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Exercise-induced AMPK activation and IL-6 muscle production are disturbed in adiponectin knockout mice

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ABSTRACT

Background: Adiponectin exhibits anti-inflammatory actions and is mainly expressed in adipose tissue. However, recent studies have shown that adiponectin can also be secreted by skeletal muscle fibers with autocrine and paracrine effects.
Objectives: To analyze the role of adiponectin in the metabolic and inflammatory response of skeletal muscle after acute exhaustive aerobic exercise.
Methods: C57BL/6 (WT) and adiponectin knockout (AdKO) mice underwent four days of treadmill running adaptation and at the fifth day, they performed an incremental maximum test to determine the maximum speed (Vmax). Acute exercise consisted of one hour at 60% Vmax. Mice were euthanatized 2 and 24 h after acute exercise session.
Results: Serum and gastrocnemius adiponectin increased after 2-hours of acute exercise. NEFA concentrations were lower in non-exercise AdKO, and decreased 2-hours after exercise only in WT. No differences were found in muscle triacylglycerol content; however, glycogen content was higher in AdKO in non-exercise (p-value = 0.005). WT showed an increase in AMP-activated protein kinase (AMPK) phosphorylation 2-hours after exercise and its level went back to normal after 24-hours. Otherwise, exercise was not able to modify AMPK in the same way as in AdKO. WT showed an increase in the phosphorylation of ACC (Ser79) 2-hours after exercise and return to normal after 24-hours of exercise (p-value < 0.05), kinects that was not observed in AdKO mice. IL-10 and IL-6 concentration was completely different among genotypes. In WT, these cytokines were increased at 2 (p-value < 0.01) and 24 h (p-value < 0.001) after exercise when compared with AdKO. NF-κBp65 protein and gene expression were not different between genotypes.
Conclusion: Adiponectin influences muscle metabolism, mainly by the decrease in exercise-induced AMPK phosphorylation, inflammatory profile and IL-6 in the muscle.

1. Introduction

Skeletal muscles are the major organ in a body that in the past were recognized for their biomechanical ability. Nowadays, it is known that the skeletal muscle can release a diversity of proteins, peptides and hormones, which can influence other tissues, changing their function responses, as a result, it is acceptable to recognize the skeletal muscle as an endocrine organ [1].

In this context, some researchers have found many related proteins produced from this tissue, called as myokines [2,3]. Exercise-induced muscle contractions, mainly aerobic ones, could influence the profile of myokines released [3,4], which can act by regulating anti-inflammatory response and catabolic pathways [4]. A protein released by the skeletal muscle in response to exercise is the widely researched interleukin-6 (IL-6) [5]. Studies have shown that this myokine have a metabolic function during exercise [5], influencing glycogen depletion from liver, promoting an increase in blood glucose levels, offering energy to skeletal muscle and another tissues [6].

Adiponectin was first identified as a protein produced only by adipose tissue, having anti-inflammatory and metabolic functions, such as glucose and fatty acid (FA) oxidation [7]. However, although almost all adiponectin is secreted from adipocytes, skeletal muscle fibers are also

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likely to release this protein. Previous studies have observed that adiponectin concentrations increase after acute exercise, mainly aerobic ones [8–10]. Indeed, in vitro and *in vivo* studies have shown that adiponectin can be secreted by skeletal muscle [11,12], exerting metabolic functions, such as insulin sensitization and glucose uptake [13].

Therefore, the main objective of the present study was to analyze the role of adiponectin in muscle metabolic and inflammatory phenotypes after a bout of aerobic exhaustive exercise. It was hypothesized that the lack of adiponectin can disrupt the metabolic and inflammatory phenotypes in skeletal muscle after acute exercise.

2. Materials and methods

2.1. Animals

The experimental procedures were approved by the Ethics Research Committee on Animal Use – CEUA, University of São Paulo, Institute of Biomedical Sciences (protocol 46/2016). Male C57BL/6J wild type (WT) and Adiponcetin knockout (AdKO) were obtained of The Jackson Laboratory, Sacramento, California, USA. Mice were housed in animal facility conditioned with 12-h:12-h light/dark cycles (lights on at 06:00) and allowed free access to normal chow (Nuvilab CR1, Nuvital, Colombo, Brazil) with *ad libitum* water supply.

2.2. Experimental groups

WT and AdKO mice aged 8–10 weeks of age were divided into two groups: exercise and non-exercise. Exercise groups were subdivided into two groups according to the time of euthanasia after exercise session: exercise 2 and 24 h. Each of the exercise groups was matched with a control group (non-exercise 2 and 24 h), due to possible circadian changes [14] (Fig. 1).

