Biochemical and Biophysical Research Communications 373 (2008) 20–24

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Systemic elevation of interleukin-15 *in vivo* promotes apoptosis in skeletal muscles of young adult and aged rats

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ARTICLE INFO

Article history:
Received 21 May 2008
Available online 12 June 2008

Keywords:
Interleukins
Aging
Sarcopenia
Apoptosis

ABSTRACT

In this study, we tested the hypothesis that systemic elevation of IL-15 would attenuate apoptosis in skeletal muscles of aged rats. IL-15 was administered to young adult ($n = 6$) and aged ($n = 6$) rats for 14 days. Apoptosis was quantified using an ELISA assay and verified through TUNEL staining of muscle sections. As expected, apoptosis was greater in muscles from aged control rats, compared to age-matched control. Apoptosis was also greater in the muscles from young adult and aged rats treated with IL-15. These increases in apoptosis were associated with decreases in muscle mass of IL-15 treated rats. These data do not support our initial hypothesis and suggest that systemic elevation of IL-15 promotes apoptosis in skeletal muscle. The proposed anti-apoptotic property of IL-15 may be specific to cell-type and/or the degree of muscle pathology present; however, additional research is required to more clearly decipher its role in skeletal muscle.

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Interleukin-15 (IL-15) is a pleiotropic cytokine with numerous proposed roles in both lymphoid and non-lymphoid tissues (reviewed in [1]). Skeletal muscle expresses mRNAs for both IL-15 [2] and the three components of the IL-15 receptor (IL-15R: IL-15Rα, IL-2R/IL-15Rβ, γc) [3]. While conditions that promote muscle atrophy (i.e. aging, disuse) have been shown to alter IL-15 mRNA concentrations within skeletal muscle [3], the role that the IL-15/IL-15R axis plays in skeletal muscle remains not fully elucidated. Production of mature IL-15 protein is tightly regulated at the levels of protein translation and vesicle trafficking, explaining in part, why mature IL-15 protein has been difficult to measure in biological fluids [4]. Recent evidence suggests that the predominant mode of action of IL-15 occurs when a complex is formed between IL-15 and IL-15Rα within the cell, which is then transported and either anchored to the cell membrane or secreted. In this manner, IL-15 can be presented at the cell surface and interact with cells expressing the IL-2/IL-15R β and γc chains (i.e. trans presentation) as well as cause reverse signaling through soluble IL-15Rα [5].

In an effort to decipher specific roles of IL-15, cytokine concentrations have been experimentally modulated both *in vitro* and *in vivo* [6–11]. One consistent finding is that IL-15 can act as an apoptosis-inhibitor in response to inflammatory conditions and/or pathological states in lymphoid cells such as CD8+ T-cells [12], non-lymphoid cells such as fibroblasts [7], and tissues such as spleen [6], liver [6], and skeletal muscle [9]. This anti-apoptotic role seems to be protective against the pro-apoptotic property of another cytokine, TNF-α, as well as FasL [6,7,13]. The incidence of apoptotic nuclei within skeletal muscle increases as a result of aging and our laboratory and others, have provided evidence that the extrinsic apoptotic pathway is active in aged skeletal muscles [14,15]. This may be in response to an age-related increase in circulating TNF-α concentrations [16]. Interestingly, experiments have shown that IL-15 can attenuate this signaling pathway *in vitro* in a fibroblast cell line by disrupting the activation of caspase-8 at the cytoplasmic portion of the type I TNFR, providing a possible mechanism for the anti-apoptotic property of IL-15 [7]. In the current experiments, we hypothesized that systemic elevation of IL-15 *in vivo* would attenuate the apoptosis observed within aged skeletal muscles.

