Antioxidants Facilitate High–intensity Exercise IL–15 Expression in Skeletal Muscle

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Key words
myokines, IL-15, sprint exercise, glycolysis, oxidative stress

accepted 19.10.2018

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ABSTRACT

Interleukin (IL)-15 stimulates mitochondrial biogenesis, fat oxidation, glucose uptake and myogenesis in skeletal muscle. However, the mechanisms by which exercise triggers IL-15 expression remain to be elucidated in humans. This study aimed at determining whether high-intensity exercise and exercise-induced RONS stimulate IL-15/IL-15Rα expression and its signaling pathway (STAT3) in human skeletal muscle. Nine volunteers performed a 30-s Wingate test in normoxia and hypoxia (P_IO_2 = 75 mmHg), 2 h after placebo or antioxidant administration (α-lipoic acid, vitamin C and E) in a randomized double-blind design. Blood samples and muscle biopsies (*vastus lateralis*) were obtained before, immediately after, and 30 and 120 min post-exercise. Sprint exercise upregulated skeletal muscle IL-15 protein expression (ANOVA, P = 0.05), an effect accentuated by antioxidant administration in hypoxia (ANOVA, P = 0.022). In antioxidant conditions, the increased IL-15 expression at 120 min post-exercise (33 %; P = 0.017) was associated with the oxygen deficit caused by the sprint (r = –0.54; P = 0.020); while, IL-15 and Tyr^{705}-STAT3 AUCs were also related (r = 0.50; P = 0.036). Antioxidant administration promotes IL-15 protein expression in human skeletal muscle after sprint exercise, particularly in severe acute hypoxia. Therefore, during intense muscle contraction, a reduced PO_2 and glycolytic rate, and possibly, an attenuated RONS generation may facilitate IL-15 production, accompanied by STAT3 activation, in a process that does not require AMPK phosphorylation.

Introduction

In skeletal muscle, interleukin (IL)-15 and its cognate receptor alpha (IL-15Rα) promote mitochondrial biogenesis, glucose uptake, fat oxidation and myogenesis [1, 13, 22, 24, 25, 29, 30]. Exercise has been shown to upregulate IL-15 expression and its signaling pathway in rodents [3, 13, 14, 24, 25]. In humans, despite IL-15 mRNA being primarily expressed in type II muscle fibers [21] and its protein expression increased after endurance training [27], a single session of resistance [21, 23] or endurance exercise [27] did not alter IL-15 protein expression in skeletal muscle. However, the mechanisms by which exercise may alter the IL-15/IL-15Rα axis protein expression in skeletal muscle remain unknown in humans.

Rodent data indicates that skeletal muscle IL-15 secretion during prolonged exercise requires 5' AMP-activated protein kinase (AMPK) pathway activation by unidentified mechanisms [3]. Oxidative stress reduces IL-15 mRNA expression in C2C12 cells [11], while hypoxia exerts a similar effect in astrocytes [10]. During exercise, reactive oxygen and nitrogen species (RONS) are produced in human skeletal muscle depending on the duration, intensity and training state and inspiratory oxygen pressure (P_IO_2) [15]. Although RONS and hypoxia play an important role in skeletal muscle me-

tabolism during exercise [15, 16], it remains unknown whether RONS and hypoxia intervene in the regulation of the IL-15/IL-15Rα axis in the exercising human skeletal muscle.

Therefore, the purpose of this study was to determine whether high-intensity (sprint) exercise stimulates IL-15/IL-15Rα expression in human skeletal muscle. Since sprint exercise facilitates RONS production [15], mainly when the sprint is performed in severe acute hypoxia [19], the current study also aimed to determine whether RONS play a role in the regulation of IL-15/IL-15Rα expression in human skeletal muscle. It is hypothesized that the RONS overproduction during sprint exercise would blunt IL-15/IL-15Rα axis expression as well as its associated signaling pathway via STAT3, while antioxidant intake would upregulate the expression of IL-15 in human skeletal muscle.

