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IL-15 improves skeletal muscle oxidative metabolism and glucose uptake in association with increased respiratory chain supercomplex formation and AMPK pathway activation

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Abstract

Background—IL-15 is believed to play a role in the beneficial impact of exercise on muscle energy metabolism. However, previous studies have generally used supraphysiological levels of IL-15 that do not represent contraction-induced IL-15 secretion.

Methods—L6 myotubes were treated acutely (3h) and chronically (48h) with concentrations of IL-15 mimicking circulating (1–10 pg/ml) and muscle interstitial (100 pg/ml–20 ng/ml) IL-15 levels with the aim to better understand its autocrine/paracrine role on muscle glucose uptake and mitochondrial function.

Results—Acute exposure to IL-15 levels representing muscle interstitial IL-15 increased basal glucose uptake without affecting insulin sensitivity. This was accompanied by increased

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Conflict of interest

The authors declare that they have no financial conflict of interest.

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mitochondrial oxidative functions in association with increased AMPK pathway and formation of complex III-containing supercomplexes. Conversely, chronic IL-15 exposure resulted in a biphasic effect on mitochondrial oxidative functions and ETC supercomplex formation was increased with low IL-15 levels but decreased with higher IL-15 concentrations. The AMPK pathway was activated only by high levels of chronic IL-15 treatment. Similar results were obtained in skeletal muscle from muscle-specific IL-15 overexpressing mice that show very high circulating IL-15 levels.

Conclusions—Acute IL-15 treatment that mimics local IL-15 concentrations enhances muscle glucose uptake and mitochondrial oxidative functions. That mitochondria respond differently to different levels of IL-15 during chronic treatments indicates that IL-15 might activate two different pathways in muscle depending on IL-15 concentrations.

General significance—Our results suggest that IL-15 may act in an autocrine/paracrine fashion and be, at least in part, involved in the positive effect of exercise on muscle energy metabolism.

Keywords

Interleukin-15; myokine; oxidative phosphorylation; mitochondrial supercomplexes; myotubes; insulin sensitivity

1. Introduction

Interleukin 15 (IL-15) is a helical cytokine [1] expressed in several non-lymphoid tissues such as placenta, heart, lung, liver, kidney, brain, testis and skeletal muscle [1–3]. Since skeletal muscle expresses high levels of IL-15 mRNA, it is believed that skeletal muscle is an important source of this cytokine in circulation. In this sense, it has been reported that circulating IL-15 levels are increased immediately and/or in the first hour following an acute physical exercise in mice and humans [4–9]. Therefore, it has been suggested that IL-15 is a myokine that contributes to the beneficial effects of exercise on energy metabolism. Research in rodents treated with IL-15 or in mice over-secreting IL-15 due to muscle-specific IL-15 overexpression confirms this hypothesis. Indeed, high doses of circulating IL-15 trigger weight loss and reduce white adipose tissue mass without compromising food intake in rodents [10–14]. Furthermore, high IL-15 levels protect against high-fat diet (HFD)-induced obesity and HFD-induced glucose intolerance and insulin resistance [15–17].

In C2C12 myotubes, IL-15 increases basal and insulin-stimulated glucose uptake [18,19], GLUT4 translocation [20] and GLUT4 mRNA levels [18], confirming that IL-15 targets skeletal muscle to induce positive effects on glucose metabolism. Besides, IL-15 induces remarkable effects on different markers of muscle oxidative metabolism. Indeed, increased circulating IL-15 levels in rodents were associated with a shift toward a more oxidative muscle phenotype [21], increased mitochondrial DNA (mtDNA) content and cytochrome C oxidase activity [6] as well as higher mRNA expression of several markers that promote oxidative metabolism in skeletal muscle, such as silent information regulator of transcription (sirtuin)-1 (SIRT1), peroxisome proliferator-activated receptor (PPAR) δ , PPAR gamma coactivator 1 (PGC-1) α and β , carnitine palmitoyltransferase (CPT) 1 and 2, and medium-

and long-chain acyl-CoA dehydrogenase [11,16,17,21]. Interestingly, the positive effects of exercise on PPAR δ and SIRT1 protein expression are dependent on muscle IL-15 expression [9]. It therefore seems clear that IL-15 increases pro-oxidative mediators in skeletal muscle. However, to our knowledge, there is a dearth of information regarding IL-15 effects on muscle mitochondrial function. While one study showed that IL-15 increases muscle fatty acid oxidation in rats [11], another demonstrated that C2C12 myotubes chronically exposed to supraphysiological IL-15 levels had a PPAR δ -dependent increased mitochondrial activity [22]. One of the ways in which IL-15 could affect the electron transport chain (ETC) activity is through altering the association of the individual ETC complexes into supercomplexes [23–25]. Defects in ETC supercomplexes have been previously associated with animal models of ageing [26] and in human type 2 diabetes [27]. Moreover, an elegant recent report demonstrated marked increases in muscle ETC supercomplexes in aged human following a bout of exercise [28]. The molecular mechanisms leading to enhanced ETC supercomplexes during exercise remain unknown. We hypothesize that IL-15 could be at least in part involved in increased ETC supercomplexes formation.

It is possible that the improved oxidative characteristics and glucose homeostasis induced by IL-15 partly explains the beneficial effects of physical exercise on muscle metabolism. However, the effects of IL-15 have mainly been tested with supraphysiological levels of IL-15 in rodents or in muscle cell lines. Apart from one study that tested IL-15 levels that mimic physiological elevations of IL-15 in response to a bout of exercise in mice [6], IL-15 concentrations in other studies performed in rodents or in muscle cell lines were 18 to 10,300 times higher [10,11,14,29–31] than IL-15 circulating levels in humans [4,5,7,8]. It is therefore possible that the phenotypes observed may not represent the physiological action of IL-15. Exercise increases circulating levels of IL-15 by ~40% to reach 10 pg/ml in human and 60–100 pg/ml in mice [6,32]. In humans, it has been shown that resting IL-15 concentration is 10–15 times higher in the interstitial space surrounding skeletal muscle than in the circulation [7]. Therefore, muscle fibers are exposed to higher levels of IL-15 than levels measured in the circulation. Unfortunately, muscle interstitial levels of IL-15 during physical exercise have never been measured. It is therefore possible that muscle interstitial IL-15 levels in response to physical exercise are much higher than what is measured in the circulation. We estimate that muscle interstitial levels of IL-15 following exercise are 10 to 200 times higher than in the circulation.