Materials and methods

Systemic elevation of IL-15 in vivo. Recombinant human IL-15 was generously provided by Amgen, Inc. (Thousand Oaks, CA) at a concentration of 4.41 mg ml^{-1}. Fischer Brown Norway rats, an approved model for the study of aging related factors [17], were randomly divided into four groups: young adult control (YC, $n = 6$), young adult IL-15 (Y15, $n = 6$), aged control (AC, $n = 6$), and aged IL-15 (A15, $n = 6$). Mini-osmotic pumps were implanted subcutaneously (s.c.; Model #2002, Alzet, Cupertino, CA) and used to deliver IL-15 systemically at a rate of 0.51 μl h^{-1} over a 14 day period. Following the 14 d intervention, blood and tissue samples were collected and stored at −80 °C. All procedures followed the guidelines of the National Institutes of Health, and were approved

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doi:10.1016/j.bbrc.2008.05.188

by the Institutional Animal Care and Use Committee of the West Virginia University School of Medicine.

Enzyme-linked immunosorbant assay (ELISA). Plasma samples were used to verify a systemic elevation of human IL-15 using a sensitive ELISA kit (R&D Systems, Minneapolis, MN) according to manufacture's instructions.

Cell death ELISA and calculation of apoptotic index. Cytoplasmic protein extracts were used to quantify the extent of DNA fragmentation in skeletal muscles using a commercially available ELISA kit according to manufacturer's instructions (Cell Death Detection ELISA, Roche Diagnostics, Mannheim, Germany). The resulting optical density (OD) was normalized to the protein concentration of each sample and recorded as the apoptotic index (OD_{405}·mg protein^{-1}).

In situ TdT-mediated dUTP nick end labeling (TUNEL). Apoptotic nuclei of myogenic origin were visualized using a TdT-mediated dUTP nick end labeling (TUNEL) detection kit (Roche Applied Science, Indianapolis, IN). Cross-sections of the plantaris and soleus were incubated in TUNEL reaction mixture in a humidified chamber at 37 °C for 1 h in the dark. Sections were then incubated in an anti-laminin mouse monoclonal antibody followed by an anti-mouse Texas Red secondary antibody (1:200 dilution, Jackson Immunoresearch Laboratories, Incorporated) and then mounted with 4′,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingham, CA). Images were visualized using an Axiovert 200M fluorescent microscope with apatome at an objective magnification of 40× (Zeiss Micro-Imagining, Thornwood, NY).

Hematoxylin and eosin staining. Muscle histology was accessed using Mayer's hematoxylin (Sigma–Aldrich, St. Louis, MO) and eosin-Y (Sigma–Aldrich, St. Louis, MO). Tissue sections were imaged using an Olympus BX51 stereology microscope at an objective of 40× (Olympus C.A.S.T. Stereology System, Olympus America Inc., Center Valley, PA).

Statistics. Statistical analyses were performed using the SPSS software package, version 10.0. Data are presented as means ± SD. Data were analyzed using a 2 × 2 ANOVA to examine the main effects of age and IL-15 treatment as well as the age × treatment interaction. When appropriate, treatment effects were examined using Student's *t*-test. For all experiments, statistical significance was set at $p < 0.05$.

Results

Systemic elevation of IL-15 in vivo promotes apoptosis in skeletal muscle

Homogenates from the plantaris (Fig. 1A) and soleus (Fig. 1A') skeletal muscles were used to measure changes in apoptosis using a cell death ELISA kit. This assay quantifies fragmented DNA by measuring the cytosolic histone-associated mono- and oligonucleosomes. The presence of fragmented DNA followed a similar pattern of change, with control muscles from aged rats exhibiting greater levels of DNA fragmentation when compared to control muscles from young adult rats, supporting previous data [14,18,19]. Specifically, the apoptotic index was 2.2-fold greater in the aged plantaris and 92% greater in the aged soleus. Unexpectedly, IL-15 treatment exacerbated the apoptotic index in muscles from young adult and aged rats when compared to age-matched controls. In the IL-15 treated young adult rats, the apoptotic index was 79% greater in the plantaris and 1.2-fold greater in the soleus. Similarly, in the IL-15 treated aged rats, the apoptotic index was 1.3-fold greater in the plantaris and 42% greater in the soleus. This pattern of change in the apoptotic index was robust, as similar changes were noted across muscles of differing fiber type composition and activity patterns.

