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Effects of aging and life-long moderate calorie restriction on IL-15 signaling in the rat white adipose tissue

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Abstract. – OBJECTIVE: Phosphorylation of insulin receptor substrate (IRS) 1 by tumor necrosis factor alpha (TNF-α) has been implicated as a factor contributing to insulin resistance. Administration of IL-15 reduces adipose tissue deposition in young rats and stimulates secretion of adiponectin, an insulin sensitizing hormone that inhibits the production and activity of TNF-α. We aimed at investigating the effects of age life-long moderate calorie restriction (CR) on IL-15 and TNF-α signaling in rat white adipose tissue (WAT).

MATERIALS AND METHODS: Thirty-six 8-month-old, 18-month-old, and 29-month-old male Fischer344´Brown Norway F1 rats (6 per group) were either fed *ad libitum* (AL) or calorie restricted by 40%. The serum levels of IL-15 and IL-15 receptor α-chain (IL-15Rα) were increased by CR controls regardless of age. An opposite pattern was detected in WAT. In addition, CR reduced gene expression of TNF-α and cytosolic IRS1 serine phosphorylation in WAT, independently from age.

RESULTS: IL-15 signaling in WAT is increased over the course of aging in AL rats compared with CR rodents. Protein levels of IL-15Rα are greater in WAT of AL than in CR rats independently from age. This adaptation was paralleled by increased IRS1 phosphorylation through TNF-α-mediated insulin resistance. Adiponectin decreased at old age in AL rats, while no changes were evident in CR rats across age groups.

CONCLUSIONS: IL-15 signaling could therefore represent a potential target for interventions to counteract metabolic alterations and the deterioration of body composition during aging.

Key Words:
Adipose tissue, Aging, Calorie restriction, IL-15, IL-15 receptor, TNF-α, NF-κB, IRS1.

Abbreviations

IRS 1: Insulin Receptor Substrate 1; TNF-α: Tumor Necrosis Factor alpha; IL-15: Interleukin-15; WAT: White Adipose Tissue; AL: Ad Libitum; CR: Calorie Restricted; IL-15R α: IL-15 Receptor α-chain; γ_c: common γ chain; IL2Rβ: IL2 Receptor β chain; F344×BNF1: Fischer$_{344}$×Brown Norway; DEPC: diethylpyrocarbonate; PVDF: polyvinylidene difluoride; ANOVA: two-way analysis of variance; RW/BW ratio: retroperitoneal adipose tissue weight/body weight; PPARs: Peroxisome proliferator-activated receptors.

Introduction

Obesity is a major public health problem in industrialized countries, due to its association with

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a broad range of negative health outcomes (e.g., diabetes mellitus and cardiovascular disease)[1]. Insulin resistance is a common feature of obesity and contributes to obesity-related adverse effects[2]. The prevalence of overweight and obesity is increasing in older populations[3]. Furthermore, regardless of weight gain, advancing age is accompanied by detrimental changes in body composition, including reductions in lean body mass and relative increases in adiposity, particularly visceral fat[4].

Adipose tissue fat redistribution occurring with age, in turn, is a major risk factor for insulin resistance[5]. Indeed, interventions counteracting fat accumulation with age have shown positive effects on insulin sensitivity and overall health[6-10].

Therapeutic exploitation of IL-15 signaling has emerged as a possible strategy against obesity and obesity-related health outcomes due to its catabolic properties on adipose tissue[11]. Although IL-15 expression has not been described in adipose tissue[12], this cytokine is believed to modulate adipose tissue metabolism, since white adipose tissue (WAT) expresses mRNA for all three IL-15 receptor subunits: IL-15 receptor α chain (IL-15Rα), common γ chain (γ_c), and IL2 receptor β chain (IL2Rβ). IL-15Rα, is responsible for IL-15 specificity and high affinity binding[13]. The other two subunits are shared with IL2[14]. Notably, obese *fa/fa* rats express lower γ_c levels compared with lean Zucker rats[15]. In addition, IL-15 administration induced remarkable reductions in WAT deposition in young rats, without affecting food intake[11]. In support to the direct action of IL-15 on fat metabolism, an *in vitro* study[12] showed that exposure to IL-15 inhibited lipid accumulation and stimulated secretion of adiponectin by differentiated adipocytes. Adiponectin suppresses tumor necrosis factor (TNF)-α mRNA expression in endotoxin-treated 3T3-L1 adipocytes[16]. It is worth mentioning that TNF-α, besides its roles in immunomodulatory and inflammatory responses[17], may represent a mechanistic link between obesity and insulin resistance[18]. Indeed, TNF-α signaling induces a state of insulin resistance by impairing insulin actions at the level of the insulin receptor substrate (IRS) proteins. Interestingly, IL-15 stimulates tyrosine phosphorylation of IRS-1 in human T cells[19]. The opposing actions of IL-15 and TNF-α on adiponectin and IRS-1 suggest that IL-15 might counteract TNF-α-mediated insulin resistance in adipose tissue.

Based on these premises, we investigated the effects of aging and life-long moderate calorie restriction (CR) on IL-15 expression and the insulin signaling in the rat WAT. We hypothesized that advancing age would be associated with decreased systemic levels of IL-15 and reduced adipose tissue IL-15Rα expression concomitant with lower secretion of adiponectin and increased signaling through the TNF-α-mediated pathway of insulin resistance. We further hypothesized that CR would increase the expression of IL-15 and IL-15Rα along with adiponectin and downregulate TNF-α-mediated IRS1 serine phosphorylation.