2.3. Exercise performance test

All animals were adapted to a treadmill for 4 days (10 min/day – 10 m/min) and at the fifth day, they performed a maximum speed test composed of 5 min of warm-up at 10 m/min increasing 3 m/min each minute until exhaustion detected by biomechanical alteration [15].

2.4. Acute exercise

After a 72-h resting period, exercise groups performed an exhaustive exercise session of 60% maximum velocity (Vmax) for 1 h. This session intensity and duration was chosen in accordance to previously studies involving alterations in healthy untrained subjects [16,17]. Treadmill speed initiated at 10 m/min during 10 min, and raised 1 m/min until reaching 60% Vmax. All exercise sessions initiated at 11:00 and 12:00 am [15].

Table 1
Sequences of forward and reverse primers used for RT-PCR.

Primer name	Left	Right
GAPDH	CAAGCTCATTTCCTGGTATGACA	GCCTCTCTTGCTCAGTGTCC
AdipoR1	CAGCCAAGGCTXAGGAAGA	CTTGCAGAGGAAGCGTCAG
AdipoQ	AGGAGATGCAGGTCTTCTTGGT	TCTCCAGGCTCTCCTTTCCT
Stat3	ATGCGGAGAAGCATTGTAG	CTTCCAGTCAGCCAGCTCTT
F4/80	GAATCCTGTGAAGATGTGGATG	GGCATGAGCAGCTGTAGGA
AMPK	AGCCGACTTTGGTCTTTCAA	GCCTGCGTACAATCTTCCTG
IL-6	GTTGTGCAATGGCAATTCTG	CCAGTTTGGTAGCATCCATC
NFKB	CCAACTGGCAGGTATTTGAC	GCTGCTTCATGTCCCCTTG

Table 2
Adiposity index and performance in maximum speed test between genotypes.

Variable	WT	AdKO	p-value
Adiposity index (%)	1.82 ± 0.07	1.63 ± 0.05	0.031
Muscle index (%)	1.11 ± 0.10	1.07 ± 0.20	0.365
Max speed ($m.min^{-1}$)	23.70 ± 0.33	22.70 ± 0.44	0.088

Data were presented as mean ± standard deviation. AI: adiposity index; Max speed: maximum speed. n = 28 mice per genotype.

2.5. Euthanasia

Animals were euthanized 2 and 24 h after the exercise session with their respectively non-exercise peers. All groups were fasted for 6 h during euthanasia. Mice were weighted and adipose tissues (epididymal, subcutaneous and retroperitoneal), gastrocnemius and soleus muscles were collected and weighted. Gastrocnemius and soleus muscles were used to calculate the muscle index. The adiposity index (AI) was calculated by the sum of white adipose tissue deposits divided by body mass × 100 and was expressed as adiposity percentage [18].

2.6. Metabolic profile

Glucose, triacylglycerol and lactate were analyzed by colorimetric method, obtained from Labtest® (Labtest, Lagoa Santa, MG, Brazil), Brazil, and non-esterified fatty acids (NEFA) were analyzed using WAKO® kit (Wako Diagnostic, Richmond, VA), according to manufacturer's instructions.

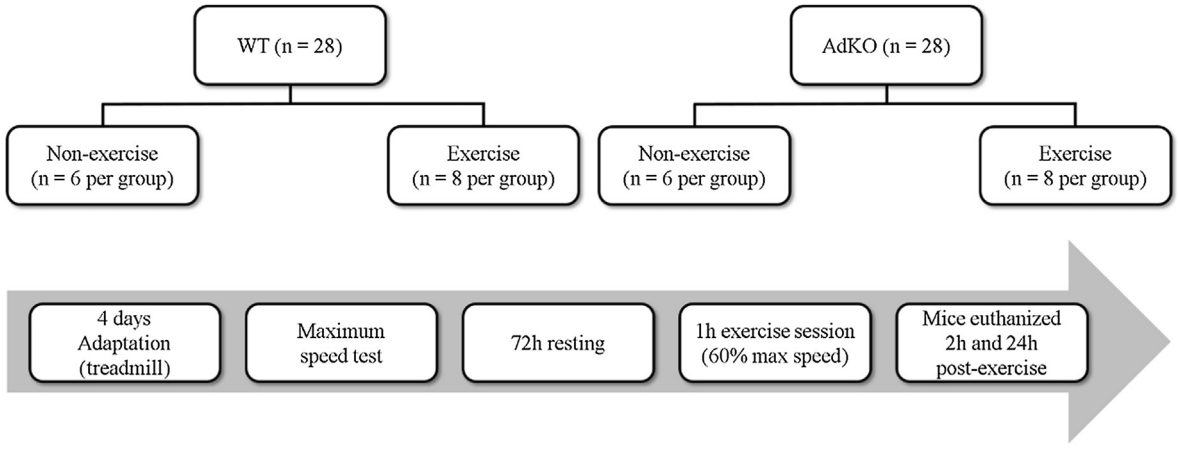

Fig. 1. Experimental design.