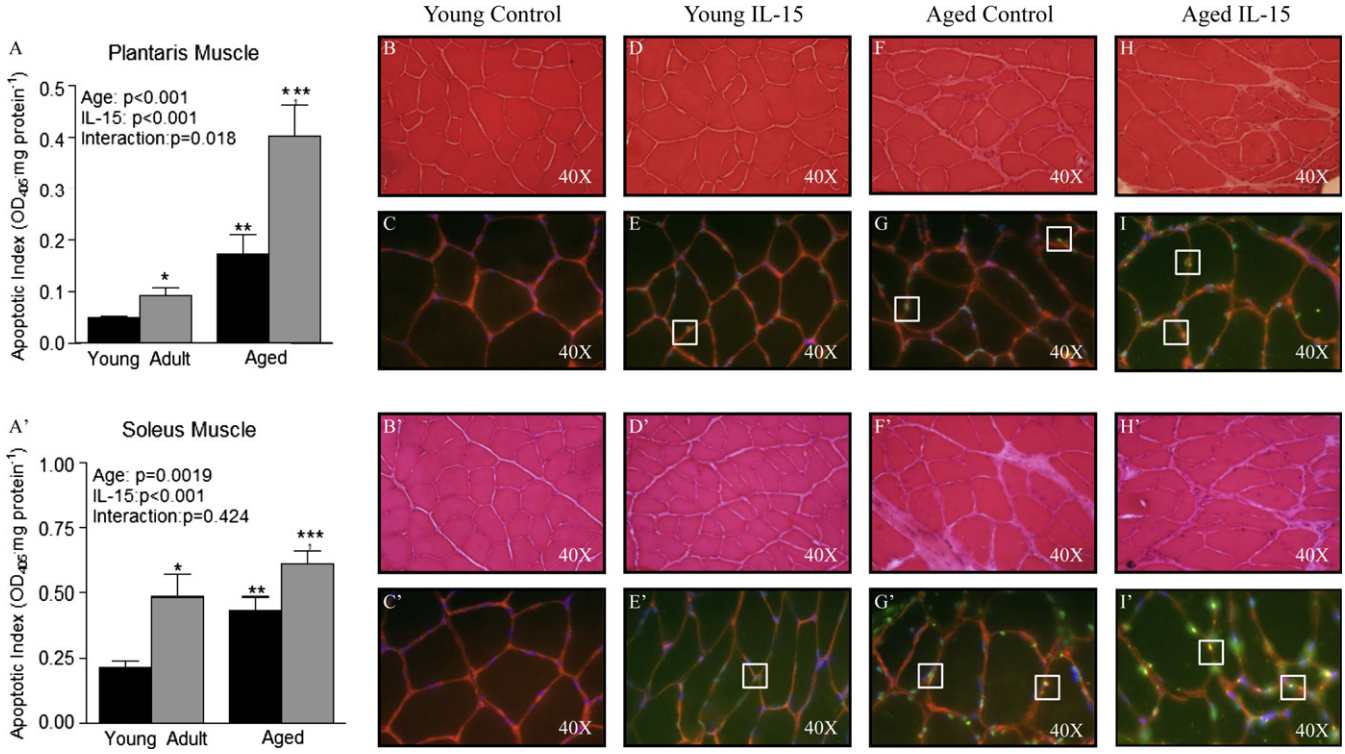

Fig. 1. Incidence of apoptosis. Apoptosis was greater as a result of age and IL-15 treatment—plantaris muscle (A), soleus muscle (A'). Results are presented as means ± SD. Black bars represent young adult and aged control rats treated with PBS; gray bars represent young adult and aged rats treated with IL-15. *Significant affect of IL-15 treatment; **significant affect of age. There were no apoptotic nuclei visualized in the plantaris (C) or soleus (C') samples from young control animals. Apoptotic nuclei under the basal lamina were observed in the plantaris (E) and soleus (E') samples from IL-15 treated young animals. In both aged groups, there was a greater number of apoptotic nuclei visualized in the plantaris (G,I) and soleus muscles (G',I').