Materials and Methods

Animals

Thirty-six 8-old male, 18-old male, and 29-old male $Fischer_{344}$×Brown Norway (F344×BNF1) hybrid rats were purchased from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN, USA). The ages were chosen from published growth and survival data[20] to reflect a young age (8 months), adulthood (18 month), and advanced age (29 months). In each age group, 6 animals were fed ad libitum (AL) and 6 were calorie restricted by 40%. 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 h before the onset of the dark period. All rats had free access to tap water. Rats were individually housed and maintained on a 12-h light/dark cycle, at constant temperature and humidity, in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care at the University of Florida (Gainesville, FL, USA). 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 National Institutes of Health and reviewed and approved by the University of Florida's Animal Care and Use Committee (USA) before commencement.

Preparation of Cytosolic and Nuclear Fractions

Rats were sacrificed by rapid decapitation and retroperitoneal, epididymal and perirenal WAT was removed and weighed. Immediately afterward, WAT was frozen in liquid nitrogen and subsequently stored at -80°C. Experiments were conducted on the retroperitoneal WAT portion.

Approximately 500 mg of WAT were minced in 1:1.5 w/v ice-cold lysis buffer (20 mM Tris, 5 mM EDTA, 10 mM sodium phosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine, 1mM PMSF, pH 7.5), homogenized on ice in a glass-glass Duall homogenizer, rotated for 1 h at 4°C and centrifuged for 10 min at 14,000 × g at 4°C to pellet cellular debris and nuclei. The supernatant was collected after removing the top layer of lard-like fat. The resulting supernatant, representing the cytosolic fraction, was collected, aliquoted, and stored at -80°C for later biochemical analyses. Preparation of nuclear extracts was performed as previously described[21].

Serum Collection

Trunk blood was collected using a funnel, and serum was obtained using plastic Vacutainer® 8.5 mL clot activator serum collection tubes (#367991, Becton Dickinson; Franklin Lakes, NJ, USA). The tubes were inverted (2-3 times) and blood was allowed to clot at room temperature for 1 h, after which the tubes were spun for 10 min at 2,000 × g, at 4°C. The serum was eventually collected, transferred into 2-mL tubes, and frozen at -20°C.

Western Blot Analysis

Prior to loading, samples were boiled at 95°C for 5 min in Laemmli buffer (62.5 mM Tris×HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue, pH 6.8; Bio-Rad, Hercules, CA, USA) with 5% β-mercaptoethanol. Proteins were separated using 5%, 10%, and 15% pre-cast Tris×HCl gels (Bio-Rad, Hercules, CA, USA). For the quantification of TNF-R1, adiponectin, ERK 1, and p-IRS1 expression, equal volumes of WAT extracts were loaded. Nuclear content of NF-κB p65 was determined by applying 60 μg of protein, whereas 100 μg were loaded to quantify IL-15 and IL-15Rα in serum. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, 0.45 μm, Millipore, Billerica, MA, USA) using a semidry blotter (Bio-Rad, Hercules, CA, USA). Transfer efficiency was verified by staining the gels with GelCode Blue Stain Reagent (Pierce Biotechnology, Rockford, IL, USA) and the membranes with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA). Ponceau S staining of total protein was also used as a loading control. For adiponectin, NF-κB, ERK-1, pIRS-1, IL-15 and IL-15Rα, nuclear PPARγ experiments, membranes were blocked in Starting Block Tris-Buffered Saline (TBS) Blocking Buffer with 0.05% Tween-20 (Pierce Biotechnology, Waltham, MA, USA) for 1 h at room temperature, washed in TBS, and incubated overnight at 4°C in rabbit polyclonal anti-adiponectin (Abcam, Cambridge, MA, USA), 1:200; mouse monoclonal anti-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200; rabbit polyclonal anti-p-ERK1/2(Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200; rabbit polyclonal anti-p-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200; rabbit polyclonal anti-IL-15 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200; rabbit polyclonal anti-IL-15Rα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200. Membranes were then washed in TBS with 0.05% Tween-20 (TBS-tween) and eventually incubated with alkaline phosphatase-conjugated secondary anti-rabbit (1:30,000; Sigma-Aldrich, St. Louis, MO, USA) or mouse (1:30,000; Sigma-Aldrich, St. Louis, MO, USA) antibodies, at room temperature for 1 h. The membranes were washed in TBS-tween, rinsed in TBS, and soaked in Tris×HCl (100 mM, pH 9.5). Finally, the DuoLux chemiluminescent/fluorescent substrate for alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) was applied, and the chemiluminescent signal was captured with an Alpha Innotech Fluorchem SP imager (Alpha Innotech, San Leandro, CA, USA). Digital images were analyzed using AlphaEase FC software (Alpha Innotech, San Leandro, CA, USA). Spot density of the target band was normalized to the total lane of Ponceau-stained membrane and expressed in arbitrary optical density (OD) units. For the analysis of TNF-R1, the Vectastain ABC-AmP immunodetection kit (Vector Laboratories, Segrate, Milan, Italy) was used, according to the manufacturer's instructions. A rabbit polyclonal anti-TNF-R1 antibody (Abcam, Cambridge, MA, USA) was used at 1:1,000 dilution. Generation of the chemiluminescent signal, digital acquisition, and densitometry analysis were performed as described above.