Materials and Methods

Subjects

Nine healthy and physically active young males (age: 25.2 ± 4.7 yr, height: 176.0 ± 5.1 cm, body mass: 80.2 ± 9.9 kg, body fat: 18.3 ± 6.7 %, normoxic VO_{2peak}: 3.850 ± 0.308 L · min^{-1}, and hypoxia VO_{2peak}: 2.586 ± 0.208 L · min^{-1}) participated in this study after they provided informed written consent. The study was approved by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2010-01), in accordance with the latest version (7th) of the Declaration of Helsinki and the ethical standards of the International Journal of Sports Medicine [8].

Experimental procedures

After familiarization [17], subjects were randomly assigned to perform a single 30-second isokinetic Wingate test at 100 RPM in four occasions under different conditions: a) normoxia after placebo intake (NP); b) hypoxia after placebo intake (HP); c) normoxia after antioxidants intake (NA); d) hypoxia after antioxidants intake (HA). On each trial day, volunteers reported to the laboratory in fasted state (~12 h). Upon arrival (7:00 a.m.), the first blood sample from a forearm vein and muscle biopsy from *vastus lateralis* were obtained. Then, participants were sat on the cycle ergometer for 4 min before the trial start, and they breathed either room air (normoxia) or a hypoxic gas mixture from a Douglas bag (hypoxia). At the end of these 4 min, subjects performed the 30-s all-out Wingate test at 100 RPM (Excalibur Sport 925900, Lode, Groningen, the Netherlands). To ensure a fixed pedaling rate of 100 RPM, the cycle ergometer was set on isokinetic mode and the braking force was servo-controlled. Within the 10-s post-exercise, a second muscle biopsy and blood sample were obtained. Two more muscle and blood samples were taken at 30 and 120 min post-exercise, changing the direction of the needle [6].

Antioxidant and placebo ingestion

Antioxidants or a placebo (microcrystalline cellulose) were provided in capsules of similar taste, color and appearance, and they were distributed in 2 doses (120 and 30 min pre-exercise) following a double-blind design. The first dose of the antioxidant cocktail was composed of 300 mg α-lipoic acid, 500 mg vitamin C and 200 IU vitamin E; while the second dose consisted of the same amount of α-lipoic acid and vitamin C plus 400 IU of vitamin E. The antioxidant cocktail selection was based on previous efficacy reducing free radical levels at baseline and in response to exercise in humans [26].

Ergospirometric variables in hypoxia and normoxia conditions

Cylinders containing 10.4 % O_2 in N_2 (Carburos metálicos, gas mixture 206030, Las Palmas de Gran Canaria, Spain) were used as a source of hypoxic gas, which was delivered through a Douglas bag. VO_{2peak} was measured using a metabolic cart (Vmax N29, SensorMedics, Yorba Linda, CA, USA). Respiratory variables were analyzed breath by breath and averaged every 5-s during the Wingate test, and every 20-s during the incremental tests performed during the preliminary assessments. The highest 20-s average of VO_2 was recorded as VO_{2peak}.

Muscle tissue analysis

Protein extraction and Western Blot procedures

Initially, total protein extraction and content quantification were performed as previously reported elsewhere [17]. Proteins were solubilized in a buffer containing 0.00625 M Tris-HCl (pH 6.8), 2.3 % (wt/vol) SDS, 10 % (vol/vol) glycerol, 5 % (vol/vol) beta-mercaptoethanol, and 0.001 % (wt/vol) bromophenol blue, electrophoresed on 7.5–10 % SDS-PAGE gels (Bio-Rad Laboratories, Hemel Hempstead Hertfordshire, UK), and transferred to Hybond-P membranes. Loading and transfer efficiency across membranes were controlled by incubating membranes with a monoclonal mouse antiα-tubulin antibody diluted in TBS-T with 5 % blotto blocking buffer. No significant changes were observed in α-tubulin protein expression (data not shown). Subsequently, membranes were incubated with primary antibodies against IL-15, IL-15Rα (Santa Cruz Biotechnology, Dallas, TX, USA), anti-AS160, anti- Tyr^{705}-STAT3 and anti-STAT3 (Cell Signaling Technology, Denver, MA, USA), and anti-Thr^{642}-AS160 (MLB International Corporation, Woburn, MA, USA), diluted in a BSA-blocking buffer containing 4 % bovine serum albumin in Tris-buffered saline with 0.1 % Tween 20. Antibody-specific labeling was revealed by incubation with an HRP-conjugated goat anti-rabbit (1:5,000–20,000) or donkey anti-mouse (1:5,000–10,000) (Jackson ImmunoResearch, West Grove, PA, USA), both diluted in 5 % blotto blocking buffer. Then, band visualization and quantification were carried out in Immun-Start™ WesternC™ using ChemiDoc XRS system and Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA, USA), respectively. Control samples (from a healthy young man) and a total protein staining-technique method (reactive brown) were used to accurately quantify for loading control. Muscle-signaling data was represented as arbitrary units (AU).

