pathway were not accompanied by a significant increase in TNF-α/IL-15. It is possible that small changes in the levels of those cytokines had occurred, but were not detected by our multiplexed analysis. Alternatively, it may be hypothesized that production of inhibitory isoforms of IL-15Rα (Bulanova et al. 2007) might have taken place over the course of aging, resulting in a partial loss of IL-15 activity, in spite of its unaltered levels. However, our Q-PCR analysis was limited to total IL-15Rα gene expression and therefore we were unable to test this hypothesis.

With regard to the mechanisms whereby IL-15 may inhibit apoptosis, it has been shown that IL-15Rα can compete with TNF-R1 for DISC assembly (Bulfone-Paus et al. 1999), thus preventing the activation of the caspase cascade. Additionally, binding of IL-15 to IL-15Rα induced NF-κB activation via phosphorylation and subsequent degradation of the inhibitory subunit I-κBα (Bulfone-Paus et al. 1999). Notably, NF-κB can promote the expression of anti-apoptotic genes such as cIAPs and $FLIP_L$ (Wang et al. 1998;Micheau et al 2001). However, results from our study do not support the involvement of NF-κB activation as a protective mechanism against age-related skeletal muscle loss. In fact, nuclear levels of p65, a subunit of

the NF-κB heterodimer were not correlated with either protein content of IL-15 or IL-15Rα gene expression. In addition, content of $FLIP_L$ and cIAP-1/2 remained unchanged over the course of aging. The discrepancies between our study and findings from Bulfone-Paus et al. (1999) can be explained by the different experimental models used (i.e., gastrocnemius muscle vs. cultured fibrosarcoma cells). In addition, it is possible that deflection of TNF-α signaling toward NF-κB might represent an acute anti-apoptotic response induced by IL-15. In this context, the finding by Pistilli et al. (2007) of increased IL-15 mRNA abundance following muscle unloading may be interpreted as an acute anti-apoptotic adaptation. In contrast, life-long CR in our study could have induced a chronic IL-15 stimulation, which may have resulted in downregulation of TNF-R1 expression and subsequent mitigation of the extrinsic pathway of apoptosis. The occurrence of such an adaptation was indeed reported by Figueras et al. (2004) following one week of IL-15 administration to tumor-bearing rats.

Elucidation of the actions of IL-15 on skeletal muscle is complicated by the existence of several splice variants of both the cytokine and IL-15Rα (Quinn 2008). Furthermore, translation of IL-15 mRNA is intrinsically inefficient, which may explain the lack of correlation reported between its mRNA and protein levels (Tagaya et al. 1996, 1997;Meazza et al. 1997;Riechman et al. 2004), which was also apparent in our study. Furthermore, it was demonstrated that co-expression of IL-15 and IL-15Rα resulted in increased stability of the two molecules (Bergamaschi et al. 2008). It may therefore be hypothesized that the age-related decrease in IL-15Rα expression observed in senescent AL rats might have resulted in a decline in IL-15 protein levels without corresponding changes in mRNA abundance. Taken together, our findings confirm that IL-15 protein levels cannot be inferred from assays of IL-15 mRNA expression.

Based on our results and the current literature, it might be hypothesized that preservation of IL-15 signaling late in life may counteract sarcopenia at least partly by mitigating the death receptor-mediated pathway of apoptosis (Figure 8). Specifically, chronic IL-15 stimulation may result in downregulation of TNF-R1 and subsequent prevention of TNF-α-mediated apoptosis. Sustained expression of IL-15Rα appears to be required for IL-15 signal stabilization (Bergamaschi et al. 2008). In addition to participating in the heterotrimeric signaling complex, IL-15Rα can also appear on the cell surface independently of the γ_c/IL-2Rβ. In this case, IL-15Rα can present IL-15 to adjacent cells (and perhaps flanking regions of a muscle fiber) expressing the γ_c/IL-2Rβ heterodimer in a juxtacrine mode of action (Budagian et al. 2006;Bulfone-Paus et al. 2006). Moreover, IL-15Rα can also exist in a soluble form (sIL-15Rα), which appears to be important for secretion, stabilization, and activity of IL-15 (Bergamaschi et al. 2008). A role for membrane-associated or soluble IL-15/IL-15Rα complexes is equally consistent with our data, and compatible with the hypothesis that decreased expression of IL-15Rα in muscle tissue during aging may be involved in increased apoptosis and sarcopenia.