Quantitative Polymerase Chain Reaction (qPCR)

To determine the relative gene expression of TNF-α, IL-15 Rα, IL2Rα, IL2Rβ, γ_c in WAT, Q-PCR analysis was performed. Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). Briefly, ~500 mg of tissues was homogenized in 1 mL of TRI Reagent with a motorized mortar and pestle. The homogenate was cleared by centrifugation, and RNA isolated from

the supernatant according to the manufacturer's instructions. Total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and quantified spectrophotometrically (i.e., 260/280 ratio). To remove possible contaminating DNA, DNAse digestion was performed using the TURBO DNAfree kit (Ambion, Foster City, CA, USA). First-strand cDNA synthesis was achieved from 2 µg of RNA using the MasterScript kit (5 Prime, Hamburg, Germany). Briefly, total RNA, 1 µL 0.5 µg oligo d(T) primer, 1 µL 50 ng/µL random hexamers, 1 µL 10 mM dNTP mix and DEPC water were mixed and heated to 65°C for 5 min. Samples were then placed on ice and 4 µL 25 mM RT-PCR buffer, 1 µL 0.75 U/µL RT enzyme, 0.5 µL RNAse inhibitor and DEPC water added. Samples were subsequently heated to 42°C for 1 h and the reaction was terminated by heating to 85°C for 5 min. QPCR was performed using an Applied Biosystems 7300 Real Time-PCR System (ABI, Foster City, CA, USA). TaqMan Universal PCR Master Mix (2×) (Roche, Branchburg, NJ, USA), as well as 0.2 µM primers and TaqMan probe mix (ABI) were used for each 25 µL reaction. Amplification of TNF-α (NM_012675), γ_c (NM_080889), IL-2Rα (NM_013163), IL-2Rβ (NM_013195), IL-15Rα (DQ_157696), and β-actin (NM_031144, endogenous control) was achieved using predesigned primers and probes (ABI), employing the following PCR cycling conditions: enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, anneal/extend at 60°C for 1 min. All samples were examined in triplicate, with the young AL group used as a calibrator. For all genes, negative controls (i.e., no template and no reverse transcriptase) were also included and run in triplicate. Differences in the expression of the target genes were explored by the $2^{-\Delta\Delta CT}$ method[22] with β-actin as the housekeeping gene.

Statistical Analysis

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

Results

Morphological Characteristics

Adipose tissue weight

WAT wet weight increased with advanced age (age effect: $p<0.0001$) (Figure 1a). However, changes in adipose tissue weight across ages were substantially different in the two diet groups (age × diet interaction: $p<0.0001$) (Figure 1a). In AL rats, WAT weight increased significantly only at old age (29 months). In contrast, in the CR group, WAT weight increased significantly between 8 and 18 months and remained unchanged thereafter. As expected, CR animals showed lower WAT weight in comparison to their AL counterparts at all ages (diet effect: $p<0.0001$).

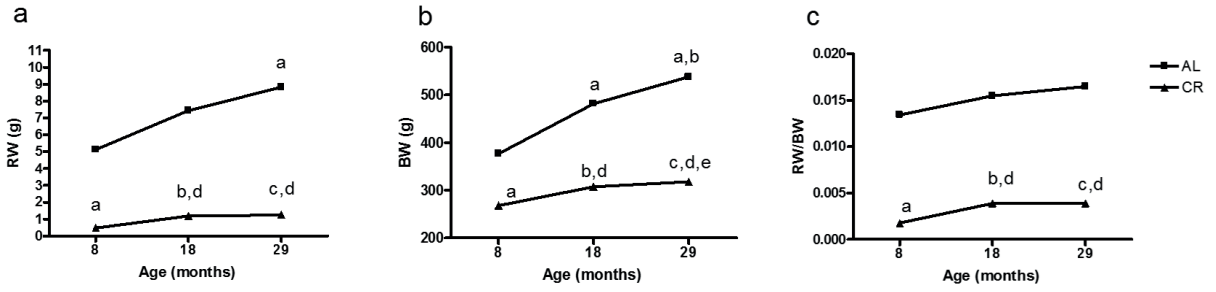

Figure 1. Retroperitoneal adipose tissue absolute wet weight (**a**), body weight (**b**) and retroperitoneal adipose tissue weight to body weight ratio (MW/BW (**c**) of 8-month-old, 18-month-old and 29-month-old Fischer$_{344}$×Brown Norway rats. Letters indicate significant differences ($p<0.05$) 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. Values are mean ± SEM (n=6/group).

Body weight

Within each age group, CR rats weighed less than their AL-fed counterparts (diet effect: $p<0.0001$; Figure 1b). However, the trajectory of changes in body weight over time was different between diet groups (age×diet interaction: $p<0.0001$). In fact, in AL rats, body weight increased progressively from 8 to 29 months of age (age effect: $p<0.0001$). In contrast, in the CR group body weight remained relatively stable over time, with the exception of a small increase observed between 18 and 29 months of age. Moreover, when WAT weight was normalized to body weight (RW/BW ratio) (Figure 1c), AL rats displayed a significantly higher ratio relative to CR rodents in all age groups (diet effect: $p<0.0001$). Changes in RW/BW with age were substantially different in the two diet groups (age × diet interaction: $p<0.0001$). In fact, RW/BW increased between 8 and 18 months in CR rats (age effect: $p<0.05$), while remained unchanged in AL animals.