In this context, the aim of the present study was to investigate how IL-15 at levels that mimic circulating and muscle interstitial IL-15 concentrations affect muscle glucose uptake, insulin sensitivity, and mitochondrial oxidative functions.

2. Methodology

2.1 Cell culture

The L6 myoblast cell line was chosen since it represents a good model to study muscle metabolism and insulin signaling pathways [33]. L6 and L6-GLUT4myc myoblasts were kindly provided by Dr. Amira Klip (Sick Kids Hospital, Toronto). Myoblasts were seeded at a 50,000 cells/ml and were cultured in growth medium (alpha-minimal essential medium (α MEM) (Wisent) supplemented with 10% Fetal Bovine Serum (FBS) (Wisent), 1X

antibiotic/antimycotic (Wisent) and 3 µg/ml gentamicin (Life Technologies)). When cells reached 80% confluence, growth medium was changed to differentiation medium (αMEM supplemented with 2% FBS, 1X antibiotic/antimycotic, 3 µg/ml gentamicin). The experiments were performed on day 7 of differentiation.

2.2 IL-15 treatments

L6 myotubes were treated with recombinant rat IL-15 (rIL-15) (Sigma-Aldrich, Product #: SRP4172) in two conditions: a) acute treatment (3h, to mimic the acute effect of exercise) and b) chronic treatment (48h, IL-15 renewed after 24h, to mimic the chronic effect of exercise training) at levels mimicking circulating IL-15 (1–100 pg/ml) and higher concentrations that may represent muscle interstitial levels of IL-15 (1–20 ng/ml). The 3h incubation was performed in serum-free medium in order to isolate the effect of IL-15 from the effects of other cytokines present in serum. The 48h incubation was performed in differentiation medium (2% FBS) as we observed that L6 myotubes cannot survive serum starvation for more than 10h.

2.3 Glucose uptake

Glucose uptake was determined as previously described [34]. Briefly, myoblasts were seeded in 24-well plates and differentiated. Then, myotubes were serum-starved for 3h prior to experiment to obtain optimal response to insulin as growth hormones present in medium are known to stimulate insulin signaling pathway [35]. For 3h IL-15 treatments, IL-15 incubation was performed during the serum starvation step.

During the last 20 min of the 3h serum starvation, half of the wells were treated with 100 nM insulin, which triggers glucose uptake in L6 myotubes at near maximal capacity [33]. Cytochalasin B (5 µM) was used to determine non-specific transport. Wells were then washed 3 times with hepes buffered saline (HBS; 140 mM NaCl, 20 mM Hepes-Na, 5 mM KCl, 2.5 mM MgSO₄, 1.0 mM CaCl₂, pH 7.4) to subsequently receive 0.2 ml of transport solution (HBS with 10 µM 2-deoxy-glucose and 0.5 µCi/ml [³H] 2-deoxyglucose) adjusted to the corresponding IL-15 concentration for 10 minutes. Transport solution was quickly removed, and myotubes were washed 3 times with ice-cold PBS. Myotubes were lysed in 0.5 ml of 0.05 M NaOH. 0.4 ml of myotube lysate was mixed with 5 ml scintillation cocktail (CytoScint, #882453) and counted twice for 1 minute using a Tri-Carb2910TR scintillation counter (PerkinElmer). 0.1 ml of the remaining cell lysate was used to measure protein concentrations using a Bradford assay (Bio-Rad). Glucose transport activity is expressed as pmol of 2-deoxy-glucose taken up/min/µg of total protein.

2.4 GLUT4 translocation

GLUT4 translocation was determined using a colorimetric assay developed by Dr. Amira Klip [36]. Briefly, L6 expressing GLUT4 tagged with Myc epitope (L6-GLUT4myc) were seeded in 48-well plates and differentiated. Myotubes were serum deprived for 3h prior to experiments, as described above. Half of the wells were treated with 100 nM insulin during the last 20 min of the 3h serum starvation step. Cells were subsequently fixed on ice with 3% paraformaldehyde in PBS for 10 min and washed twice with PBS, before quenching the reaction with 0.1 M glycine for 10 min. After 3 more PBS washes, cells were blocked with

5% goat serum in PBS for 15 min. Myc epitopes were targeted by 1 µg/ml anti-Myc polyclonal antibody (Santa Cruz, #sc-789). Surface-bound Myc epitopes were revealed by exposing myotubes to *o*-phenylenediamine dihydrochloride reagent (OPD, Sigma, #P9187) for 30 min. OPD is a colorimetric substrate that detects peroxidase activity. Reaction was stopped using 3 M HCl and GLUT4 translocation was quantified by measuring absorbance at 462 nm.

2.5 Western blots

After differentiation and treatments, myotubes were lysed in ice-cold RIPA buffer (Thermo Scientific) supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM EGTA) and protease inhibitors (ThermoScientific, PI88666) and kept at -80°C prior to western blot analysis.

Wild-type and IL-15 overexpressing mice (IL-15 Tg) used in the present study have been described elsewhere [31]. Details about IL-15 Tg construct can be found in reference [31]. Mouse muscle samples (soleus, extensor digitorum longus (EDL), gastrocnemius) were first cut into smaller pieces in an ice-cold lysis buffer (20 mM Tris, 50 mM NaCl, 250 mM sucrose, 1% 100X triton, 50 mM Na-fluoride, 5 mM Na-pyrophosphate, 1 mM Na-orthovanadate and one pill of anti-protease/10 mL (Thermo Scientific)) then homogenized at 30 Hz for 3 minutes using the Tissue Lyser II machine (Qiagen). Cell and tissue protein content was then quantified using the DC protein assay (Bio-Rad).