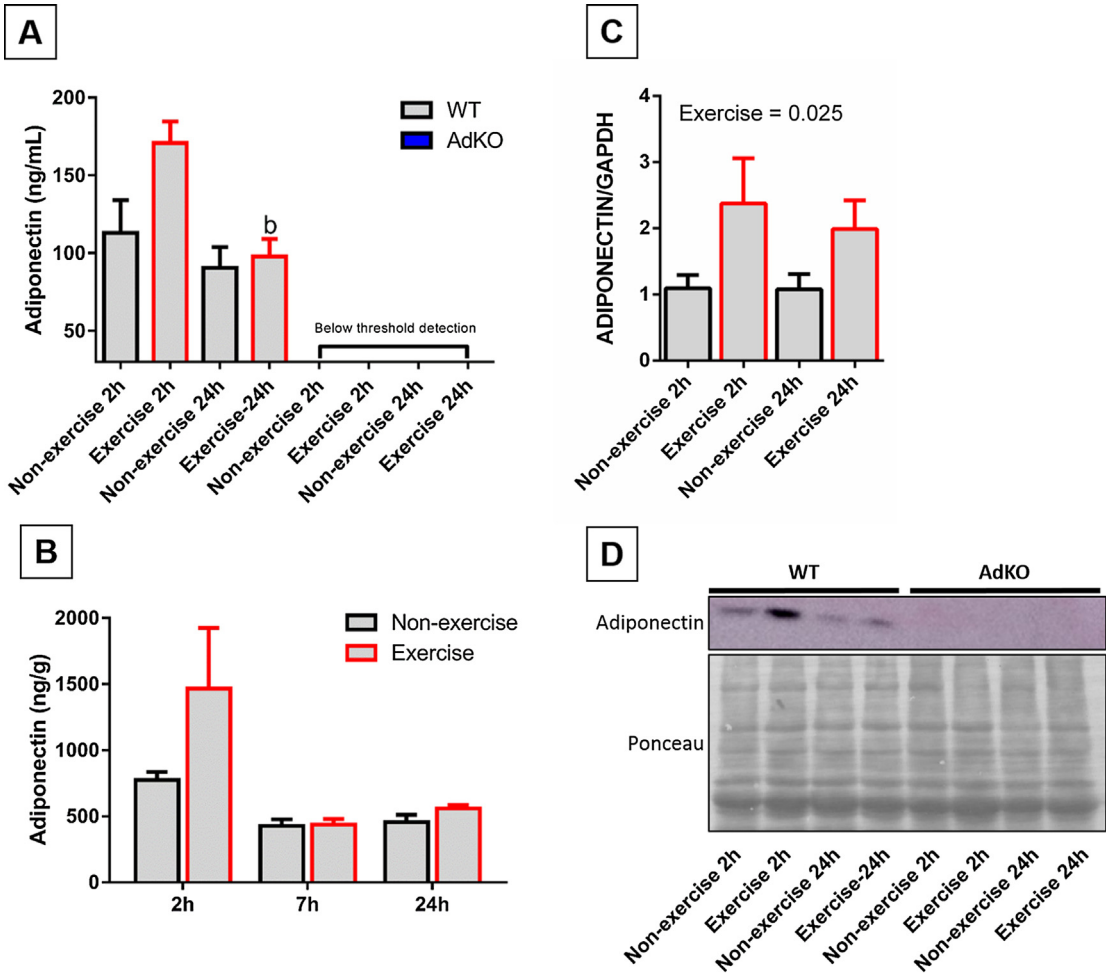

Fig. 2. Effect of acute aerobic exercise on adiponectin protein content and gene expression in serum and gastrocnemius muscle of AdKO and WT mice. (A) Serum adiponectin concentration. (B) Gastrocnemius muscle adiponectin concentration in WT mice. (C) Gastrocnemius muscle adiponectin gene expression in WT mice. (D) Representative immunoblot for adiponectin protein expression in gastrocnemius muscle. Data were presented as mean ± standard error. b = significantly different from exercise 2 h within the genotype. n = 4–8 mice per group.