Expression of IL-15 and IL-15 receptor subunits

Because little or no IL-15 mRNA has been detected in either undifferentiated pre- or differentiated adipocytes[12], we measured IL-15 protein levels in serum to obtain a direct measurement of IL-15. Moreover, since IL-15 actions are mediated by its interaction with a trimeric receptor comprising γ_c, IL-2Rβ, and IL-15Rα subunits, their gene expression levels were determined in WAT.

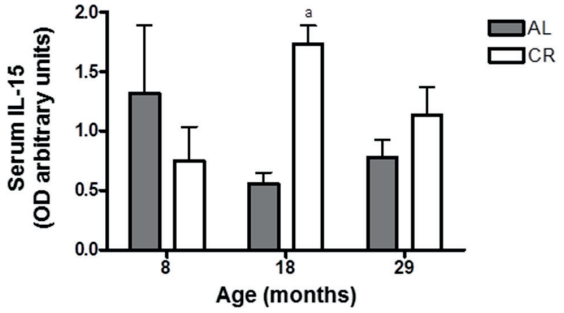

Figure 2. Protein levels of IL-15 in serum. In serum, IL-15 levels vary with advancing age in an opposite manner depending on the diet regimen ($p<0.03$) and are higher in CR compared with AL rats. The letters indicate statistically significant differences ($p<0.05$) within the groups, and between AL and CR rats of the same age. [a]Significantly different from 18-month AL. Values are expressed as averages ± SE (n = 6 / group).

IL-15

The trajectory of changes in serum IL-15 protein levels across groups showed an age × diet interaction ($p<0.03$) (Figure 2). No significant effects of age ($p=0.81$) or diet ($p=0.18$) were detected.

IL-15 receptor subunits

In WAT, gene expression of γ_c and IL-2Rβ were significantly higher in AL animals compared with CR rats (diet effect: $p=0.0035$ and $p=0.0045$, respectively) (Figure 3a, b). For the IL-2Rβ subunit, we also detected an age effect ($p=0.0061$) and

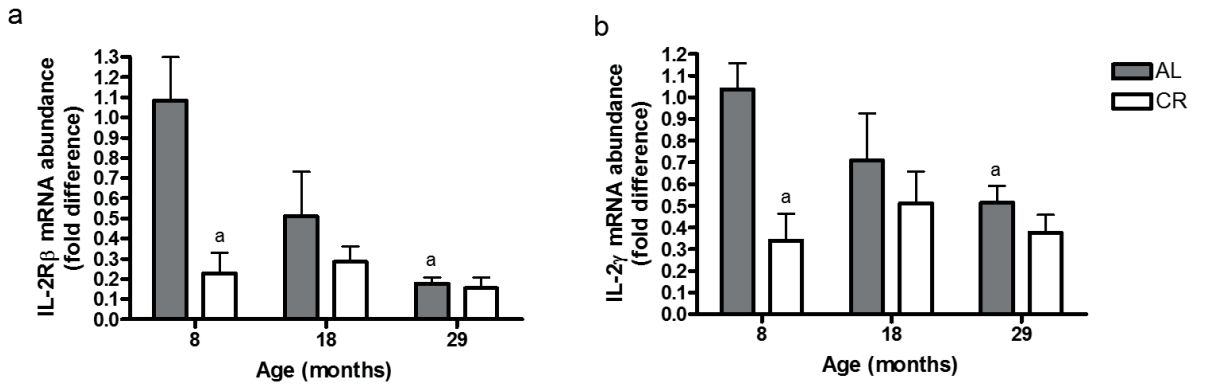

Figure 3. Relative gene expression of IL-2Rβ (**a**) and IL2Rγ (**b**) in adipose tissue. The gene expression of IL-2Rβ and γc is significantly lower in CR animals compared to AL rats ($p < 0.01$). In addition, γc expression shows a progressing decline with age ($p<0.01$) and a significant diet × -age interaction ($p<0.05$). The letters indicate statistically significant differences ($p < 0.05$) within the groups, and between rats AL and CR of the same age. [a]Significantly different from 8-months AL; [b]significantly different from 29-months AL. Values are expressed as averages ± SE (n = 6/group).