Protein carbonylation assessment, muscle metabolites and glycolytic rate

Skeletal muscle and plasma protein carbonylation, muscle metabolites and glycolytic rates have been previously reported [17, 18, 28].

Statistical analysis

Data collected were analyzed using the statistical package SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA), and Graph Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA). Firstly, the Shapiro-Wilks test was used to evaluate the distribution of the data. Non-normally distributed variables ($P < 0.05$) were logarithmically transformed. A three-

way repeated-measures ANOVA was used with three within-subject factors: F_IO_2 (with two levels: normoxia and hypoxia), antioxidants (with two levels: antioxidants and placebo), and time (with four levels: pre-exercise, end-exercise, 30 min recovery and 120 min recovery). The Mauchly's test of sphericity was run before the ANOVA, and in the case of violation of the sphericity assumption, the degrees of freedom were adjusted according to the Huynh-Feldt test. Holm-Bonferroni correction was used as a *post hoc* test when significant differences were detected. Finally, the area under the curve (AUC) was calculated using the trapezoid method, and linear regression analysis was used to determine the relationship between variables. Values are reported as mean ± standard deviation of the mean (SEM) (unless otherwise stated); a $P \leq 0.05$ was considered statistically significant.

Results

The effects on performance (▶ **Table 1**), metabolism and carbonylated proteins in plasma and skeletal muscle have been previously reported [17–20] and are only summarized here. Skeletal muscle carbonylated protein expression increased in hypoxia compared to normoxia in both skeletal muscle and plasma (ANOVA hypoxia effect, $P = 0.032$ and $P < 0.001$, respectively), and this effect was attenuated by the ingestion of antioxidants [19, 20].

Skeletal muscle signaling

Skeletal muscle IL-15 and IL-15Rα expressions are illustrated in ▶ **Fig. 1**. Sprint exercise stimulated the expression of IL-15 (ANOVA time effect, $P = 0.05$). This effect was accentuated by the administration of antioxidants when the sprint was performed in hypoxia (ANOVA antioxidants x F_IO_2 x time interaction, $P = 0.022$) (▶ **Fig. 1b**). In contrast, skeletal muscle IL-15Rα expression was not altered by the interventions (ANOVA antioxidants x F_IO_2 x time interaction, $P = 0.72$) (▶ **Fig. 1c**).

IL-15/IL-15Rα downstream signaling events, STAT3 and AS160, were further explored. Compared to pre-exercise levels, the Tyr^{705}-STAT3 ratio to total STAT3 ratio was reduced 30 min after the sprints ($P < 0.001$), while a 4.2-fold increase was observed at the 120th min of the recovery period ($P < 0.001$) (▶ **Fig. 2c**). This response was influenced neither by the ingestion of antioxidants nor by F_IO_2 (ANOVA antioxidants x F_IO_2 x time interaction, $P = 0.74$). This Tyr^{705}-STAT3 to total STAT3 ratio upregulation occurred despite a reduction of total STAT3 expression at the 120th min after the sprints (ANOVA time effect, $P < 0.001$) (▶ **Fig. 2d**). In addition, the $pThr^{642}$-AS160 to total AS160 ratio was significantly reduced immediately after the sprints ($P = 0.02$), while it was increased above pre-exercise levels at 30 min post-sprint ($P < 0.001$). Total AS160 expression remained unchanged during the recovery period until 120 min post-sprint, when a significant decrease was found (ANOVA time effect, $P = 0.003$). Neither the antioxidant ingestion nor the F_IO_2 had a significant influence on total AS160 expression or phosphorylation state.