Other aspects of our study deserve further discussion. First of all, we conducted our analyses on a predominantly type II muscle. Therefore, our findings may not be generalized to predominantly slow-myosin-containing muscles. Additionally, the increased extent of apoptosis observed in the gastrocnemius muscle of senescent CR rats (Figure 5), albeit much less severe as compared to the AL animals, was not accompanied by caspase-3 cleavage (Figure 3d). This may suggest the involvement of caspase-independent apoptogenic mediators such as apoptosis inducing factor (AIF) and endonuclease G (Marzetti et al. 2008c). Furthermore, in the 37-month-old CR rats, caspase-8 cleavage was not coupled with elevations in cleaved caspase-3. $FLIP_L$ (Micheau et al. 2002) and c-IAPs (Wang et al. 1998) have been shown to inhibit caspase-8 activity. However, expressions levels of those proteins were not reduced in senescent CR rats as compared to younger pair-fed animals. Alternatively, inhibition of caspase-8 activity may be attributable to the apoptosis repressor with caspase recruitment

domain (ARC) (Koseki et al. 1998), whose cytosolic levels were increased in the gastrocnemius muscle of old CR F344 rats compared to AL controls (Dirks and Leeuwenburgh 2004). Finally, the assay employed to determine apoptosis provided a comprehensive quantification of DNA fragmentation. Therefore, it cannot be excluded that apoptosis in other cell types (e.g., endothelial and interstitial cells) may have contributed to the apoptotic index. However, studies employing TUNEL staining have confirmed that the extent of apoptosis is indeed elevated in skeletal muscles of old F344×BNF1 rats (Leeuwenburgh et al. 2005;Rice and Blough 2006;Pistilli et al. 2006b).

Conclusions

Results from our study indicate that production of IL-15 and expression of IL-15Rα in gastrocnemius muscle decrease over the course of aging, which might contribute to the loss of muscle mass. Furthermore, our findings suggest that preservation of IL-15 signaling late in life may represent an additional means underlying the protective effect of CR against age-related muscle loss, possibly via downregulation of TNF-α-mediated apoptosis.

Unexpectedly, in a very recent study, continuous infusion of human IL-15 into aging rats for two weeks appeared to stimulate, rather than suppress, muscle apoptosis (Pistilli and Alway 2008). It is possible that heterologous IL-15 may have functioned as an IL-15 antagonist in the rat system, or that continuous infusion of IL-15 further downregulated IL-15Rα in that study (Kumaki et al. 1996). Additionally, it may be hypothesized that, with reduced signaling or stabilization conferred by IL-15Rα in old rats (Rubinstein et al. 2006;Bergamaschi et al. 2008), IL-15 infusion had paradoxical effects on apoptosis. These conflicting findings underlie the importance of examining expression of all components of the IL-15 signaling apparatus, including IL-15 and its three receptor subunits. Clearly, further research is required to elucidate the complexity of IL-15 activity in aged skeletal muscle and to test whether manipulation of IL-15 signaling may serve as a novel strategy to restore muscle mass and function at old age.

Acknowledgments

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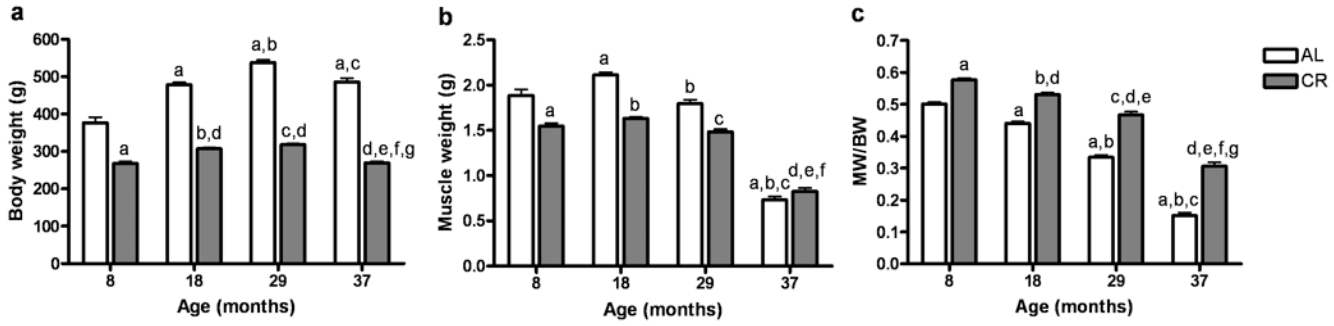

Figure 1.