The cell death ELISA assay used to quantify the degree of apoptosis, measures fragmented DNA in whole muscle homogenates. The presence of apoptotic nuclei from tissue types other than skeletal muscle (i.e. smooth muscle, connective tissue) can contribute to the changes observed. Therefore, to verify that apoptotic nuclei were of myogenic origin, frozen sections of the plantaris and soleus muscles were used for TUNEL staining to visualize the location of apoptotic nuclei. We defined apoptotic nuclei of myogenic origin as those TUNEL positive nuclei that lie under the basal lamina, which was stained using an anti-laminin antibody and Cy3 secondary antibody. The acquired TUNEL images support the apoptotic index data, showing an increase in apoptosis as a result of aging and of IL-15 treatment. Specifically, no apoptotic nuclei were visualized in the plantaris and soleus muscles from young control rat (Fig. 1C and 1C'). However, a few apoptotic nuclei under the basal lamina were visualized in the young muscles that had been exposed to IL-15 treatment (Fig. 1E and E'). The plantaris and soleus muscles from control and IL-15 treated aged rats showed a greater number of apoptotic nuclei under the basal lamina (Fig. 1G–I). Histological examination of the plantaris and soleus muscles by H&E staining revealed an aging-associated increase in non-muscle area surrounding muscle fibers (Fig. 1B, D, F, and H).

Changes in body weight in response to systemic elevation of IL-15

At the end of the 14 day intervention, the change in body weight of YC rats averaged 35.5 ± 4.9 g. This supports previous observations in this rat strain showing steady increases of bodyweight at this age [17]. In contrast, the change in body weight of the Y15 rats only averaged 25.9 ± 2.9 g, a 27% difference that tended toward significance ($p = 0.11$). At the end of the 14 day intervention, the aged control rats lost an average of 9.9 ± 1.9 g, again supporting previous data in this rat strain at this age [17]. In contrast, the IL-15 treated aged rats lost an average of 25.4 ± 6.1 g, a 61% difference that was statistically different ($p = 0.035^*$). All aged rats were significantly heavier than all young rats (aged: 596.7 g ± 36.3; young adult: 322.2 g ± 23.4; $p < 0.001$).

Changes in skeletal muscle weights in response to systemic elevation of IL-15

The pattern of change was similar for all five skeletal muscles analyzed in both the young adult and aged rats, with some reaching statistical significance and some showing trends toward significance. In general, muscle wet weight from IL-15 treated rats were less than muscles from age-matched controls. Specifically, the plantaris was 9.5% less in the young adult rats ($p = 0.052$) and 9.0% less in the aged rats ($p = 0.027^*$) (Fig. 2A and F); the soleus was 12.3% less in the young adult ($p = 0.029^*$) and 9.0% less in aged ($p = 0.035^*$) (Fig. 2B and G); the medial gastrocnemius was 10.5% less in young adult ($p = 0.019^*$) and 7.2% less in aged ($p = 0.13$) (Fig. 2C and H); the tibialis anterior (TA) was 8.2% less in young adult ($p = 0.071$) and 7.9% less in aged ($p = 0.034^*$) (Fig. 2D and I); and the extensor digitorum longus (EDL) was 8.2% less in young adult ($p = 0.089$) and 6.4% less in aged (0.079) (Fig. 2E and J).