2.7. Enzyme-linked immunosorbent assay

Gastrocnemius muscle samples (80–100 mg) were carefully homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid at pH 7.4) containing 10 mg/ml protease and phosphatase inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Homogenates were centrifuged and the supernatant was used to determine the protein concentration via Bradford assays (Bio-Rad, Hercules, CA), and the protein levels of adiponectin (31.3–2000 pg/mL), IL-6 (15.6–1000 pg/mL), IL-1β (15.6–1000 pg/mL), IL-10 (31.3–2000 pg/mL) and TNF-α (31.3–2000 pg/mL) were measured by ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN). According manufacturer, no cross-reactivity or interference were found with these proteins.

2.8. Western blot

The gastrocnemius muscle was homogenized and protein concentrations were measured as previously described. Aliquots of each sample with the same total protein concentration (30 mg) were then diluted in Laemmli buffer (10x), submitted to electrophoresis on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred from the gel to a nitrocellulose membrane. Membranes were incubated with antibodies against total and phosphorylated NF-κB (Ser536), total and phosphorylated acetyl-CoA carboxilase (ACC [Ser79]) total and phosphorylated AMP-activated protein kinase (AMPKα [Thr 172]) purchased from Cell Signaling (Danvers, MA). All primary antibodies were incubated overnight (4 °C) with 1:1000 dilution. Subsequently, membranes were incubated, by one hour, with anti-IgG antibody conjugated with peroxidase (1:5000 dilution) (Cell Signaling), followed by peroxidase substrate (SignalFire Plus ECL Reagent, Cell signaling), being immediately revealed (Amersham™ Imager 680, GE Healthcare Life Sciences). The intensities of bands were quantified by densitometry (ImageJ, 1.50i, National Institutes of Health) and normalized optical densitometry of bands incubated with ponceau (load control) [19].

2.9. RNA isolation, reverse transcription, and qRT-PCR

Total gastrocnemius muscle RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA), according to method described by Chomczynski and Sacchi [20]. Reverse transcription to cDNA was performed using the high-capacity cDNA kit (Applied Biosystems, Foster, CA, USA). Gene expression was evaluated by real-time PCR according to Higuchi et al. [21], using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, EUA) as fluorescent dye. Primer sequences are shown in Table 1. Gene expression quantification was carried out using the GAPDH gene as internal control [22], as previously described by Liu and Saint [23].