Associations between muscle signaling and metabolic responses

The IL-15 expression at 120 min was negatively related to the oxygen deficit incurred during the sprint exercise after antioxidant ingestion ($r = -0.54$; $P = 0.020$, $n = 20$). Likewise, in the antioxidant conditions, the IL-15 AUC was associated with Tyr^{705}-STAT3 AUC ($r = 0.50$; $P = 0.036$, $n = 20$).

Discussion

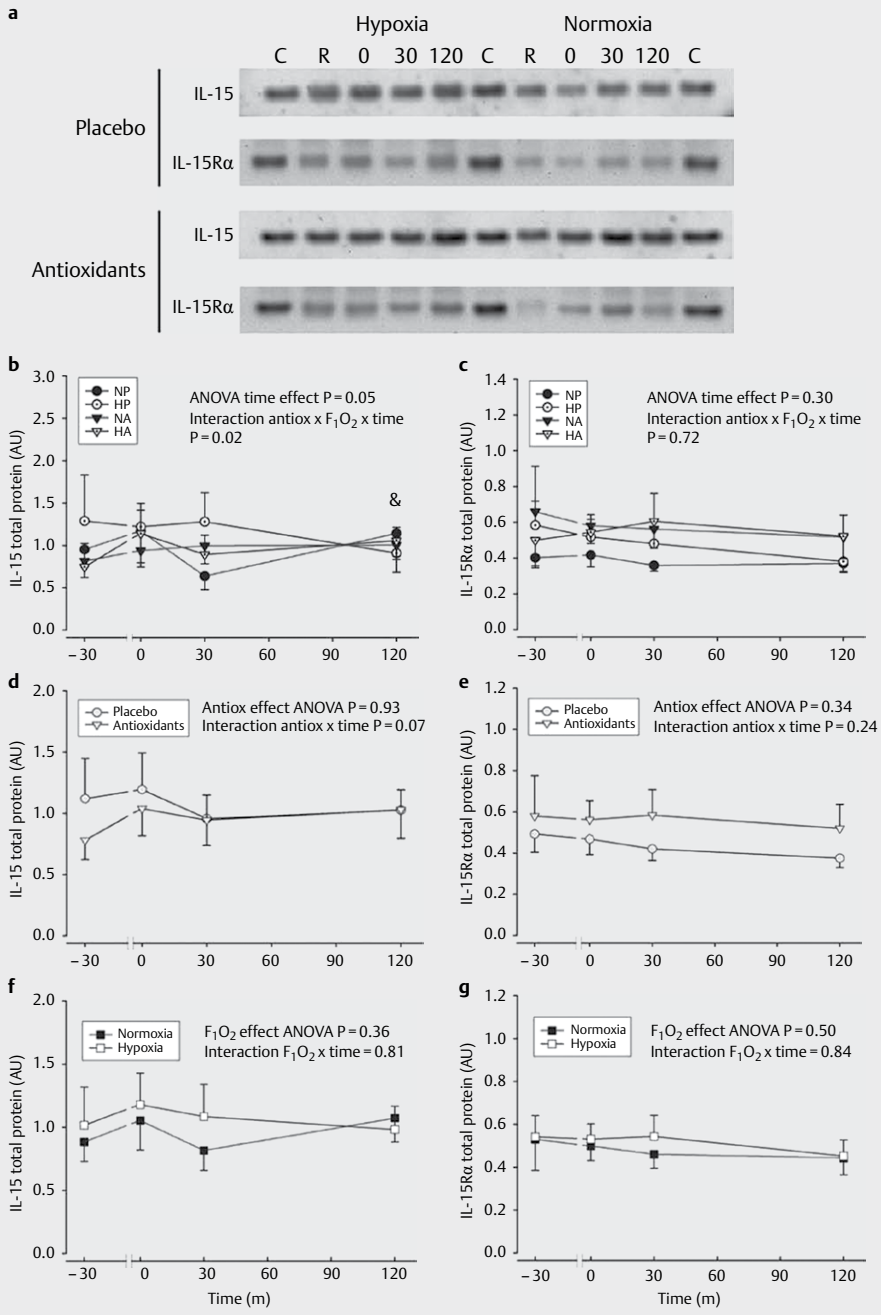
The present study demonstrates that antioxidant ingestion attenuates oxidative stress [15, 19, 20] and glycolytic rate in response to high-intensity exercise [17, 18], facilitating the expression of IL-15 protein, and possibly, IL-15-mediated signaling through STAT3 in human skeletal muscle. Moreover, skeletal muscle IL-15 expression was inversely associated with the magnitude of the oxygen deficit, which represents the total amount of energy provided by phosphagens (phosphocreatine and ATP) and the glycolysis (anaerobic). Since the administration of antioxidants reduced the glycolytic rate, these findings suggest that the high glycolytic rate attained during sprint exercise, and possibly associated excessive RONS production, as indicated by the increased protein carbonylation [17], blunts the skeletal muscle IL-15 protein expression.