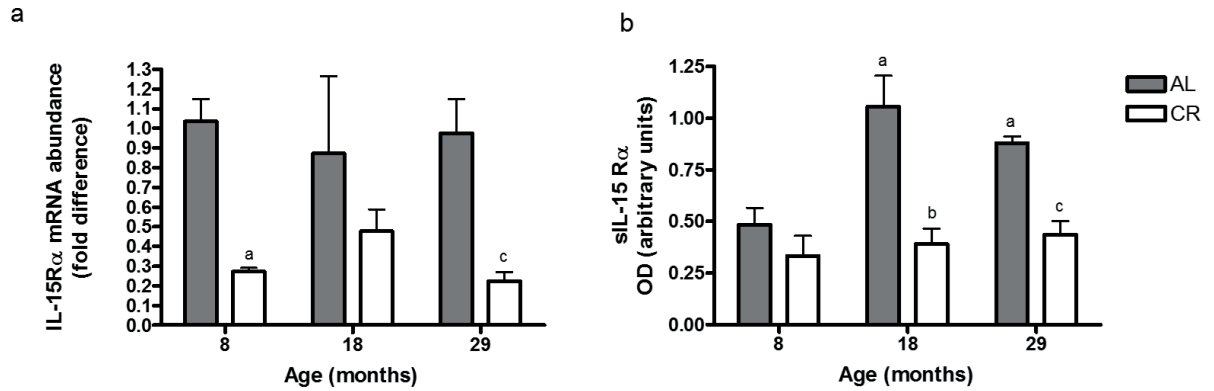

Figure 4. Adipose tissue gene expression (**a**) and protein content (**b**) of IL-15Rα increased in AL fed animals compared to CR rats. Letters indicate significant differences ($p<0.05$) within age groups, and between AL and CR rats of the same age. [a]Significantly different from 8-mo AL; [b]significantly different from 18-mo AL. [c]Significantly different from 29-mo AL; [d]significantly different from 8-mo CR. Values are mean ± SEM (n=6/group).

an age × diet interaction ($p=0.0160$) (Figure 3a). Indeed, the relative mRNA abundance of IL2Rβ decreased with advancing age in the AL group, which was not evident in CR rats. Likewise, we detected a significant higher IL-15Pα gene expression in AL animals compared with their CR counterparts (Figure 4a).

High affinity α-chain of the IL-15 receptor exists not only in membrane-anchored but also in soluble forms[23]. Three distinct IL-15Pα soluble isoforms have been described[24]. The two soluble IL-15Pα sushi domain isoforms potentiate IL-15 action[25]. Conversely, a full-length sIL-15Pα ectodomain, released by TNF-α-converting enzyme (TACE)-dependent proteolysis, inhibits IL-15 activity[24]. To assess if the increased gene expression of IL-15Pα in AL animals was mostly attributable to the soluble form of IL-15Pα with inhibitory properties, WAT full-length sIL-15Ra protein levels were quantified by Western blot analysis.

sIL-15Ra

Protein levels of sIL-15Rα were significantly higher in WAT from AL animals compared with CR rats (diet effect $p<0.0001$) (Figure 4b). However, changes in protein content of sIL-15Pα across ages were substantially different in the two diet groups (age × diet interaction: $p=0.0257$). In fact, in AL rats, sIL-15Rα increased significantly from 8 to 18 months of age (age effect; $p=0.0031$)

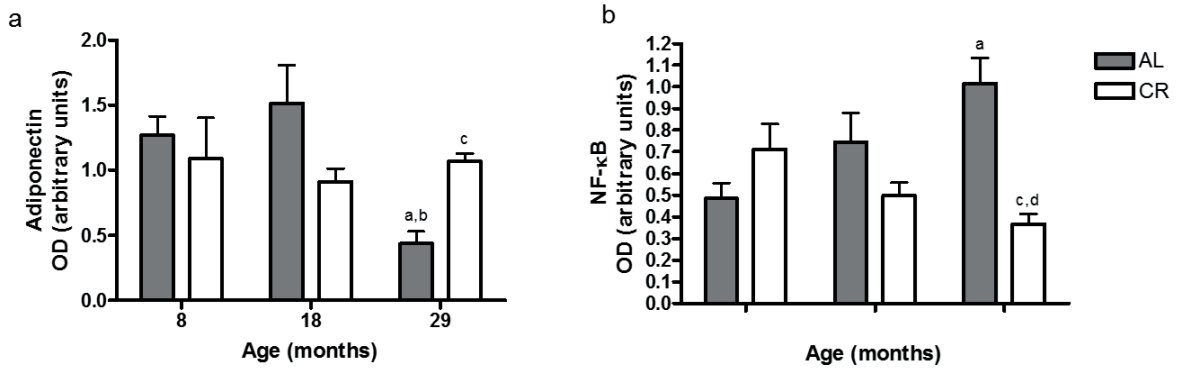

Figure 5. Protein levels of adiponectin (**a**) and nuclear NF-κB in adipose tissue (**b**). Adiponectin protein levels are significantly reduced with age ($p = 0.01$) and are influenced by the dietary regimen ($p<0.01$). NF-kB is increased in AL compared with CR rats ($p <0.01$), with different trajectories depending on the diet administered ($p <0.001$). The letters indicate statistically significant differences ($p<0.05$) within the groups, and between AL and CR rats of the same age. [a]Significantly different from 8-month AL; [b]significantly different from 18-month AL; [c]significantly different from 29-month AL; [d]significantly different from 8-month RC. Values are expressed as averages ± SE (n = 6 / group).

and remained elevate thereafter. In contrast, no significant changes across ages were detected in the CR group.

Adipokines

Adiponectin is an anti-inflammatory cytokine[26] that is expressed almost exclusively in adipose tissue[27] with significant effects on insulin sensitivity[28-30]. Interestingly, IL-15 administration stimulates secretion of adiponectin by differentiated 3T3-L1 adipocytes[12]. Thus, adiponectin protein levels were determined by Western blot analysis.

Adiponectin

In WAT, changes of adiponectin levels displayed a different trajectory over the course of aging in AL and CR rats (age × diet interaction: p=0.0029) (Figure 5a). Specifically, in the AL group, adiponectin content decreased at 29 months (age effect: p=0.01), whereas, no changes were evident in CR animals across age groups.

Activation of NF-kB

In aortic endothelial cells adiponectin has shown to block TNF-α signaling by preventing the activation and translocation of NF-κB[31]. A similar action of adiponectin has been confirmed in pig macrophages[32] and adipocytes[16]. In order to evaluate the impact of age and CR on NF-κB activation, nuclear levels of p65 were determined by Western blot analysis.