Cell and tissue lysates were analyzed by western blotting. Briefly, proteins were separated by 8% or 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The following primary antibodies were used: P-AMPKα (Thr172) (#2535), AMPKα (#5831), P-ACC (Ser79) (#11818), ACC (#3676), P-STAT3 (#9145), STAT3 (#4904), GAPDH (14C10) (#2118), caveolin 1 (#3238), GLUT4 (1F8) (#2213), and α-Tubulin (#3873) (Cell Signaling Technologies), diluted 1:500 or 1:1000 in TBST-BSA (5%). Primary antibodies for ETC complexes were Complex I [NDUFA9] (459100, Invitrogen – 1:2,000), Complex II [Fp] (459200, Invitrogen – 1:10,000), Complex III [UQCRC2] (Ab14745 MitoSciences – 1:2,000), Complex IV [subunit I] (459600, Invitrogen – 1:2,000), Complex V [ATP5A] (Ab14748, MitoSciences – 1:2,000). Antibodies were diluted in TBST-milk (5%). The secondary antibodies were anti-rabbit and anti-mouse coupled to horseradish peroxidase diluted 1:5000 in TBST-Milk (Santa Cruz Biotechnologies Inc. or Promega). Proteins were visualized using an enhanced chemiluminescent kit and exposed to UV light in a ChemiDoc system for (P-)AMPK, (P-)ACC and α-tubulin, or exposed on X-Ray film for ETC supercomplex levels. Protein bands were quantified by density analysis using Image J Launcher Software (NIH, USA).

2.6 Cellular oxygen consumption

L6 myoblasts were seeded on XF24-well or XF96e-well plates (Seahorse; Agilent Inc.). For the chronic IL-15 treatment condition, myotubes were treated for 48h with IL-15 as described above, then washed 3 times with HCO₃-free assay buffer (XF medium) (DMEM, 4 mM glutamine, 1 mM pyruvate and 5.5 mM glucose, no IL-15 (pH 7.4)), and exposed to XF medium at 37°C for 40 min. Myotube oxygen consumption rates (OCR) were then

measured in XF24 or XF96e analyzers as described in [37]. After the basal OCR measurement, myotubes were treated with 600 ng/ml oligomycin to measure OCR due to mitochondrial proton leak (state 4). Subsequently, maximum uncoupled and non-mitochondrial OCRs were determined by treating myotubes with 1 μ M FCCP and 4 μ M antimycin A, respectively. All mitochondrial inhibitors were from Sigma-Aldrich. Myotubes were then lysed in 0.05 M NaOH, and cellular protein content was measured using a Bradford protein assay. Antimycin OCR values (non-mitochondrial OCR) were subtracted from total OCR values (basal, oligomycin, and FCCP OCR) to present mitochondrial OCR.

For acute treatments, after 7 days of differentiation, myotubes were serum-deprived in Seahorse medium for 40 min. Myotube OCR was then assayed under resting state. IL-15 was injected in wells for a final IL-15 concentration of (in pg/ml): 0, 1, 10, 100, 1 000, 10 000 or 20 000. OCR was measured every 15 min for 3h. Uncoupled, maximal and non-mitochondrial OCR were then measured as described above.

2.7 Mitotracker staining

Mitochondrial content was measured with Mitotracker Deep Red FM staining (Invitrogen, Molecular Probes Inc.). After differentiation and IL-15 treatments, live L6 muscle cells were incubated with Mitotracker Deep Red FM at 100 nM for 15 min in 1% FBS phenol red-free DMEM at 37°C before observations on the Zeiss Z1 AxioObserver with the 20X long working distance objective. For every condition, 3 pictures were taken in 2 different replicates (n=3 independent experiments) with consistent exposure times and randomly chosen fields of observation. Images were analyzed for quantification with ImageJ software. Images were analyzed by first establishing a threshold for each independent experiment. This threshold was maintained for the measure of the pixels within the threshold limit in each picture with the “analyse particles” menu of ImageJ software. The number of pixels representing the stained mitochondria was then normalized to the number of nuclei in each picture.

2.8 Mitochondrial DNA quantification

Mitochondrial DNA from L6 myotubes was isolated using the Qiagen DNeasy Blood & Tissue Kit (69504) according to protocol except DNase free RNase A was added after step 1 and incubated for 2 min at room temperature before proceeding. DNA concentrations were determined using a Nanodrop 1000 and samples were diluted to 2 ng/ul in DNase free water for QPCR. A custom Rat 12S rRNA taqman assay (APFVNA3) was measured on an ABI 7500 FAST using h 18S assay as an endogenous control. Briefly, 10 ng of DNA was mixed with Taqman Fast Universal PCR mastermix (4352042) and either R-12S or H-18S assays in triplicate in a 12 ul reaction. Assays were measured using standard run conditions using comparative δ CT to assess expression levels.

2.9 Electron transport chain supercomplex levels

ETC supercomplexes were measured by Blue-Native PAGE electrophoresis (BN-PAGE) as described in Wittig et al, 2006 [38]. Protein extraction and electrophoresis were done on ice or at 4°C with whole cells or muscle homogenates (quadriceps) from wild-type and IL-15 Tg mice. L6 cells were differentiated and treated with IL-15 as described above in 60 mm

plates, and then scraped in PBS. Cells were centrifuged at 1,000 g for 5 min and pellets were resuspended in solubilisation buffer (50 mM imidazole/HCl pH 7.0, 50 mM NaCl, 5 mM 6-aminohexanoic acid, 1 mM EDTA) with 2% digitonin (final digitonin to cell wet weight ratio of 1:4 w/w – experimentally determined). Extraction was done on ice for 30 min with gentle mixing. For quadriceps samples, the muscle was first homogenized in sucrose buffer (250 mM sucrose, 20 mM imidazole/HCl, pH 7.0). The membrane fraction, including mitochondria, was pelleted at $10,000 \times g$ for 10 minutes and resuspended in solubilisation buffer with 1% digitonin (final digitonin to equivalent tissue wet weight ratio of 1:8 w/w - experimentally determined). Proteins from cells or tissues were cleared at 10,000 g for 30 min. 5% glycerol and a 1:10 dye:digitonin ratio of Coomassie blue G-250 were added to the supernatants, and samples were loaded on 3–13% gels. Gels were run at 150V, using high Coomassie Blue cathode buffer for the first 2h and then in low dye cathode buffer overnight at 200V. Proteins were transferred to nitrocellulose membranes at 400 mA for 2.5h. Membranes were then analyzed by western blot analysis. ETC complexes and supercomplexes were analyzed based on size and banding pattern, as previously described [27,39], using accepted nomenclature as previously confirmed by 2D-BN-PAGE [40].