In rodents, both endurance [3, 24, 25] and resistance exercise [14] promote IL-15 protein expression. In humans, exercise-induced skeletal muscle IL-15 expression has been suggested to be stimulated in an intensity-dependent manner [27]; however, the IL-15 response to high-intensity exercise has not been previously investigated. In the current study, skeletal muscle IL-15 protein expression was explored in response to a 30-s all-out Wingate test, which elicits greater metabolic perturbation compared to previous exercise models [21, 23, 27], mainly when the sprints are performed in severe acute hypoxia [2, 19]. Since IL-15 production was

▶ **Table 1** Ergospirometric responses during a 30-s all-out sprint (Wingate test) in normoxia (P_IO_2 = 143 mmHg) and hypoxia (P_IO_2 = 75 mmHg), after the ingestion of antioxidants or placebo.

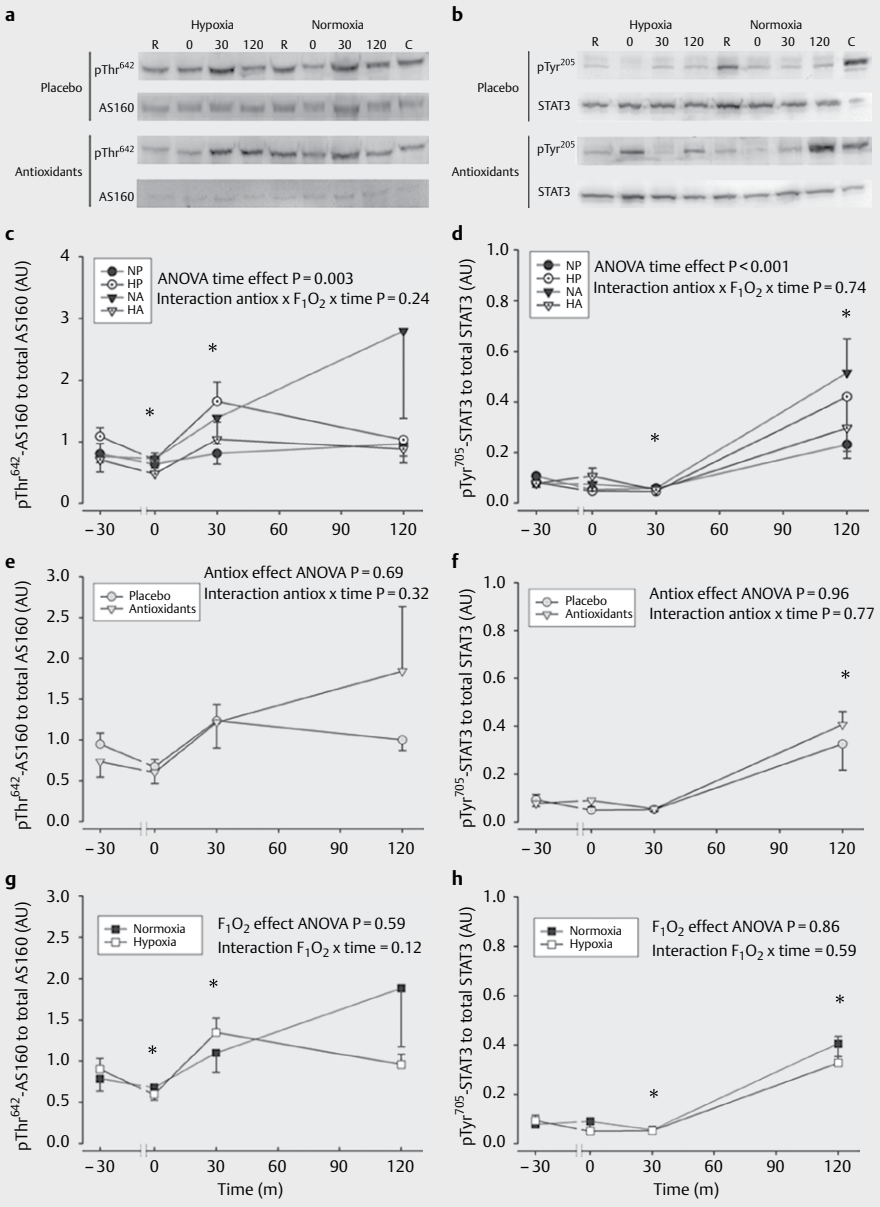
	Placebo		Antioxidants		Antiox	F_IO_2	Antiox x F_IO_2