Verification of IL-15 Bioactivity in vivo

Plasma samples were used to measure human IL-15 in experimental animals. Human IL-15 was detected in the serum of IL-15 treated rats, although differences were noted when comparing young adult and aged rats (Y15: 8.3 ± 1.0 pg ml^{-1}; A15: 174.4 ± 48.1). Values for the young adult and aged control animals were below the lowest standard used in the assay (3.9 pg ml^{-1}), indicating specificity for human IL-15 (YC: 3.3 ± 0.01; AC: 2.8 ± 0.16). To determine IL-15 bioactivity, serum IL-15 values ($\log_{10}$) and rat spleen weights were fitted to a linear regression curve (Fig. 3). There was a significant linear correlation noted in this analysis ($r^2 = 0.51$; $p = 0.0002$), indicating biological activity of IL-15 in this experiment.

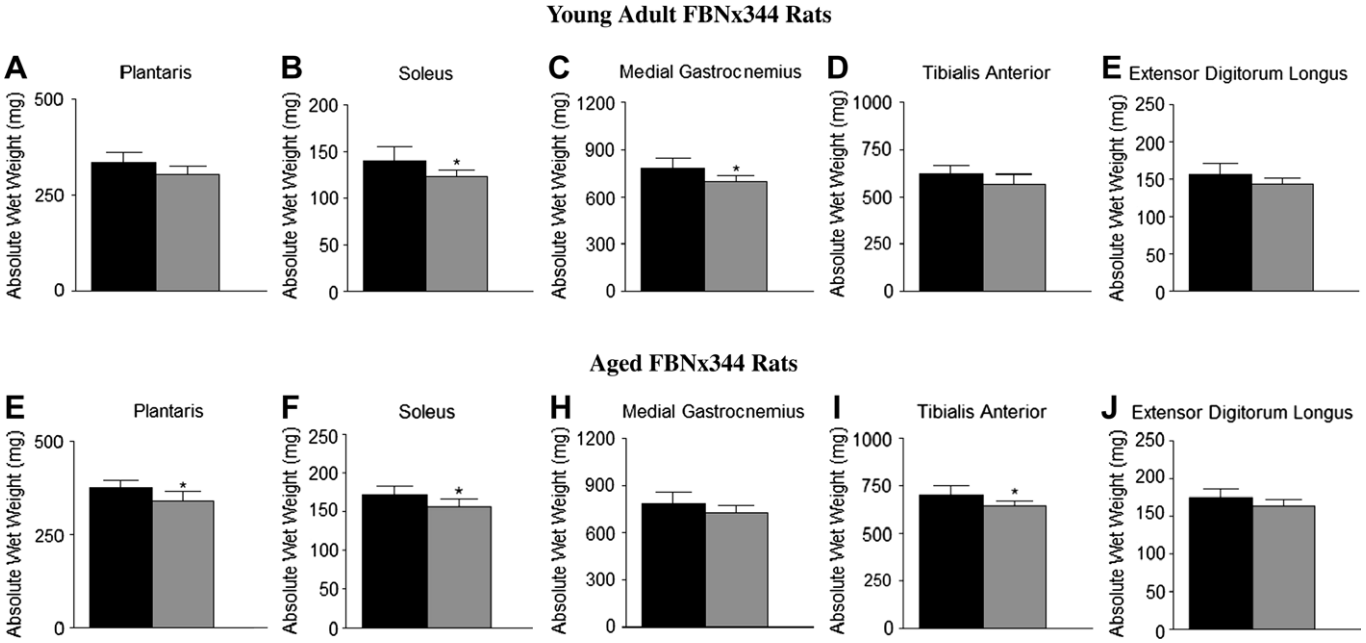

Fig. 2. Muscle weights. In the young adult group, the soleus muscle and medial portion of the gastrocnemius muscle from IL-15 treated rats weighed less than muscles from age-matched control rats, while the plantaris, tibialis anterior, and extensor digitorum longus muscles showed a trend for this effect. Similarly, in the aged group, the plantaris muscle, soleus muscle, and tibialis anterior muscle weighed less than muscles from age-matched control rats, while the medial portion of the gastrocnemius muscle and the extensor digitorum longus muscle showed a trend for this effect. Results are presented as means ± SD. Black bars represent young adult and aged control rats treated with PBS; gray bars represent young adult and aged rats treated with IL-15. Statistical significance was set at $p < 0.05$; *significant affect of IL-15 treatment.