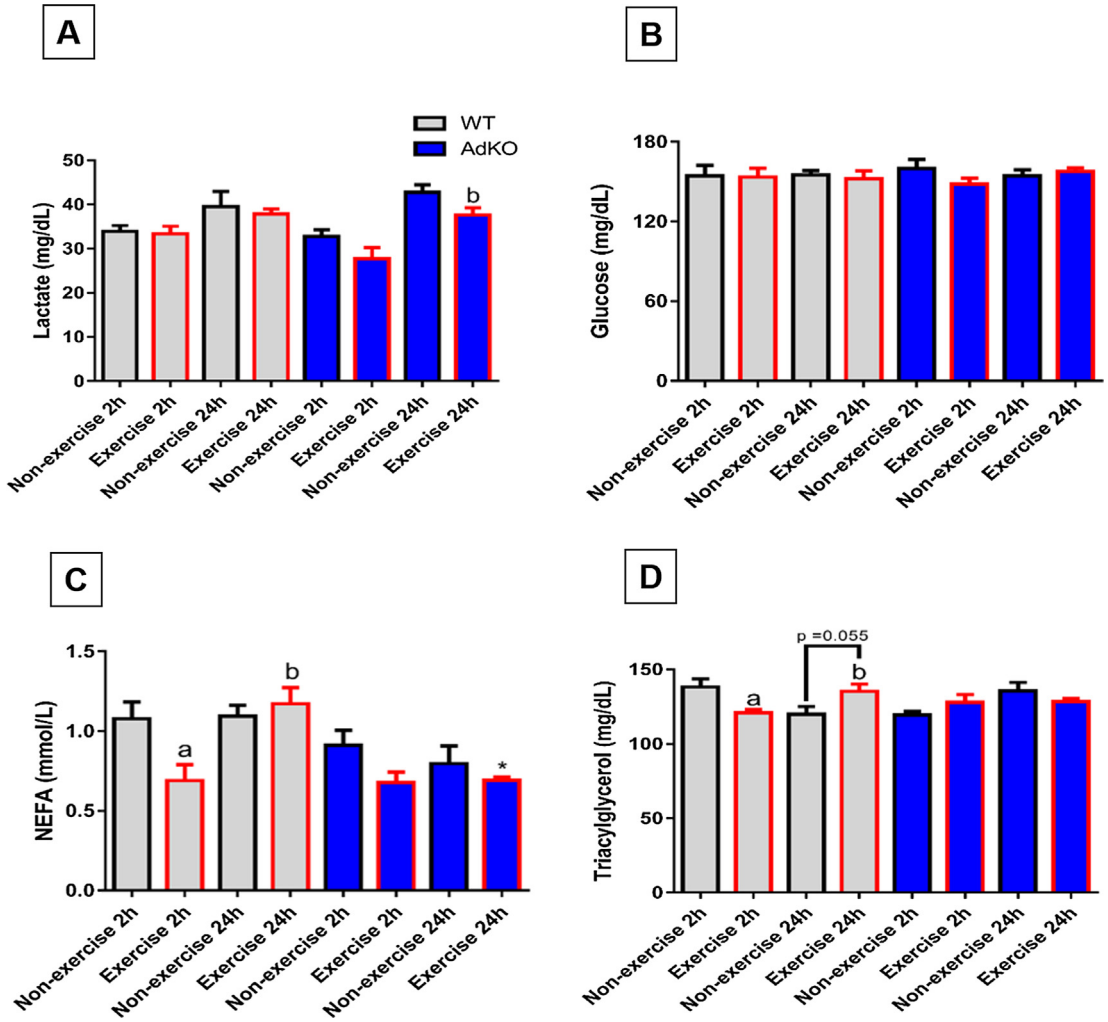

Fig. 3. Differences between genotypes in metabolic profile. (A–D) Exercise-mediated changes in serum lactate, glucose, NEFA and triacylglycerol concentration. Data were presented as mean ± standard error. a = significantly different from non-exercise 2 h within the genotype. b = significantly different from exercise 2 h within the genotype. * = significantly different between given genotype/time n = 6–8 mice per group.

2.10. Histological analysis

For histological analysis, gastrocnemius muscle was collected and fixed under a cork disk, with OCT (Tissue-Tek®, Sakura® Finetek, CA, USA) and Tragacanth (Sigma Aldrich, MO, USA) in the vertical position and frozen in liquid nitrogen. Cross-sections (10 μm-thick) were obtained on cryostat, mounted on StarFrost hot slides (Knittel Glass®, Braunschweig, Germany) and stained with hematoxylin-eosin (HE) to describe morphology, Sudan Black (SB) to evaluate lipid deposition, and periodic acid-schiff (PAS) to visualize glicogen deposits. Images were captured on a Nikon Eclipse E800 microscope. ImageJ software (ImageJ, 1.50i, National Institutes of Health) was used to quantify the average mean intensity of SB and PAS.

2.11. Triacylglycerol content in gastrocnemius muscle

The extraction of lipids from gastrocnemius muscle used the Folch and Sakura [24] method. Briefly, chloroform and methanol at ratio of 2:1 (v/v) were added to the sample placed into glass tube and homogenized. Samples were centrifuged at 2.000 rpm for 10 min. The organic lower phase was collected and added of 2 ml of water. This mixture was centrifuged at 1.500 rpm for 5 min. Then, the extracted lipids were collected and naturally dried. Samples were reconstituted in 300 ml of Triton 3% for storage. Triacylglycerol concentrations were analyzed by colorimetric method (Labtest®, Lagoa Santa, MG, Brazil).

2.12. Statistical analysis

Results are presented as mean ± SEM (standard error of the mean), and statistical differences between groups were determined by two-way ANOVA and independent *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Acute aerobic exercise increases serum and protein adiponectin concentration

WT mice showed increased adiposity index when compared with AdKO mice, without statistical difference in aerobic power (Table 2).

Two hours after aerobic exercise, there was an increase in serum adiponectin concentration (151%) (Fig. 2**A**). The same pattern was observed in gastrocnemius muscle adiponectin concentration, which increased two hours after exercise (189%) without changes in seven and 24 h after exercise (Fig. 2**B and D**). Regarding gene expression, adiponectin was upregulated two and 24 h after the exercise bout (Fig. 2**C**).