	Hypoxia	Normoxia	Hypoxia	Normoxia			
W_{peak} (W)	944 ± 131	999 ± 129	959 ± 115	979 ± 114	0.875	0.162	0.478
W_{mean} (W)	540 ± 69 *	575 ± 61	547 ± 64	572 ± 61	0.813	0.031	0.159
O_2 Demand ($L \cdot min^{-1}$)	7.830 ± 0.765 *	8.390 ± 0.798	8.137 ± 0.920	8.257 ± 0.838	0.595	0.039	0.126
Accumulated VO_2 (L)	0.847 ± 0.123 *	1.192 ± 0.406	0.740 ± 0.328 †	1.207 ± 0.391	0.531	0.002	0.277
O_2 deficit (L)	3.068 ± 0.399 *	3.003 ± 0.498	3.328 ± 0.601 †	2.921 ± 0.513	0.319	0.010	0.020
O_2 deficit/W_{mean} ($mL \cdot W^{-1}$)	5.7 ± 0.4	5.2 ± 0.8	6.1 ± 0.8	5.1 ± 0.7	0.461	0.005	0.048

Values are means ± SD. W_{peak}, peak power output during the Wingate test; W_{mean}, mean power output during the Wingate test; O_2 Demand, demand of O_2 during the Wingate test; Accumulated VO_2, amount of O_2 consumed during the 30-s Wingate test. * $P < 0.05$ Compared with normoxia placebo; † $P < 0.05$ Compared with normoxia antioxidants; $n = 9$.