Body weight (a), gastrocnemius absolute wet weight (b) and gastrocnemius muscle weight to body weight ratio (MW/BW, c) of 8, 18, 29 and 37-month-old Fischer344×Brown Norway rats. Letters indicate significant differences ($p<0.05$) within age groups, and between AL and CR rats of the same age. [a] significantly different from 8-mo AL; [b] significantly different from 18-mo AL; [c] significantly different from 29-mo AL; [d] significantly different from 8-mo CR; [e] significantly different from 18-mo CR; [f] significantly different from 29-mo CR; [g] significantly different from 37-mo AL. Values are mean ± SEM (n=9/group).

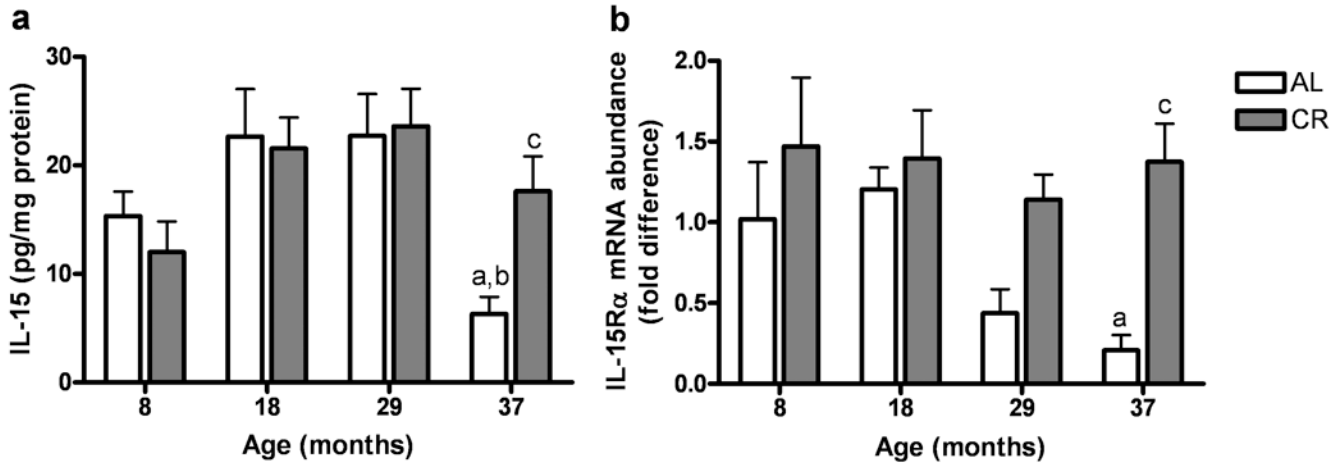

Figure 2.

Muscle expression of IL-15 and IL-15Rα. Gastrocnemius protein content of IL-15 (a) decreased with aging in AL, but not in CR rats (age: p=0.0008; diet=0.39; interaction: p=0.11). Advanced age was also associated with reduced gene expression levels of IL-15Rα (b) which was prevented by CR (age: p=0.01; diet: p=0.04; interaction: p=0.02). Letters indicate significant differences ($p<0.05$) within age groups, and between AL and CR rats of the same age. [a] significantly different from 18-mo AL; [b] significantly different from 29-mo AL; [c] significantly different from 37-mo AL. Values are mean ± SEM (n=5-7/group).