2.10 Statistics

Data are means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using StatView 5.0 (SAS Institute Inc. Cary, NC, USA). One and 2-way ANOVAs with Fisher's protected least significant difference (PLSD) posthoc tests. Since a Fisher's PLSD test does not correct for multiple comparisons, with a p-value of 0.05, the probability of finding a significant difference by chance alone is 5% (i.e. one out of 20 comparisons could have been different simply by chance). We only compared treatments to the control. A t-test was used to assess statistical significance for the comparison of wild-type and IL-15 Tg mice. A p-value <0.05 was considered significant.

3. Results

3.1 Acute IL-15 treatment stimulates basal glucose uptake in L6 myotubes

Glucose uptake was measured by following the uptake of radioactively labeled [^3H] 2-deoxyglucose in response to acute or chronic exposure to different IL-15 concentrations. Under basal condition (no insulin), acute IL-15 exposure (3h) at concentrations higher than 0.75 ng/ml tend to increase 2-deoxyglucose uptake with a significant 34% increase at 20 ng/ml IL-15 (Figure 1A). However, acute IL-15 treatments did not increase insulin-stimulated 2-deoxyglucose uptake (Figure 1B). Chronic IL-15 treatments (48h) at all concentrations did not significantly influence glucose uptake under basal or insulin-stimulated conditions (Figure 1C and D).

Since acute IL-15 treatment at 20 ng/ml significantly increased basal glucose uptake in L6 myotubes, we tested whether GLUT4 translocation was altered at this concentration. Membrane GLUT4 translocation was measured in L6-GLUT4myc myotubes. Acute IL-15 treatment did not influence GLUT4 translocation under basal condition (Figure 1E) nor in the presence of insulin (Figure 1E). GLUT4 translocation in response to insulin followed a 1.5 fold increase, consistent with previous reports [41]. We also tested whether IL-15

treatment altered the expression of total GLUT4. As shown in Figure 1F, 3h-IL-15 treatment at 10 or 20 ng/ml did not significantly alter total GLUT4 expression.

It has been recently demonstrated that a 24h-treatment with 100 ng/ml of IL-15 in C2C12 myotubes resulted in increased glucose uptake through the Jak3/STAT3 signaling pathway [20]. We therefore tested whether IL-15 exposure at levels that might mimic IL-15 levels in circulation and in muscle interstitium similarly increased the phosphorylation of STAT3 in L6 myotubes. As presented in Figure S1, acute (3h) or chronic (48h) IL-15 treatment did not significantly increase STAT3 phosphorylation in L6 myotubes.

3.2 Acute and chronic IL-15 exposures increase L6 myotube mitochondrial oxidative function and capacity

To determine the effect of acute IL-15 exposure on mitochondrial oxidative functions, OCR of L6 myotubes was measured throughout a 3h IL-15 exposure. Whereas concentrations of IL-15 that represent circulating levels of IL-15 did not significantly alter resting OCR (Figure 2A), higher concentration (10–20 ng/ml) increased resting OCR by 20–30% from 30 to 180 min after IL-15 exposure (Figure 2C). Proton leak (state 4 respiration) and maximal or spare respiratory capacities were not significantly altered by acute IL-15 exposure (Figure 2B and C).

To determine the effect of chronic IL-15 exposure on mitochondrial function, OCR was also measured in L6 myotubes exposed for 48h to different IL-15 concentrations. Interestingly, a 48h exposure to concentrations that mimic circulating levels of IL-15 (1 and 10 pg/ml) significantly increased resting OCR by 49 and 38%, respectively (Figure 2E). However, resting OCR remained at control levels when L6 myotubes were exposed for 48h to 100–750 pg/ml of IL-15, whereas higher concentrations (>1,000 pg/ml) significantly increased resting OCR by 35–40% (Figure 2E). This biphasic profile was also observed for proton leak and maximal and spare mitochondrial capacities (Figure 2E).

3.3 Chronic IL-15 treatment in myotubes affects ETC supercomplex assembly without increasing mitochondrial biogenesis

To determine whether increased mitochondrial function in myotubes chronically exposed to IL-15 was the result of increased mitochondrial content, L6 myotubes were treated for 48h with different concentrations of IL-15 and were stained with Mitotracker Deep Red FM (Figure 3A). Using this approach, mitochondrial content was demonstrated to be unchanged. This result was confirmed with other indicators of mitochondrial content, including no change in mtDNA (Figure 3C), as well as no change in the expression of individual ETC complexes (data not shown).

Since there were no changes in mitochondrial content following IL-15 treatments, one of the ways that ETC function could be regulated is through altered ETC supercomplex formation. We therefore measured ETC supercomplex formation in response to acute or chronic IL-15 treatments. Acute IL-15 treatments (1,000 and 20,000 pg/ml) significantly increased the formation of complex III-containing supercomplexes while no significant changes were observed for the formation of other ETC supercomplexes (Figure 4). Chronic IL-15 treatment resulted in interesting effects on ETC supercomplex formation. Levels of IL-15

representing circulating IL-15 levels (1 pg/ml) induced a significant increase in the formation of complex IV-containing supercomplex and a trend for increased complex I-containing supercomplex (Figure 5A, B and D). Surprisingly, with higher IL-15 concentrations (>100 pg/ml) ETC supercomplex levels were similar to the control conditions and even sometimes lower (Figure 5).