▶ **Fig. 1** Skeletal muscle IL-15 and IL-15Rα responses to sprint exercise in normoxia vs. hypoxia and after placebo and antioxidant intake. Representative western blots for IL-15 and IL-15Rα protein expression in response to a single 30-s sprint exercise in normoxia vs. hypoxia, and after placebo or antioxidant intake **a**. IL-15 **b, d, f**, and IL-15Rα total protein expressions **c, e, g** before, immediately after, and 30 and 120 min following the end of a single 30-s all-out sprint exercise. **b** and **c** show F_IO_2 (normoxia vs. hypoxia) and antioxidants (antioxidants vs. placebo) comparison for IL-15 and IL-15Rα protein expressions, respectively. NP, normoxia placebo (black circles); HP, hypoxia placebo (open circles, F_IO_2: 0.105); NA, normoxia antioxidants (black triangles); HA, hypoxia antioxidants (open triangles; F_IO_2: 0.105). **d** and **e** show antioxidant (antioxidants vs. placebo) comparison for IL-15 and IL-15Rα protein expression, respectively. Placebo, the 2 placebo conditions averaged (gray circles); Antioxidants, the 2 antioxidants conditions averaged (gray triangles). **f** and **g** show F_IO_2 (normoxia vs. hypoxia) comparison for IL-15 and IL-15Rα protein expression, respectively. Normoxia, the 2 normoxic conditions averaged (black squares); Hypoxia, the 2 hypoxic conditions averaged (open squares). AU, arbitrary units; & $P < 0.05$ hypoxia antioxidants in comparison to its resting value (interaction antioxidants x F_IO_2 x time). n = 9 for all variables.

increased only after the ingestion of antioxidants, which attenuate skeletal muscle protein carbonylation (a marker of oxidative stress) [19, 20], our findings suggest that RONS exert an inhibitory influence in skeletal muscle IL-15 expression. This concurs with the reported decrease of IL-15 mRNA in C2C12 exposed to H_2O_2 [11], indicating the potential existence of a negative feedback loop by



▶ **Fig. 2** Skeletal muscle pThr^{172}-AS160 to total AS160 ratio and pTyr^{705}-STAT3 to total STAT3 responses to sprint exercise in normoxia vs. hypoxia, and after placebo and antioxidant intake.
Representative western blots for Thr^{642}-AS160 phosphorylation and AS160 total protein expression **a** and Tyr^{705}-STAT3 phosphorylation and STAT3 total protein expression **b** in response to 30-s sprint exercise in normoxia vs. hypoxia and after placebo or antioxidant intake. Thr^{642}-AS160 phosphorylation to AS160 total protein expression **c, e, g** and Tyr^{705}-STAT3 phosphorylation to total STAT3 total protein expression **d, f, h** before, immediately after, and 30 and 120 min following the end of a single 30-s all-out sprint exercise. **c** and **d** show F_IO_2 (normoxia vs. hypoxia) and antioxidants (antioxidants vs. placebo) comparison for AS160 and STAT3 phosphorylation to total ratios, respectively. NP, normoxia placebo (black circles); HP, hypoxia placebo (open circles, F_IO_2 : 0.105); NA, normoxia antioxidants (black triangles); HA, hypoxia antioxidants (open triangles; F_IO_2 : 0.105). **e** and **f** show antioxidants (antioxidants vs. placebo) comparison for AS160 and STAT3 phosphorylation to total ratios, respectively. Placebo, 2 placebo conditions averaged (gray circles); Antioxidants, 2 antioxidants conditions averaged (gray triangles). **g** and **h** show F_IO_2 (normoxia vs. hypoxia) comparison for AS160 and STAT3 phosphorylation to total ratios, respectively. Normoxia, 2 normoxic conditions averaged (black squares); Hypoxia, 2 hypoxic conditions averaged (open squares). AU, arbitrary units; * $P < 0.05$ in comparison to resting (ANOVA, time effect). n = 9 for all variables.

which intracellular RONS in skeletal muscle inhibits IL-15 production, which, if excreted, could exacerbate local RONS production by an autocrine mechanism [11].

The main difference between normoxia and hypoxia Wingate tests is the greater metabolic perturbation and the lower mean capillary PO_2 in hypoxia compared to normoxia [2]. The fact that, once the effects of RONS were attenuated with antioxidants, a neg-

ative correlation was observed between the oxygen deficit and the change in IL-15 production at 120 min after the sprints, is compatible with a negative influence of high anaerobic energy turnover on IL-15 production in human skeletal muscle.

It seems that a great metabolic perturbation does not elicit IL-15 expression in our experimental conditions. In agreement with our findings, no upregulation of IL-15 protein expression was observed after a strength training session [21, 23]. This type of training is also eliciting a remarkable activation of the anaerobic metabolism [5].