3.2. AdKO mice show different metabolic profile after acute aerobic exercise

After 24 h of exercise, lactate levels were increased in AdKO mice

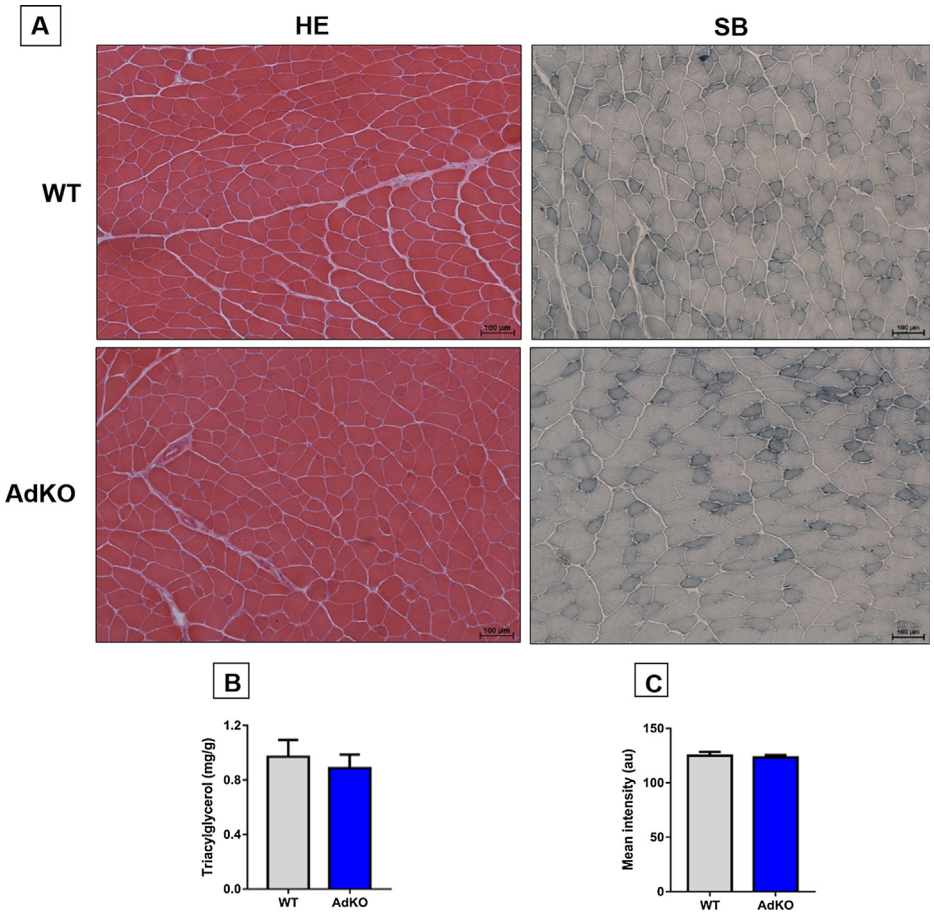

Fig. 4. Differences between genotypes in metabolic profile. (A) Histological aspects of WT and AdKO mice under basal conditions. (B) Triacylglycerol content of gastrocnemius muscle. (C) Average triacylglycerol intensity of Sudan Black staining (SB). Data were presented as mean ± standard deviation. n = 6–8 mice per group.

compared to exercise 2 h (p-value = 0.005) (Fig. 3**A**), whereas no difference between genotypes was observed regarding glucose levels (Fig. 3**B**). After two hours, aerobic exercise induced a more robust decrease in NEFA concentration in WT mice when compared to AdKO mice, and the values were restored 24 h later. (Fig. 3**C**). Similarly, triacylglycerol levels decreased two hours after exercise and was restored after 24 h of rest only in WT mice (Fig. 3**D**).

Sudan black staining and Folch analysis showed no differences in the triacylglycerol content of gastrocnemius muscle between genotypes (Fig. 4**A–C**).

Total and phosphorylated AMPK varied between genotypes (Fig. 5). AdKO mice at basal condition showed increased phospho-AMPK levels compared to WT mice (p-value = 0.030) (Fig. 5**A [upper board]).** WT mice tended to increase AMPK phosphorylation (Thr172) two hours after exercise, despite not statistical significant, and its level went back to normal after 24 h. On the other hand, exercise was not able to modify the AMPK phosphorylation similarly to AdKO mice. Moreover, AMPK activation was increased after 24 h of exercise in AdKO mice compared to WT mice (p-value = 0.033) (Fig. 5**A-B**). Relative AMPK gene expression also differed between genotypes, in which AdKO mice showed less expression 24 h after exercise (Fig. 5**C**).

At basal condition, AdKO mice showed increased in the phosphorylation of ACC (p-ACC) compared to WT mice (p-value = 0.002) (Fig. 5**D [upper board]).** In WT mice, the p-ACC:Total ratio was increased after 2-hours of exercise (p-value = 0.012) and returned to basal levels after 24-hours (p-value < 0.001) (Fig. 5**E**). Moreover, there was a statistical significant difference between genotypes after 2-hours of exercise (p-value = 0.002) (Fig. 5**E**).