3.4 IL-15 activates the AMPK signaling pathway in L6 myotubes

The AMP-dependent protein kinase (AMPK) pathway is known to increase muscle glucose uptake independently of insulin [42] and to improve mitochondrial function [43]. Furthermore, it has been previously shown that treatment with 1 ng/ml IL-15 for 2h increases AMPK phosphorylation in C2C12 myotubes [19]. We therefore investigated whether acute and chronic treatment with IL-15 were able to activate the AMPK pathway. AMPK is activated when phosphorylated, which in turn phosphorylates acetyl-CoA carboxylase (ACC), the main target of AMPK. Acute treatment (3h) with 10 to 20000 pg/ml of IL-15 significantly increased ACC phosphorylation in L6 myotubes (Figure 6B and D). Similarly, AMPK phosphorylation tended to be increased by acute IL-15 treatment in a dose-dependent manner (Figure 6A and C), but it did not reach statistical significance. L6 myotubes exposed to 20 ng/ml IL-15 from 15 to 180 min displayed a trend for an increase in the phosphorylation of AMPK and ACC, without reaching statistical significance (Figure 6E and F). We also tested whether chronic treatment with IL-15 altered the activity of the AMPK pathway. As shown in Figure 6G and H, whereas P-AMPK levels were not affected by chronic IL-15 treatment, 1000 and 10000 pg/ml of IL-15 for 48h significantly increased ACC phosphorylation.

3.5 Chronic high levels of IL-15 in circulation activate the AMPK signaling pathway in soleus muscle but do not influence the formation of ETC supercomplexes

Our results in L6 cells suggest that chronic exposure to high levels of IL-15 activates the AMPK pathway (Figure 6H), but does not increase ETC supercomplex content (Figure 5). In order to determine whether the muscle of mice that are chronically exposed to very high levels of IL-15 show similar alterations, we measured ETC supercomplex formation and P-AMPK and P-ACC levels in muscle samples from IL-15 Tg mice [31]. IL-15 Tg mice overexpress and oversecrete IL-15 specifically from skeletal muscle and display supraphysiological IL-15 circulating levels [31]. In agreement with results obtained in L6 myotubes treated chronically with high levels of IL-15 (Figure 5), no difference in ETC supercomplex formation was observed in the quadriceps muscle of IL-15 Tg compared to wild-type mice (Figure 7A-E). Soleus muscle from IL-15 Tg mice showed significantly higher ACC phosphorylation and a trend for higher AMPK phosphorylation compared to wild-type mice (Figure 7F and G). This increased AMPK pathway activity is similar to what we observed in L6 myotubes treated with high levels of IL-15 (Figure 6H). However, the AMPK pathway did not seem to be activated by muscle-specific IL-15 overexpression in other types of muscle (EDL or gastrocnemius) (data not shown).

4. Discussion

The secretory capacity of skeletal muscle has been known for many years [44]. However, the muscle secretome is still not well characterized. IL-15 has been classified as a novel myokine, and high concentrations trigger anti-obesity and anti-diabetic effects [45]. In light of these findings, a number of studies investigated whether IL-15 contributes to the beneficial impact of physical exercise on muscle energy metabolism. However, many studies have used supraphysiological doses of IL-15, which may not be representative of IL-15 levels following an acute bout of exercise. In the present study, we investigated the metabolic response of muscle cells to acute and chronic IL-15 treatments that might represent circulating and muscle interstitial levels of IL-15 in response to muscle contraction. As summarized in Table 1, we demonstrated that, in L6 myotubes, acute treatment with 10–20 ng/ml IL-15 increased basal glucose uptake and mitochondrial oxidative functions in association with increased activation of the AMPK pathway. Furthermore, chronic IL-15 exposure with concentration as low as 1 pg/ml increased mitochondrial oxidative function in association with increased formation of ETC supercomplexes. In contrast to our initial hypothesis, nor acute or chronic IL-15 treatment in our conditions altered L6 myotube insulin sensitivity. To the best of our knowledge, this is the first study showing that IL-15 treatment that mimics IL-15 levels in response to muscle contraction results in increased mitochondrial oxygen consumption in skeletal muscle.

A negative correlation between circulating IL-15 levels and resting blood glucose and insulin levels has been reported [16,17]. High levels of IL-15 increase whole-body insulin sensitivity in mice [15–17,37], as well as glucose uptake [18–20] and glucose oxidation [18] in C2C12 myotubes. In the present study, we observed a 34% increase in basal glucose uptake in L6 myotubes treated for 3h with 10–20 ng/ml IL-15 (Figure 1A) as previously observed in C2C12 myotubes [18]. This increased basal glucose with acute IL-15 treatment was not related to an increased GLUT4 translocation. In muscle cells, basal glucose uptake is mainly mediated by GLUT1. Interestingly, other cytokines have been shown to increase glucose uptake in non-muscle cells through GLUT1 [47,48]. It is thus possible that GLUT1 mediates the increased basal glucose uptake in IL-15 treated myotubes. This should be tested in future studies.

Acute exposure to IL-15 did not improve insulin-stimulated glucose uptake and chronic IL-15 treatment did not increase glucose uptake (Figure 1C) nor GLUT4 translocation (not shown) in L6 myotubes, in contrast with the recent study by Krolopp et al. (2016) in C2C12 myotubes. The discrepancies between the two studies could be due to the fact that the highest IL-15 concentration used in the present study (20 ng/ml) was 5 times lower than the one used in the Krolopp et al. (2016) study (100 ng/ml). Furthermore, in that study, C2C12 muscle cells were differentiated in a medium with very high concentrations of insulin (1 μ M) which might alter the insulin signaling pathway and GLUT4 translocation [49]. It is therefore possible that IL-15 treatment improves insulin sensitivity only in insulin-resistant muscle cells.