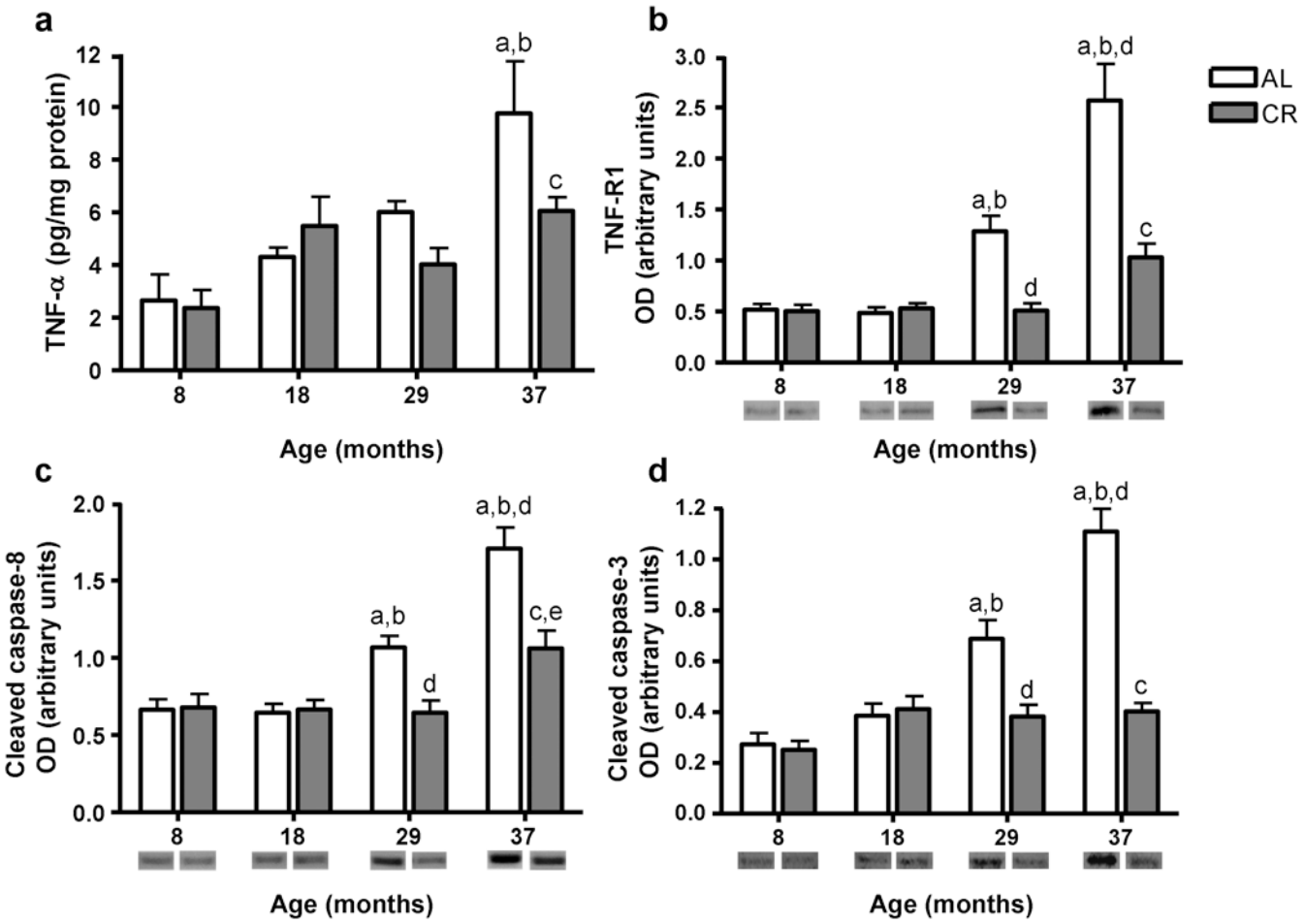

Figure 3.

Analysis of components of the TNF-α-mediated pathway of apoptosis. Gastrocnemius protein content of TNF-α (a) displayed an age-dependent increase, which was attenuated by CR (age: $p<0.0001$; diet=0.02; interaction: $p=0.26$). Content of TNF-R1 (b) increased with advancing age only in AL rats (age: $p<0.0001$; diet: $p<0.0001$: interaction: $p<0.0001$). Levels of cleaved caspase-8 (c) rose with aging in the AL group, starting at 29 months, whereas CR rats displayed an increase only during senescence (age: $p<0.0001$; diet: $p<0.0001$: interaction: $p<0.0001$). Finally, an age-dependent increase in cleaved caspase-3 content (d) was observed in AL rats, which was prevented by CR (age: $p<0.0001$; diet: $p<0.0001$: interaction: $p<0.0001$). Letters indicate significant differences ($p<0.05$) within age groups, and between AL and CR rats of the same age. [a] significantly different from 8-mo AL; [b] significantly different from 18-mo AL; [c] significantly different from 37-mo AL; [d] significantly different from 29-mo AL; [e] significantly different from 29-mo CR. Representative blots are shown for TNF-R1, cleaved caspase-8 and -3. Values are mean ± SEM (n=6-9/group).