Nuclear p65

In AL rats, the expression of nuclear p65 increased markedly at 18 months of age compared with CR animals, and even further at 29 months (diet effect: p=0.0082) (Figure 5b). Conversely, the expression of nuclear p65 remained unchanged over the course of aging in CR rats (age × diet interaction: p=0.0003). No age effect was detected (p=0.6205).

In support to the ability of adiponectin to inhibit NF-κB activation, a significant negative correlation between adiponectin and NF-κB expression in WAT was determined (Pearson r: -0.32, p <0.05; Figure 6).

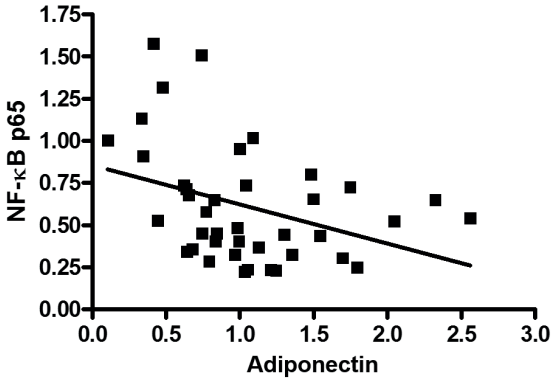

Figure 6. Correlation analysis between adiponectin and NF-κb p65. (Pearson's r: -0.32, $p < 0.05$).

PPARs

Peroxisome proliferator-activated receptors (PPARs) regulate the expression of several genes involved in lipid metabolism and play a key role in adipocyte differentiation. Three PPAR isoforms have been identified – PPAR-α, PPAR-β, PPAR-γ, with the latter being the most abundant in WAT where it regulates glucose metabolism. In particular, PPARγ activation is able to increase WAT adiponectin production[33].

To explore whether delayed reduction of adiponectin in AL rats (29 months) compared with the earlier increase of TNF-α (18 month) was linked to higher levels of PPAR-γ, the nuclear protein content of PPAR-γ was determined. We found that protein levels of nuclear PPAR-γ decreased in the AL group at 29 months (age effect: p=0.02) (Figure 7). Conversely, no changes were evident in CR animals across age groups. A previous study reported the opposite[34].

TNF-Ð-mediated pathway

In vitro studies indicate that TNF-a may contribute to decreased adiponectin production in obesity[35,36]. TNF-α acts on adipose tissue *via* two distinct cell surface receptors termed TNFR1 and TNFR2. TNFR1 mediate most of the effects of TNF-α on adipose tissue function and is the predominant receptor for mediating TNF-α-induced insulin resistance associated with obesity[37,38]. To evaluate the impact of TNF-α on adipose tissue adiponectin and IRS-1 levels, TNF-α gene expression was determined by qPCR, while TNFR1 protein levels were measured by Western blot analysis.

TNF-α

In WAT, gene expression of TNF-α was significantly higher in AL animals compared with CR rats (diet effect: p=0.037) (Figure 8a). No significant age effect (p=0.54) or age × diet interaction was detected (p=0.56).

TNF-R1

In WAT, the changes in TNF-R1 levels across ages were different in relation to diet (age × diet

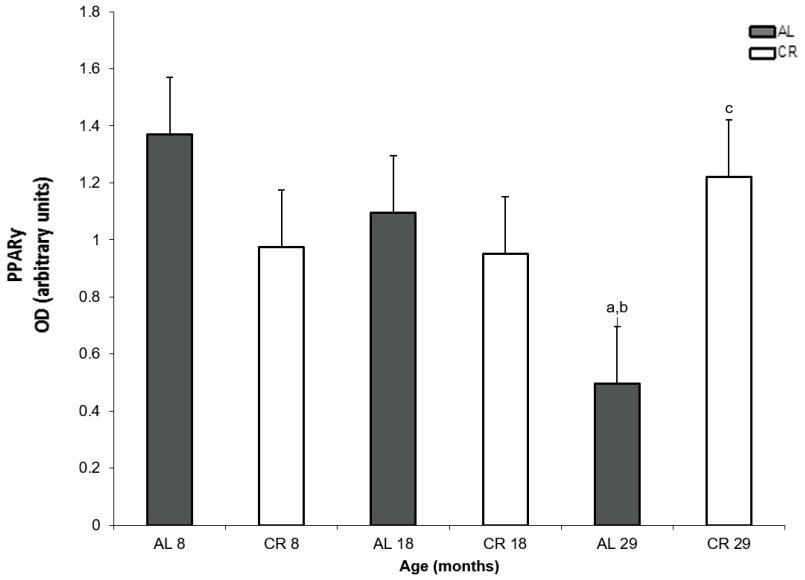

Figure 7. Nuclear levels of PPARγ in adipose tissue. In adipocytes, protein levels of nuclear PPARγ decreased in the AL group in advanced age (age effect: p=0.02). No changes were evident in CR animals across ages.

interaction: p=0.0180), resulting in a significant increase in TNF-R1 levels in AL animals starting at 18 months (age effect: p=0.0019), with no significant changes in CR rats (Figure 8b). We detected also a significant diet effect (p=0.0002). One of the mechanisms whereby IL-15 impacts adipose tissue metabolism might be the inhibition of TNF-α. Our data indicate that in AL animals the increase of TNF-R1 expression in tissue adipose occurred at the same age as the elevation of IL-15Rα protein. This might suggest a mutually inhibitory effect between IL-15 and TNF-α in adipose tissue. In support to this idea, a positive correlation was found between the expression levels of sIL-15Rα and TNF-R1 in WAT (Pearson r: 0.44, p <0.05; Figure 9).