Interestingly, Krolopp et al. (2016) demonstrated that 24h exposure of C2C12 myotubes to 100 ng/ml of IL-15 stimulated glucose uptake through the Jak3/STAT3 signaling pathway,

independently of the AMPK pathway [20]. In our study, we were unable to show any alteration in the phosphorylation of STAT3 when L6 myotubes were exposed for 3h or 48h to 1–20000 pg/ml of IL-15 (Figure S1). In contrast to Krolopp et al. (2016), we observed that high levels of IL-15 (10–20 ng/ml) were able to activate the AMPK pathway in a time-dependent manner (Figure 6 C-F), in accordance with a previous study in C2C12 myotubes [19]. We also showed that the phosphorylation of ACC was still induced when L6 myotubes were treated chronically with 1–10 ng/ml compared to the control condition (Figure 6H). Furthermore, muscle-specific IL-15 overexpressing mice that are known to have very high levels of circulating IL-15 also showed higher activation of the AMPK pathway in their soleus muscle (Figure 7F-G). Those results are in accordance with higher levels of phosphorylated ACC and AMPK in skeletal muscle of IL-15R $\alpha^{-/-}$ mice that present high level of circulating IL-15 and a phenotype similar to muscle-specific IL-15 overexpressing mice [50].

Chronic exposure to high IL-15 levels increases mitochondrial activity in C2C12 myotubes [22] and induces pro-oxidative changes *in vivo* in rodents [6,11,16,17,21]. The novelty of the present study is that we directly measured the effect of IL-15 treatment on mitochondrial function by measuring oxygen consumption rates in living cells and the concentrations of IL-15 used were representative of physiological levels of IL-15. We demonstrated for the first time that chronic IL-15 treatment in muscle cells induced a biphasic dose-response curve similar to what Crane *et al.* (2015) observed in fibroblasts. This biphasic profile was also observed for OCR related to proton leak, maximal OCR, and spare capacity. These results suggest that IL-15 activates a certain pathway that increases mitochondrial function in skeletal muscle cells at concentrations that represent circulating IL-15 (lower than 100 pg/ml). However, this signaling pathway is neutralized when muscle cells are exposed to concentrations ranging from 100 to 750 pg/ml, and a second signaling pathway that increases OCR is activated at higher IL-15 concentrations (>1,000 pg/ml). As high concentrations of IL-15 can bind and transduce a signal in cells expressing only IL-2R β and γ (in the absence of the α subunit) [51], IL-15 might potentially stimulate a second pathway through other receptor complexes that may or may not be specific to IL-15.

The improved oxidative capacity of skeletal muscle from muscle-specific overexpressing mice or of myotubes exposed to IL-15 is associated with increased PPAR δ and PGC1 α levels [9,21,22,52]. PPAR δ seems to be central to the positive effect of IL-15 on muscle oxidative metabolism. Indeed its inhibition in C2C12 myotubes prevented the positive effect of IL-15 on mitochondrial function and pro-oxidative markers [22]. In the present study, we demonstrate for the first time that IL-15 exposure also influences the ability of the mitochondrial ETC complexes to form supercomplexes. ETC supercomplexes play an important role in the stability of individual complexes [23] and they reduce the production of reactive oxygen species [24]. It has also been suggested that ETC supercomplexes increase the efficiency of the ETC [53]. Our results suggest that chronic exposure to levels of IL-15 that mimic circulating IL-15 levels in response to physical exercise promotes the formation of ETC supercomplexes composed of complex IV and to a lesser extent, complex I (not significant) (Figure 5B and D), which, importantly, also align with the increased mitochondrial oxidative activities (Figure 2E). A recent report demonstrated that a 4 month-aerobic exercise training in sedentary older adults resulted in increased muscle content of

ETC supercomplexes composed of complexes I, III and IV [28]. Interestingly, this increased ETC supercomplex pool was correlated to an increased physical capacity and efficiency, as well as an increased reliance on fatty acids during exercise, suggesting that ETC supercomplexes might have a role in efficient energy use during physical exercise [28]. In the present study, chronic IL-15 treatments that mimic circulating IL-15 levels in response to exercise resulted in increased content of ETC supercomplexes in L6 myotubes similar to skeletal muscle of exercise-trained humans. It is therefore possible that IL-15 may be, at least in part, involved in the positive effect of chronic exercise on ETC supercomplexes. More research is needed to better understand the role of IL-15 and other exercise-induced myokines in the increased ETC supercomplexes formation in response to exercise training. Interestingly, whereas chronic exposure to low levels of IL-15 tended to increase ETC supercomplex formation, the content of supercomplexes was decreased back to the control levels (even sometimes lower) with higher IL-15 concentrations (Figure 5B-E). Furthermore, in IL-15 Tg muscle, ETC supercomplex levels were not altered compared to the wild-type mice (Figure 7A-E). Thus, supraphysiological levels of IL-15 do not influence ETC supercomplex formation in the same way as physiological doses of the myokine. These data again strongly suggest that two different signaling pathways are involved in the increased mitochondrial function with low doses vs. high doses of chronic IL-15 (Figure 2E). It is also possible that muscle overexpression of IL-15 and over-secretion of IL-15 in the IL-15 Tg model results in other compensatory mechanisms that may limit the formation in ETC supercomplexes.

On the other hand, we showed that higher levels of IL-15 that might represent muscle interstitial IL-15 levels in response to muscle contraction (10–20 ng/ml) acutely increased resting oxygen consumption rates (i.e. in the first 30 min of exposure) (Figure 2C) in association with an activation of the AMPK pathway (Figure 6). As previously mentioned, PPAR δ is required to mediate the beneficial effect of IL-15 on muscle oxidative metabolism [22]. Interestingly, many studies that used PPAR agonists to mimic the beneficial effect of physical exercise demonstrated a direct or indirect activation of the AMPK pathway [54]. It is thus possible that the activation of the AMPK pathway in our model is the result of PPAR δ activation. Interestingly, this quick improvement of mitochondrial function in response to IL-15 was also linked to an increased formation of complex III supercomplexes (Figure 5C). Therefore, at high concentrations, IL-15 might quickly stimulate complex III supercomplex formation, which may be beneficial to adapt to the quick increase in ATP demand in skeletal muscle in response to acute physical exercise / muscle contraction.