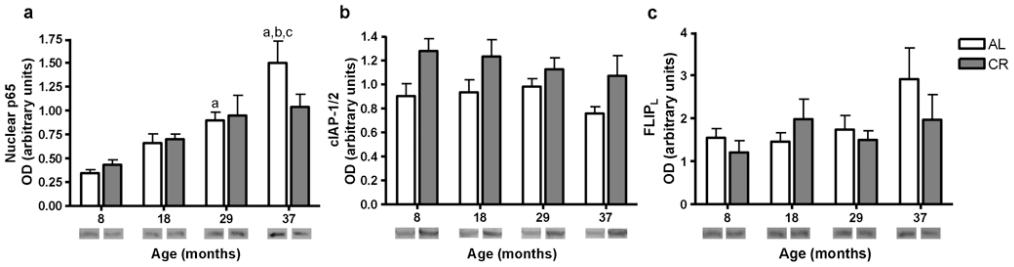

Figure 4.

Muscle levels of nuclear NF-κB and anti-apoptotic proteins cIAP-1/2 and $FLIP_L$. Gastrocnemius nuclear levels of NF-κB subunit p65 (a) displayed a progressive age-dependent increase (age: $p<0.0001$; diet: $p=0.45$; interaction: $p= 0.15$). Levels of cIAP-1/2 (b) were higher in CR rats than in their AL counterparts, regardless of age (age: $p=0.38$; diet: $p=0.0006$; interaction: $p=0.73$). Muscle content of $FLIP_L$ (c) did not change across age or diet groups. Letters indicate significant differences ($p<0.05$) within age groups. [a] significantly different from 8-mo AL; [b] significantly different from 18-mo AL; [c] significantly different from 29-mo AL. Representative blots are shown. Values are mean ± SEM (n=7-9/group).