3.3. Interleukin-6 protein expression is suppressed after exercise in AdKO mice

The acute effect of exercise was most different between genotypes in IL-6 protein expression. There was an increase two (p-value < 0.001) and 24 h (p-value < 0.001) after exercise in WT mice, whereas in AdKO mice, cytokine was completely suppressed (Fig. 6**A**). WT mice showed increased gene expression after two hours (p-value = 0.028) followed by a downregulation after 24 h of exercise when compared to AdKO mice (p-value = 0.004). In addition, AdKO mice showed a decrease in IL-6 gene expression two hours after exercise (p-value = 0.046) and an upregulation 24 h after exercise (p-value = 0.018) (Fig. 6**B**).

The quantitative glycogen content measurement in the skeletal muscle determined by PAS demonstrated that AdKO mice have higher glycogen content than WT animals (p-value = 0.005) (Fig. 6**C-D**).

3.4. Muscle inflammatory profile is disturbed in AdKO mice

Fig. 7 shows the inflammatory profile of the gastrocnemius muscle after exercise. The IL-1β concentration showed different pattern after exercise between genotypes, although no statistical differences were found (Fig. 7**A**). TNF-α was decreased two hours after exercise in AdKO mice when compared to WT mice (p-value = 0.014). In addition, cytokine levels decreased after 24 h of exercise only in WT mice (Fig. 7**B**). IL-10 concentration was completely different between genotypes. In WT mice, cytokine levels increased two (p-value < 0.001) and 24 h (p-value < 0.001) after exercise when compared to AdKO mice (Fig. 7**C**).

Regarding the inflammatory gene expression profile, STAT3 was decreased after 24 h compared to two hours in WT mice (p-value = 0.026), a behavior not observed in AdKO genotype (Fig. 7**D**).