It is worth noting that IL-15 levels in muscle interstitium have never been measured in response to a bout of exercise. Therefore, we do not know whether our highest IL-15 treatments (10 and 20 ng/ml) are representative of muscle interstitial levels of IL-15 after a bout of exercise, or represents supraphysiological levels of IL-15. It has been recently demonstrated that muscle interstitial IL-15 levels are 10–15 times higher than circulating IL-15 levels under resting conditions [7]. If the same ratio is maintained during exercise, muscle interstitial IL-15 levels should be around 0.1 ng/ml (in humans) and 1 ng/ml (in mice). This estimated concentration is 10 to 20 times lower than the highest IL-15 treatments used in the present study (10–20 ng/ml). However, it is also probable that the primary role of IL-15 when secreted during muscle contraction is to act locally in skeletal

muscle to improve muscle metabolism [55]. It is thus possible that muscle interstitial IL-15 levels during exercise are even more than 10–15 times higher than circulating IL-15 levels. Importantly, future research should determine IL-15 levels in muscle interstitium in response to exercise in order to be able to better estimate whether the highest concentrations of IL-15 used in this study are representative of levels of IL-15 surrounding skeletal muscle.

5. Conclusion

In summary, the present study demonstrates that acute exposure to high levels of IL-15 resulted in improved basal glucose uptake and mitochondrial oxidative function in relation with the activation of the AMPK pathway and the formation of mitochondrial supercomplexes. Furthermore, chronic IL-15 treatment of L6 myotubes, as an *in vitro* model of physical exercise training, had no apparent effect on glucose uptake but improved mitochondrial function in relation with increased ETC supercomplex formation only when IL-15 levels were lower than 10 pg/ml, which represents circulating levels of IL-15 in response to exercise in humans. The improved mitochondrial function with higher levels of chronic IL-15 treatment is probably the result of the activation of a different pathway, since mitochondrial supercomplexes were decreased in this situation. Taken together, these results suggest that when released during muscle contraction, IL-15 may act in an autocrine/paracrine fashion, and contribute, at least in part, to the positive effects of exercise on skeletal muscle energy metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACC	acetyl-CoA carboxylase
αMEM	alpha-minimal essential medium
AMPK	AMP-dependent protein kinase
BN-PAGE	blue-native PAGE electrophoresis
CPT	carnitine palmitoyltransferase
EDL	extensor digitorum longus
ETC	electron transport chain

FBS	fetal bovine serum
HBS	hepes buffered saline
HFD	high-fat diet
IL-15	interleukin 15
IL-15 Tg	muscle specific IL-15 overexpressing mice
mtDNA	mitochondrial DNA
OCR	oxygen consumption rates
OPD	<i>o</i> -phenylenediamine dihydrochloride
PGC	PPAR gamma coactivator 1
PLSD	protected least significant difference
PPAR	peroxisome proliferator-activated receptor
rIL-15	recombinant rat IL-15
SEM	standard error of the mean
SIRT1	sirtuin-1

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Highlights

- Acute IL-15 improves muscle glucose uptake in association with AMPK pathway activation,
- Acute IL-15 improves muscle mitochondrial oxidative functions together with ETC supercomplex formation,
- Chronic IL-15 effects on mitochondrial function/supercomplexes are concentration-dependant,
- IL-15 might activate two different pathways in muscle depending on its concentration.

Figure 1: Acute IL-15 exposure increases basal glucose uptake in L6 myotubes with no alteration of insulin sensitivity or GLUT4 translocation.

A-D. L6 myotubes were exposed to different IL-15 concentrations for 3h (**A and B**) or 48 hr (**C and D**). Myotubes were subsequently treated with no insulin (**A and C**) or with 100 nM insulin (**B and D**) for 20 min and exposed to 10 μ M 2-deoxy-glucose and 0.5 μ Ci/ml [3 H]2-deoxyglucose for 10 min. The absolute values of 2-deoxyglucose uptake under basal state (no insulin) were between 8 to 12 pmol/min/ μ g protein with a 1.5–2-fold increase in response to insulin. A and B: n=3, each independent experiment performed throughout in

triplicate; C and D: n=5, each independent experiment done in triplicate. * $P < 0.05$ vs. 0 ng/ml of IL-15. **E.** L6-GLUT4myc myotubes were exposed or not to 20 ng/ml IL-15 for 3h. Cells were then treated $\pm$ 100 nM insulin for 10 min. Cells were subsequently fixed, and cell membrane GLUT4myc was quantified using an α -Phenylenediamine dihydrochloride activity assay. n=3, each independent experiment done in triplicate. $\dagger$ $P < 0.05$ vs. no insulin. **F.** L6 myotubes were exposed to different IL-15 concentrations for 3h, treated with 100 nM insulin during the last 20 min of IL-15 treatment and total GLUT4 levels were measured by western blotting. Top panels: representative western blots of GLUT4. GAPDH was used as loading controls. Bottom panel: Quantification of GLUT4 by density analysis. n=3 independent experiments. **A-F.** Data are mean $\pm$ SEM.

Figure 2: Acute and chronic IL-15 treatments improve mitochondrial oxidative functions in L6 myotubes.