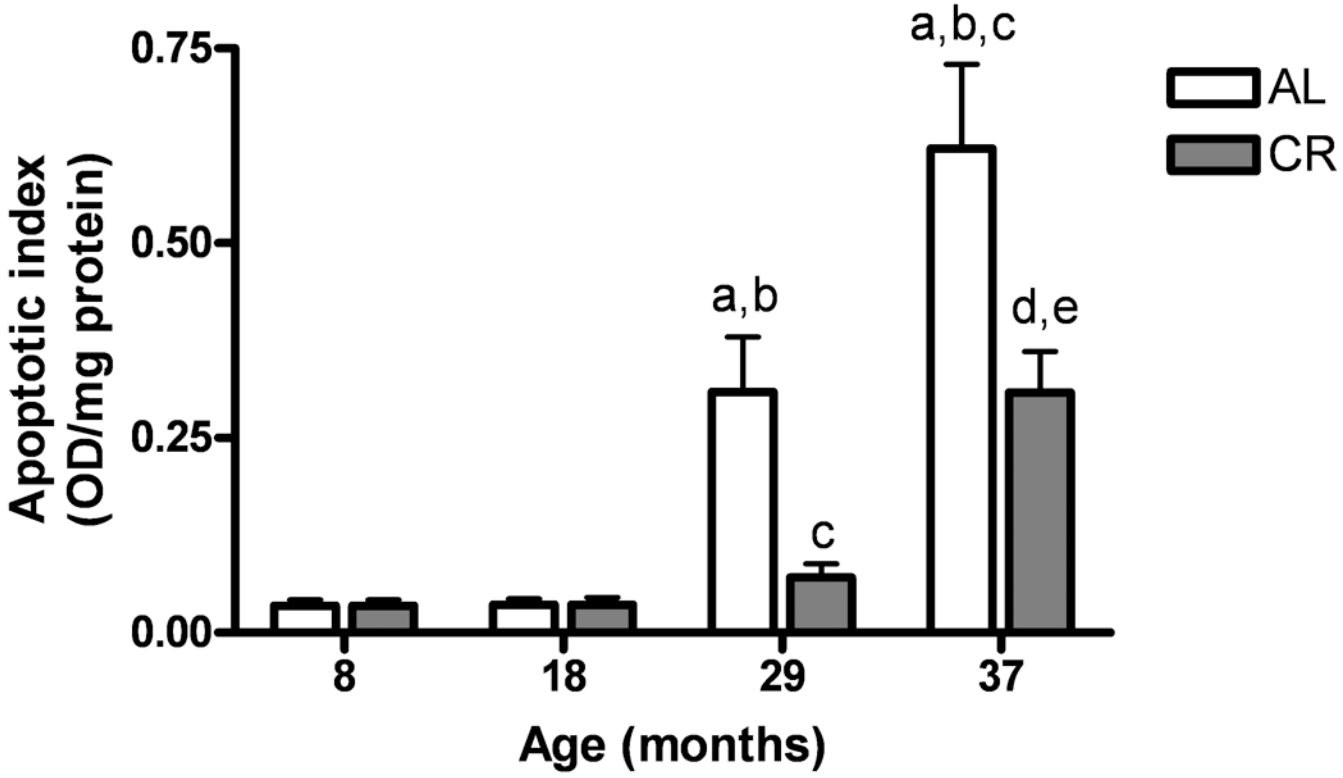

Figure 5.

Gastrocnemius apoptotic index. Apoptotic DNA fragmentation increased with advancing age in AL rats starting at 29 months, whereas CR rats displayed an increase only at 37 months (age: $p<0.0001$; diet: $p<0.0001$: interaction: $p=0.001$). Letters indicate significant differences ($p<0.05$) within age groups, and between AL and CR rats of the same age. [a] significantly different from 8-mo AL; [b] significantly different from 18-mo AL; [c] significantly different from 29-mo AL; [d] significantly different from 29-mo CR; [e] significantly different from 37-mo AL. Values are mean ± SEM (n=7-9/group).