Phosphorylation of IRS-1

TNF-α promotes serine/threonine phosphorylation of IRS-1[39]. Serine/threonine phosphorylation of IRS-1 impairs its ability to associate with the insulin receptor, which inhibits subsequent insulin-stimulated tyrosine phosphorylation[40]. Moreover, TNF-α-induced Ser[307]phosporylation is correlated with the ERK1/2 activation[41].

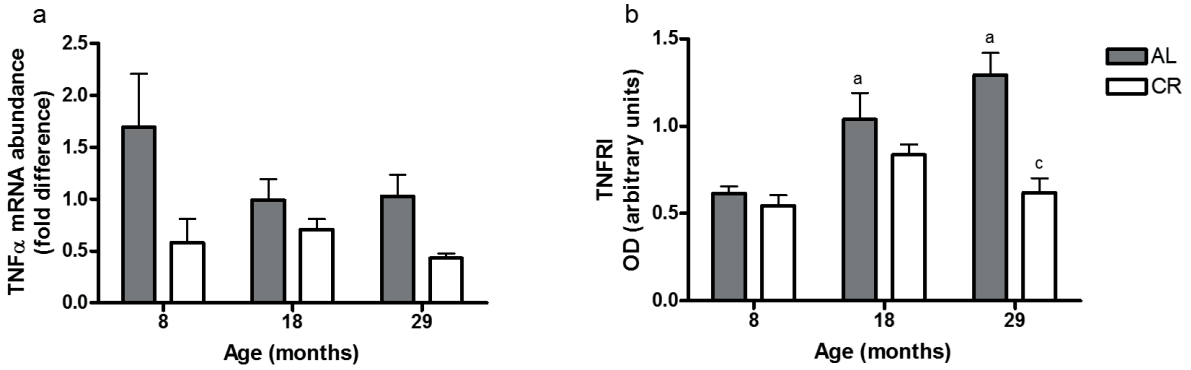

Figure 8. Relative gene expression of TNFα (**a**) and protein levels of TNF-R1 (**b**) in adipose tissue. The TNF-α gene expression and TNF-R1 protein levels are higher in the AL group than in CR rats (p <0.05 and p <0.001, respectively). Furthermore, TNF-R1 levels increase significantly with age (p <0.001) following different trajectories depending on the diet administered (p <0.01). The letters indicate statistically significant differences (p <0.05) within the groups, and between AL and CR rats of the same age. [a]Significantly different from 8-month AL. Values are expressed as averages ± SE (n = 6 / group).

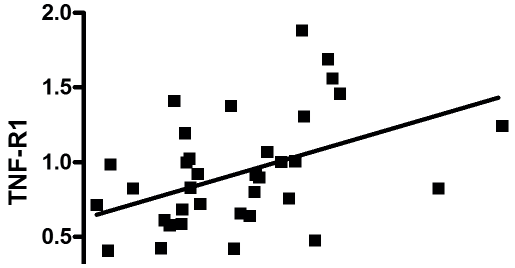

Figure 9. Correlation analysis between sIL-15Rα and TNF-R1 (Pearson r: 0.44, $p < 0.05$)

p-IRS1

In WAT, the protein content of p-IRS1 was significantly greater in AL animals compared with CR rats in all age groups (diet effect $p<0.0001$) (Figure 10a). We also detected a significant increase of p-IRS1 in AL animals starting at 18 months of age (age effect: $p=0.02$). No significant age × diet interaction was detected ($p=0.1347$).

p-ERK1

Protein expression levels of activated ERK1 were significantly higher in AL animals relative to CR rats ($p<0.0001$), with a significant age effect ($p=0.0043$) and age × diet interaction ($p=0.0437$) (Figure 10b). Indeed, in AL rats, we detected a significant increase in p-ERK1 levels starting at 18 months of age, while no significant changes were evident in CR rats.

p-ERK2

Similar to p-ERK1, the protein content of p-ERK2 was significantly higher in AL animals compared with CR rats (diet effect $p<0.0001$) (Figure 10c). No significant age effect ($p=0.21$) or age × diet interaction was detected ($p=0.30$).

Discussion

The interaction between TNF-α and IL-15 signaling in adipose tissue may have significant implications in terms of body composition changes with age and metabolic deregulation (e.g., insulin resistance, impaired glucose tolerance).

In the present study, changes in serum IL-15 levels during aging were different in relation to diet, as evidenced by higher circulating concentrations of IL-15 in CR rats (Figure 2). The mRNA abundance of IL-15Rα in WAT was unaffected by age in either rat group. Though, gene expression levels of IL-15Rα were lower in WAT from CR rats relative to their AL-fed counterpart. This finding is in contrast with our original hypothesis that aging would be associated with reduced levels of IL-15Rα and that CR would increase the expression of IL-15Pα in adipose tissue. To address this unexpected finding, we measured WAT protein levels of (sIL-15Rα, that possesses inhibitory properties on IL15[24]. Notably, we found a higher expression of IL-15Rα inhibitory isoform in AL rats. This may lead to hypothesize that the higher gene expression of IL-15Rα detected in AL rats might be mostly accounted for the inhibitor isoform of IL-15Rα.