A-D. L6 myotubes were exposed to different concentrations of IL-15 for 3h with OCR measured every 15 min during the 3h-IL-15 treatment period. Cells were treated subsequently with 600 ng/ml oligomycin, 1 μ M FCCP, and 4 μ M antimycin A to determine OCR due to proton leak, maximal, and non-mitochondrial respiration, respectively. Spare capacity was calculated by subtracting basal OCR from maximal OCR. **A-B.** OCR of L6 myotubes treated with 0 to 1,000 pg/ml IL-15. n=6, where each independent experiment was

done in 4 replicates. **C-D.** OCR of L6 myotubes treated with 0 to 20,000 pg/ml IL-15. $n=3$, where each independent experiment was done in 5 replicates. **E.** L6 myotubes were exposed to different concentrations of IL-15 for 48h. After measuring basal OCR, cells were treated subsequently with oligomycin, FCCP, and antimycin A as described in A-D. $n=3$, where each independent experiment was done in 20 replicates for 0 pg/ml, 12 replicates for 1, 10, 100 pg/ml and 6 replicates for 250, 500, 750, 1000, 10 000, 20 000 pg/ml IL-15. Data are mean $\pm$ SEM. * $P<0.05$ vs. 0 ng/ml of IL-15.

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Figure 3: IL-15 does not influence mitochondrial content in L6 myotubes.

A-B. L6 myotubes were exposed to 0–20,000 pg/ml IL-15 for 48h. Mitochondria were then stained with Mitotracker Deep Red FM and observed at 20X long distance on Zeiss Z1 Inverted Fluorescence Microscope. **A.** Representative images of stained mitochondria in myotubes exposed to the different conditions. Scale bars represent 50 μ m **B.** Quantification of mitochondrial content/nuclei relative to the control condition (0 pg/ml IL-15). n=3, for which 3 pictures were quantified per independent experiment and per condition. **C.** mtDNA quantification by qPCR relative to the control condition (0 pg/ml IL-15). n=2–3 independent experiments. **B-C.** Data are mean $\pm$ SEM.

Figure 4: Acute IL-15 exposure increases complex III supercomplex formation in L6 myotubes.

A-E. L6 myotubes were exposed to different IL-15 concentrations for 3h and levels of supercomplexes were analysed by BN-PAGE. **A.** Representative BN-PAGE blot of the indicated mitochondrial complexes (CI-V), supercomplexes (SCs), and monomers (Mon). **B-E.** Quantification of the indicated mitochondrial supercomplex normalized to complex II monomer levels. n=3 independent experiment. Data are mean $\pm$ SEM. * P<0.05 vs 0 ng/ml of IL-15.

Figure 5: The effect of chronic IL-15 exposure on ETC supercomplex assembly is concentration-dependent.

A-E. L6 myotubes were exposed to different IL-15 concentrations for 48h and levels of supercomplexes were analysed by BN-PAGE. **A.** Representative BN-PAGE blot of the indicated mitochondrial complexes (CI-V), supercomplexes (SCs), and monomers (Mon). **B-E.** Quantification of the indicated mitochondrial supercomplex normalized to complex II monomer levels. n=3 independent experiment. Data are mean $\pm$ SEM. * P<0.05 vs 0 ng/ml of IL-15.

Figure 6: IL-15 activates the AMPK pathway in L6 myotubes.

A-D. L6 myotubes were exposed to different IL-15 concentrations for 3h and P-AMPK and P-ACC levels were measured by western blotting (n=5–9 independent experiments). **E-F.** L6 myotubes were exposed to 20 ng/ml IL-15 for 0–180 min and P-AMPK and P-ACC levels were measured by western blotting (n=4 independent experiments). **G-H.** L6 myotubes were exposed to different IL-15 concentrations for 48h and P-AMPK and P-ACC levels were measured by western blotting (n=5–6 independent experiments). Top panels: representative western blots of P-AMPK (**A,C,E,G**) and P-ACC (**B,D,F,H**). AMPK and ACC were used as loading controls. α -tubulin is shown to confirm that variation in the expression of total AMPK or total ACC was following α -tubulin expression. Bottom panel: Quantification of P-AMPK (**A,C,E,G**) and P-ACC (**B,D,F,H**) by density analysis. **A-H.** Data are mean $\pm$ SEM. * $P < 0.05$ vs. 0 ng/ml of IL-15.

Figure 7: Muscle-specific IL-15 overexpression increases the activity of the AMPK pathway without affecting ETC supercomplex formation.

A. Representative BN-PAGE blot of the indicated mitochondrial complexes (CI-V), supercomplexes (SCs), and monomers (Mon), measured in the quadriceps muscle of wild-type (WT) and muscle-specific IL-15 overexpressing mice (IL-15 Tg). **B-E.** Quantification of the indicated mitochondrial supercomplex normalized to complex II monomer levels. n=7. **F-G.** P-AMPK and P-ACC were measured by western blotting in soleus homogenates from WT (n=3) and IL-15Tg mice (n=5). Top panels: representative Western blots of P-

AMPK (F) and P-ACC (G). AMPK and ACC were used as loading controls. Bottom panel: Quantification of P-AMPK (F) and P-ACC (E) by density analysis. A-G. Data are mean $\pm$ SEM. * $P < 0.05$ vs. WT.

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Table 1:

Summary of the effects of acute and chronic IL-15 treatments at different concentrations on L6 myotube metabolism.

	IL-15			
	Acute (3h)		Chronic (2*24h)	
	0–100 pg/ml(circulating)	1000–20,000 pg/ml interstitial or supraphysiologic)	0–100 pg/ml(circulating)	1000–20,000 pg/ ml(interstitial or supraphysiologic)
Glucose uptake	-	↑ (20,000 pg/mL)	-	-
Insulin sensitivity	-	-	-	-
GLUT4 translocation	-	-	-	-
Mitochondrial activity	-	↑ (20,000 pg/ml)	↑ (1–100 pg/ml)	↑ (1,000–20,000 pg/ml)
Mitochondrial content	-	-	-	-
Supercomplexes	-	↑ (1,000 and 20,000 pg/ml)	↑ (1 pg/ml)	↓ (1,000 pg/ml)
AMPK pathway	↑ (10–100pg/ml)	↑ (10,000-20,000 pg/ml)	-	↑ (1,000–10,000 pg/ml)
STAT3	-	-	-	-

↑, significant increase at the specified IL-15 concentration; ↓, significant decrease at the specified IL-15 concentration; -, no effect of IL-15