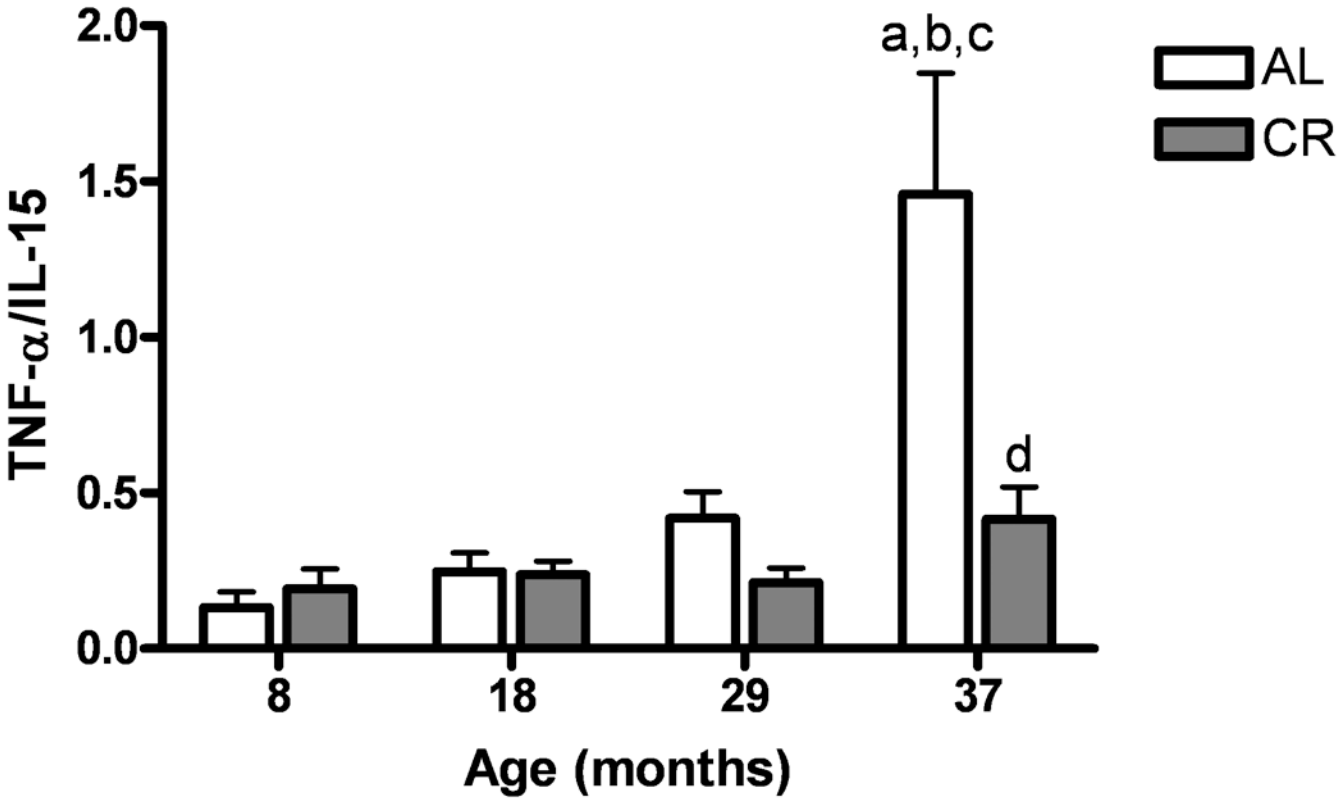

Figure 6.

An age-dependent increase in TNF-α to IL-15 ratio was detected, which was prevented by CR (age: $p<0.0001$; diet: $p=0.004$; interaction: $p=0.001$). Letters indicate significant differences ($p<0.05$) within age groups, and between AL and CR rats of the same age. [a] significantly different from 8-mo AL; [b] significantly different from 18-mo AL; [c] significantly different from 29-mo AL; [d] significantly different from 37-mo AL. Values are mean ± SEM (n=5-6/group).

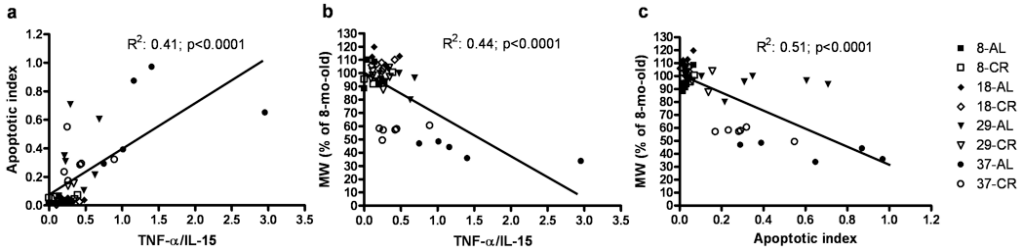

Figure 7.

Linear regression analysis revealed a negative correlation between TNF-α/IL-15 and muscle weight (MW) expressed as percentage of 8-month-old rats in the two dietary groups (a). A positive correlation was also detected between TNF-α/IL-15 and the apoptotic index (b). Finally, a negative correlation was evidenced between the apoptotic index and MW (c).