One mechanism by which IL-15 exerts positive effects both *in vitro*[42] and *in vivo*[43] is by blocking TNF-α signaling. In particular, IL-15 administration to cachectic rats resulted in decreased expression of both TNF-α receptors (TNF-R1 and TNF-R2) in skeletal muscle[43]. In our study, CR was associated with decreased TNF-R1 protein levels in WAT, while in AL animals, TNF-R1 expression increased starting at 18 months of age. To further support this finding, we observed a positive correlation between WAT content of inhibitory sIL-15Rα and TNF-R1 protein levels (Pearson's *r*: 0.44, $p=0.006$; Figure 9).

Consistent with changes in TNF-R1 expression, AL rats showed increased activation of ERK-1 and consequent phosphorylation of insulin receptor 1 (Figure 10). According to our original hypothesis and the protective role postulated for dietary restriction, CR animals did not show alterations in the expression of TNF-α receptor or phosphorylation of insulin receptor 1. Furthermore, in WAT, the protein content of p-IRS1 was higher in AL animals with a significant increase starting at 18 months of age.

Adiponectin is a major modulator of systemic insulin sensitivity and glucose metabolism. We showed that adiponectin decreased in AL animals only at old age, while in the CR group its expression remains unvaried across ages. According to reports from other groups[16,31,32], we found an increase in nuclear translocation of p65 in AL rats temporally coinciding with adiponectin reduction (Figure 5). On the other hand, CR resulted in a significant reduction in p65 levels in old rats (29 months), suggesting that this dietary regimen, even at old age, plays a protective role on mediators of inflammation such as NF-κB. In support to the ability of adiponectin to inhibit NF-κB activation, we found a negative correlation between adiponectin and WAT NF-κB expression (Pearson *r*: -0.32, $p<0.05$; Figure 6).

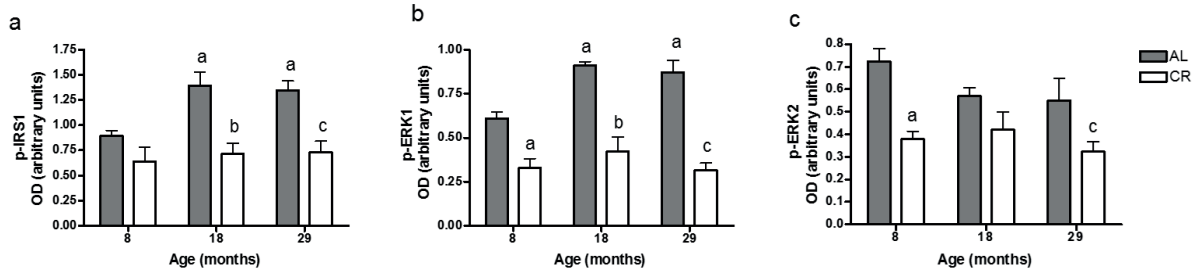

Figure 10. Protein levels of pIRS-1 (**a**), pERK-1 (**b**), and pERK-2 (**c**) in adipose tissue. Protein expression levels of pIRS-1 and pERK-1 are higher in the AL group than in CR rats ($p < 0{,}0001$), with a significant age effect ($p < 0.05$ and $p < 0.01$, respectively). The protein content of pERK-2 is greater in AL rats than in CR rodents ($p < 0{,}0001$), with no significant age effect. The letters indicate statistically significant differences ($p < 0.05$) within the groups, and between rats AL and CR of the same age. [a]Significantly different from 8-months per; [b]significantly different from 18-months to the; [c]significantly different from 29-months AL. Values are expressed as averages ± SE (n = 6/group).

However, the lack of temporal agreement among changes in TNF-R1 expression, adiponectin levels, and nuclear NF-κB p65 content suggests the existence of a compensatory mechanism to maintain insulin sensitivity in the presence of TNF-R1activation. This mechanism could involve the conserved expression of PPARγ during adulthood (18 months), since an age-related reduction of nuclear levels of these receptors has been reported[44]. In fact, the preservation of PPARγ receptor expression and their activation by specific ligands (e.g., thiazolidinediones) promote an increased secretion of adiponectin[33], by inhibiting the action of TNF-α on a specific promoter region of adiponectin[45]. Along with this finding, we showed that, in WAT, the protein levels of nuclear PPARγ decreased only in old AL rats.

Conclusions

Our findings indicate that AL-fed rats show higher expression of sIL-5Rα. In keeping with the upregulation of sIL-15Rα, TNF-α-mediated insulin-resistance signaling may occur in AL rats. CR, irrespective of age, is associated with reduced expression of sIL-15Rα and reduced activation of TNF-α signaling. IL-15 signaling could therefore represent a potential target for interventions to counteract metabolic alterations and the deterioration of body composition during aging.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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Authors' Contributions

Silvia Giovannini, Christy S. Carter, Christiaan Leeuwenburgh, and Emanuele Marzetti participated in study concept and design, data analysis and interpretation, and the preparation of the manuscript. Andrea Flex, Federico Biscetti, Drake Morgan, Alice Laudisio, Daniele Coraci,-Giulio Maccauro, Giuseppe Zuccalà, Pietro Caliandro and Roberto Bernabei participated in data analysis and interpretation with critical overview of the manuscript.

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