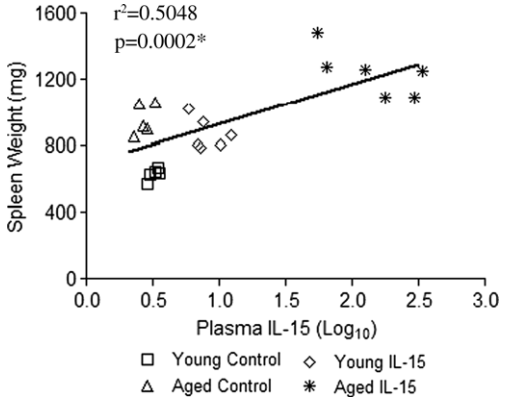

Fig. 3. An ELISA kit for human IL-15 was used to verify a systemic elevation of the cytokine *in vivo*. Significant increases in human IL-15 were measured in the serum from IL-15 treated rats. Serum IL-15 values were correlated with spleen weights indicating biological activity of IL-15 in this study.

Discussion

Our laboratory has previously suggested that skeletal muscle increases IL-15 mRNA concentrations in response to atrophic stimuli, as a molecular attempt to counter muscle loss [3]. In this study, we tested the hypothesis that IL-15 would attenuate apoptosis within aged skeletal muscles by increasing circulating levels of IL-15 protein in aged rats. The results of the current studies, however, do not support a protective role of IL-15 in skeletal muscle. In fact, when recombinant human IL-15 was elevated systemically for 14 days, muscles from young adult and aged rodents displayed increases in fragmented DNA. The systemic elevation of IL-15 within experimental animals has been performed previously [8–10]. Daily injections of IL-15 over a 7 day period into young tumor-bearing rats decreased the rate of protein degradation [8], and reduced the incidence of DNA fragmentation in skeletal muscles from tumor bearing rats [9]. *In vitro* data also provided evidence that IL-15 had positive effects in myogenic cultures when IGF-I levels were experimentally reduced [20]. Based on these data, we hypothesized that IL-15 would exert similar anti-apoptotic effects in aged animals where circulating TNF-α levels are also reported to be elevated [15] and anabolic hormone levels are typically reduced [21]. However, the incidence of DNA fragmentation was increased in all muscle types analyzed. Thus, the anti-apoptotic role of IL-15 may be tissue specific and/or dependent on the degree of pathology present. The chronic stimulus of aging may not have as dramatic an effect as an acute cancer stimulus for eliciting elevations in TNF-α or initiating pathological changes in skeletal muscle. These observations may explain why we did not observe a protective effect of IL-15 in the skeletal muscles from our aged rodents.

Interleukin-15 as an apoptosis inhibitor

Numerous cytokines have been implicated in attenuating apoptosis (reviewed in [22]). Manipulation of the IL-15/IL-15R system has been shown to inhibit apoptosis in numerous cell types, including T-lymphocytes [12,23], neutrophils [24], skeletal muscle [9], and fibroblasts [7]. Multisystem apoptosis initiated in mice via treatment with an anti-Fas antibody was suppressed with injection of a long-lasting IL-15-IgG2b fusion protein [6]. In addition, IL-15 transgenic (Tg) mice were resistant to a lethal dose of *Escherichia coli (E. coli)*. Administration of 10 μg of IL-15 intraperitonally (i.p.) into control mice also minimized the death rate from a lethal challenge of *E. coli*. These authors also demonstrated that IL-15 administration into isolated peritoneal cell cultures *in vitro*, prevented TNF-α induced apoptosis [13].