Although in rodent skeletal muscles IL-15 expression is stimulated by AMPK [3], in the present investigation, IL-15 expression was only stimulated after the administration of antioxidants, which blunted AMPK activation via Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation immediately after the sprint [20]. Without antioxidants and in normoxia, a single sprint exercise elicits AMPK activation via Thr^{172} phosphorylation at 30 min post-exercise in human skeletal muscle [19, 20]; however, when the sprint is performed in severe acute hypoxia [19], after antioxidant (current investigation) [20] or glucose administration [7], Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 is phosphorylated immediately post-exercise, blocking Thr^{172} phosphorylation. Interestingly, in the absence of AMPK activation, an upregulation of IL-15 after sprint exercise has been observed here. Thus, in human skeletal muscle IL-15 expression may not require AMPK activation in response to high-intensity exercise.

The signaling pathways triggered by IL-15 activation in the myocyte have not been delineated yet. In C2C12 cells, IL-15 activates JAK3, which phosphorylates STAT3 and promotes the translocation of $pTyr^{705}$-STAT3 to the myocyte nucleus [9]. In the present study, skeletal muscle produced IL-15 might have acted in an autocrine manner eliciting Tyr^{705} phosphorylation (activation) of STAT3 during the recovery period, as indicated by the association between the AUC of IL-15 and Ty^{705}-STAT3. In human skeletal muscle, STAT3 phosphorylation induced by leptin administration is associated with increased fat oxidation [32]. However, it remains unknown whether the IL-15 produced in skeletal muscle during contractions stimulates fat oxidation by an autocrine mechanism.

Since STAT3 may also elicit GLUT4 translocation and subsequent glucose uptake in skeletal muscle [12] , an effect that could be mediated by IL-15 according to cell culture and animal studies [3, 9, 29], we also examined whether there was an association between the changes in IL-15 and AS160 protein expression. The IL-15 expression increase observed after antioxidant ingestion was accompanied by an upregulation of AS160 phosphorylation at Thr^{642} after sprint exercise; nonetheless, several signals could have elicited the phosphorylation of AS160 [4, 31]. Until selective IL-15 blockers are available for human use, it remains unknown whether the concomitant upregulation of IL-15 and Thr^{642}AS160 phosphorylation is causal or not.

In conclusion, antioxidant administration facilitates skeletal muscle IL-15 protein expression after sprint exercise, especially in severe acute hypoxia, by attenuating the glycolytic rate and, possibly, by reducing a potentially negative influence of exercise-released RONS on IL-15 production. The current investigation also indicates that skeletal muscle IL-15 protein expression in response to sprint exercise does not require the activation of AMPK. The molecular signals triggering IL-15 protein expression in response to sprint exercise remain to be identified, but a reduced PO_2 during intense muscle contractions may play a crucial role in exercise-induced IL-15 production. The fact that IL-15 protein expression was associated with known downstream targets in skeletal muscle (STAT3) gives support to the autocrine action of IL-15 in human skeletal muscle.

Author Contributions

APL, CD, DMA, and JAC conceived and designed the experiment. CD, DMA, JAC, IPS, collected the data. APL, CD, DMA, IPS, MMR, AS, and JAC analyzed and interpreted the data. APL and JALC drafted the manuscript and DMA, APL and JAC prepared all figures. All authors read and approved the final version of the manuscript.

Acknowledgements

This study was supported by grants from the Ministerio de Educación y Ciencia (DEP2010-21866, DEP2015-71171-R, and FEDER), Programa Innova Canarias 2020 (P.PE03-01-F08), Proyecto del Programa Propio de la ULPGC (ULPGC 2015/05), III Convocatoria de Ayudas a la Investigación Cátedra Real Madrid-Universidad Europea de Madrid (2010/01RM), and Programa propio de la Universidad de Alcalá (CCG2015/BIO-069). Special thanks are given to José Navarro de Tuero for his excellent technical assistance and to Nicolas López Jessen for his correction of the English version of the manuscript.

Conflict of Interest

No conflict of interest has been declared by the authors.

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