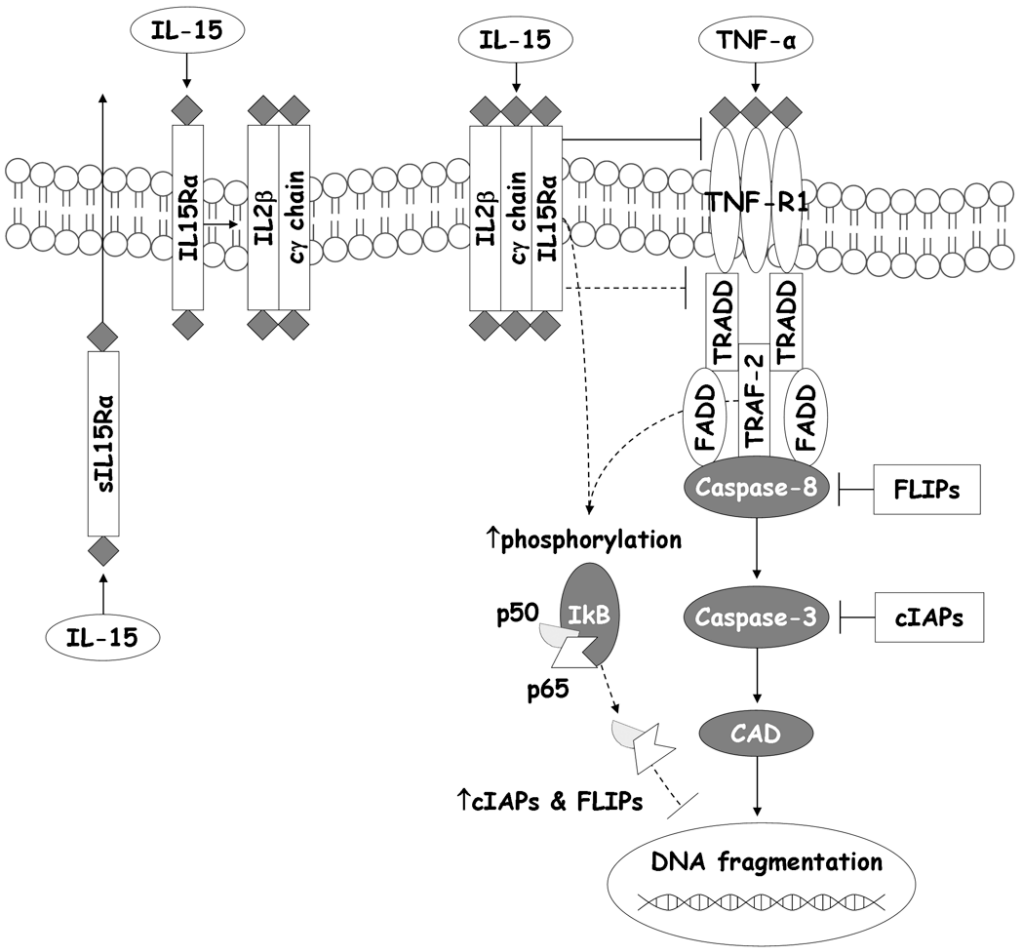

Figure 8.

Proposed interplay between IL-15 and TNF-α signaling in skeletal muscle. Binding of TNF-α to TNF-R1 triggers the extrinsic pathway of apoptosis. Chronic IL-15 stimulation may result in downregulation of TNF-R1 and subsequent prevention of TNF-α-mediated apoptosis (solid line). Actions of IL-15 may be transduced by its cell surface trimeric receptor. IL-15 may also interact with IL-15Rα on the plasma membrane and be presented to adjacent cells (or portions of a muscle fiber) expressing the γ_c/IL-2Rβ heterodimer. Soluble IL-15Rα (sIL-15Rα) may be important for secretion, stabilization and activity of IL-15. Additionally, in the presence of an acute apoptotic stimulus, IL-15Rα might counteract DISC assembly and promote the production of anti-apoptotic proteins such as FLIPs and cIAPs via NF-κB activation (dashed lines).

Table 1

Accession and catalogue numbers of the investigated genes. Amplicon length is also reported.

Gene	Accession number	Catalogue number	Amplicon length
TNF-α	NM_012675	Rn99999017_m1	108
IL-15	NM_013129	Rn00565548_m1	63
Common γ_c	NM_080889	Rn01752908_g1	86
IL-2Rβ	NM_013195	Rn00566178_m1	82
IL15Rα	DQ157696.1	Rn01487180_m1	83
β-actin	NM_031144	Rn00667869_m1	91

Table 2

Gene expression of IL-15, common γ_c, IL-2Rβ and TNF-α in gastrocnemius muscle, as determined by Q-PCR analysis. No significant differences were detected for any of the genes in either age or diet groups. Data are mean ± SEM (n=5-7/group).

	8-mo AL	8-mo CR	18-mo AL	18-mo CR	29-mo AL	29-mo CR	37-mo AL	37-mo CR
IL-15	0.90±0.17	0.51±0.16	1.11±0.33	0.52±0.17	0.56±0.12	1.18±0.29	0.47±0.17	0.45±0.12
Common γ_c	1.07±0.27	0.89±0.31	1.26±0.07	0.66±0.15	1.19±0.34	1.47±0.87	1.12±0.47	0.64±0.21
IL-2Rβ	1.02±0.09	1.40±0.39	2.98±1.11	2.39±1.11	3.69±1.48	4.65±2.30	2.63±1.03	3.94±0.89
TNF-α	1.09±0.21	0.27±0.06	1.17±0.52	0.26±0.09	0.51±0.15	1.18±0.47	1.59±0.72	0.97±0.42