A more precise mechanism for this anti-apoptotic effect was demonstrated in fibroblasts *in vitro* [7]. Fibroblast cultures stimulated with TNF-α showed increases in DNA fragmentation. However, when cultures were co-stimulated with IL-15 and TNF-α, apoptosis was attenuated. Co-immunoprecipitation experiments revealed that when IL-15 bound to the IL-15Rα in the presence of TNF-α, the caspase cascade downstream of the TNFR was inhibited. Thus, IL-15 seems to possess the ability to inhibit apoptosis by blocking the apoptotic signaling downstream of the TNFR. We have recently demonstrated that this signaling pathway is active in the skeletal muscles of 33mo. FBN rats. Caspase-8 and caspase-3 activity were both greater in aged skeletal muscles and aged muscles also had greater protein content of Fas-associated death domain protein (FADD) and BH3-interacting death domain agonist (Bid) [14]. However, increasing IL-15 systemically in a similarly aged group of rodents in the current study, did not attenuate the incidence of apoptosis in skeletal muscle.

Interleukin-15 and muscle

Initial experiments utilizing IL-15 protein *in vitro*, provided data suggesting that IL-15 was an anabolic factor for skeletal muscle. The anabolic effects of IL-15 were first demonstrated in differentiated myotubes *in vitro* [25]. Myotubes incubated in IL-15 protein ($10\ ng\ ml^{-1}$) displayed a hypertrophic morphology as well as increased myosin heavy chain protein content in culture. These results were supported upon myotube infection with a retroviral expression vector expressing murine IL-15 [26]. The anabolic effect of IL-15 *in vivo* has been demonstrated in diaphragm muscle from *mdx* mice [10]. Muscle cross sectional area (CSA) and specific force ($Po\ CSA^{-1}$) were elevated in the diaphragm muscles of *mdx* mice treated with IL-15, compared to control *mdx* mice. These data implicate a potential therapeutic role for IL-15 in muscle degenerative diseases, where skeletal muscles have a high number of degenerative fibers, regenerative fibers, and inflammatory foci [27,28].

A hypertrophic role for IL-15 *in vivo* has been difficult to demonstrate in young control animals with no overt muscle pathology, and data from previous reports are similar to the data from the current study. In a previous study, male Wistar rats that received once daily bolus injections of IL-15 ($100\ \mu g\ kg\ bw^{-1}$) for 7 days showed no hypertrophy in the gastrocnemius and tibialis anterior muscles [8]. The reported data actually show slight reductions in muscle weight in non-tumor bearing rodents that received IL-15. These data, along with the current study, suggest some degree of overt pathology must be present for IL-15 to have a positive myogenic effect, as shown in skeletal muscle during a tumor burden [8,9] and in a mouse model of muscular dystrophy [10].

Conclusions

In summary, our data suggest that IL-15 does not attenuate apoptosis in the skeletal muscles of aged animals. These data conflict with other data showing a protective effect of IL-15. However, this protective effect may take place in response to acute and dramatic increases in apoptosis, as observed following a cancer stimulus [8,9] or anti-Fas injections [6], and not during the chronic stimulus of aging. Additionally, variability in the delivery methods of IL-15, the type of IL-15 utilized, as well as ages and strains of experimental animals may have contributed to our conflicting data. The signaling pathways involving the IL-15/IL-15R system are extremely complex and the multiple isoforms of both IL-15 and IL-15Rα coupled with the widespread distribution of their mRNA transcripts require further study to fully elucidate their roles in skeletal muscle. Future studies should address the efficacy of IL-15 treatment to spare muscle mass in response to muscle

wasting disorders and in very old animals, as IL-15 has shown promise as an apoptotic inhibitor in disease conditions involving more extreme stresses than imposed in this study.

Acknowledgments

This study supported by NIH: National Institute on Aging Grant R01 AG021530 (S. Alway) and the ACSM Doctoral Student Research Grant RFG14 (E. Pistilli). The authors would like to acknowledge the generous contribution of recombinant human IL-15 by Amgen, Inc. We would like to thank Dr. Kanneboyina Nagaraju, Ramkishore Gernapudi, and Dr. Claire Mitchell for assistance in the preparation of this manuscript.

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