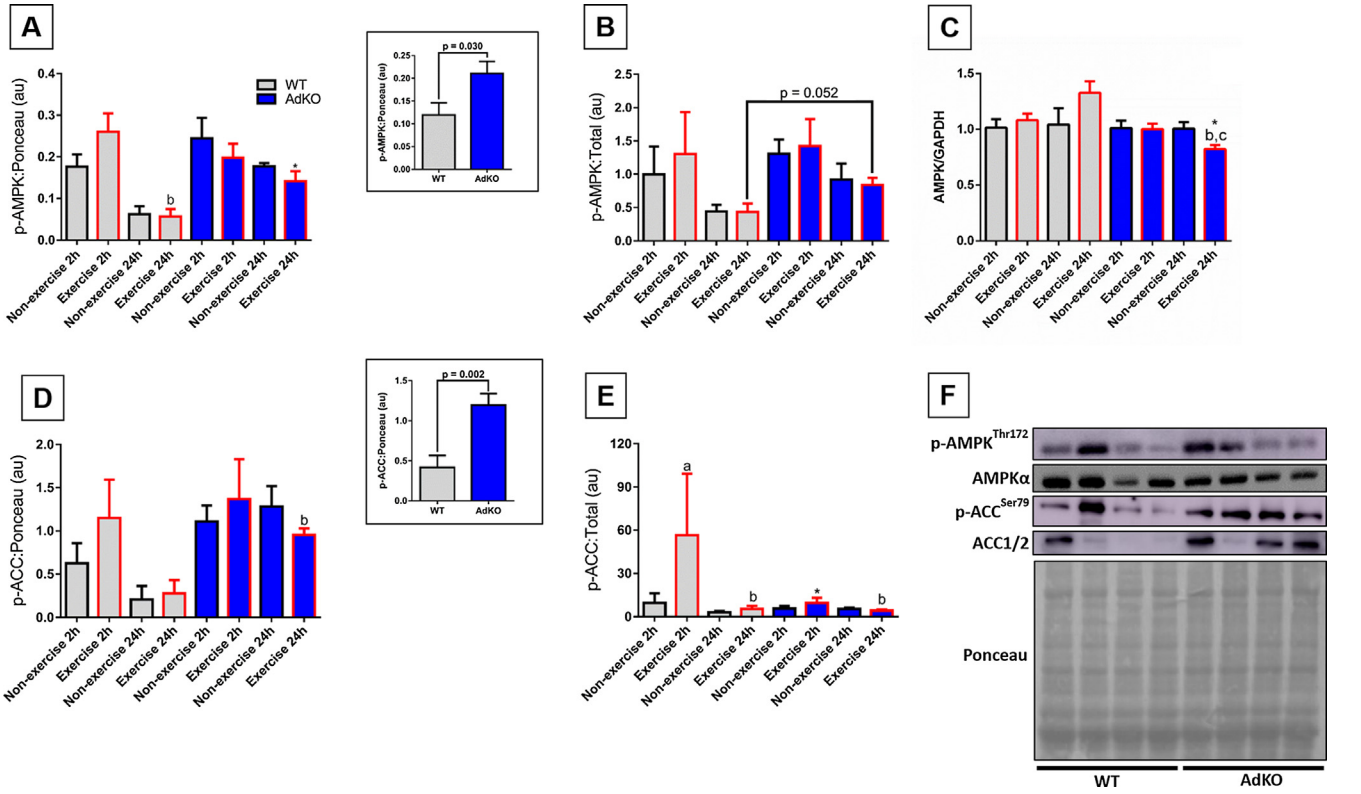

Fig. 5. AdKO mice show disturbed muscle AMPK activation after acute aerobic exercise. (A) Phosphorylated AMPKα (Thr 172) expression. (B) The ratio of p-AMPKα (Thr172) and total AMPKα protein expression. (C) AMPK relative gene expression. (D) Phosphorylated ACC (Ser79) expression. (E) The ratio of p-ACC (Thr172) and total ACC1/2 protein expression. (F) Representative immunoblot images. Data were presented as mean ± standard error. b = significantly different from exercise 2 h within the genotype. c = significantly different from non-exercise 24 h within the genotype. * = significantly different between given genotype/time n = 4 mice per group.

The relative gene expression of F4/80 was decreased after two hours of exercise only in AdKO mice (p-value = 0.032) (Fig. 7**E**). Finally, ADIPOR1 gene expression tended to decrease after 24 h of exercise in WT mice (Fig. 7**F**).

Although no statistical differences were found, WT mice showed different NF-κB protein expression pattern, such as lower NF-κB phosphorylation levels when compared to AdKO mice (Fig. 8**A**) and decrease in NF-κB content after exercise (two and 24 h), whereas no change in AdKO mice was observed (Fig. 8**B-C**). NF-κB gene expression was not different between genotypes after the exercise bout (Fig. 8**D**).

4. Discussion

In the present study, it was demonstrated that adiponectin was increased in serum and muscle after acute exercise featuring it as a myokine. Furthermore, AdKO mice presented disturbed metabolic and inflammatory phenotypes after acute aerobic exercise.

Several studies have shown that after acute exercise, mainly exhaustive ones, serum adiponectin contents increase above basal levels [8–10]. Similarly, it was found that two hours after exercise, adiponectin concentrations increased not only in serum but also in skeletal muscle. To confirm whether adiponectin was being produced by skeletal muscle, the gene expression analysis was performed and an increase in adiponectin gene expression after 2 and 24 h of exercise was also found. Therefore, this is the first study to show that physical exercise can stimulate adiponectin secretion in skeletal muscle. The study by Krause, et al. [11] was the first to identify in *in vitro* and *in vivo* assays that adiponectin is a protein produced by skeletal muscle, mainly in fibers with high intramyocellular lipids. Indeed, adiponectin can be secreted by skeletal muscle cells [25], especially under PPAR-γ agonist treatment [25,26], featuring this protein as a bona fide myokine, which could play a role in the improvement of metabolism by increasing glucose uptake and insulin sensitivity [25,26]. However, it is not yet clear how acute exercise increases systemic adiponectin levels, but it is known that hormonal changes that accompany physical exercise, such as circulating catecholamine and insulin concentrations, may be responsible for these changes [8,27,28].

Although is well known the benefits of adiponectin on glucose metabolism [25], its lack had little effects on lactate and glucose changes after the bout of exercise in both genotypes. But, the increased on lactate concentration 24 after the exercise session found only on AdKO suggesting that this mice increased the glucose utilization in glycolytic pathway. While, we observed that post-exercise NEFA changes were more robust in WT mice, demonstrating reduction of this substrate. This pattern was not observed in AdKO mice. Acute exercise at moderate-intensity (~65% VO_2max) utilizes oxidative metabolism as its predominant resource of energy, leading to fatty acid mobilization and oxidation by skeletal muscle. Therefore, since we showed that AdKO mice displayed lower serum NEFA levels, we expected that in these mice the amount of intramyocellular lipids was increased, as already describes by Krause, et al. [11], however, it was not confirmed by histological and triacylglycerol content analysis. These metabolic results showed that with reduction on adiposity index of AdKO and no changed in NEFA levels, probably this genotype is less efficient in fatty acid utilization as skeletal muscle substrate, but this mice are most efficient in glucose utilization for maintenance of energetic production on skeletal muscle.

Although in vitro studies indicate that in adipocytes adiponectin reduces lipolysis, the increased amount of glycogen observed in AdKO mice may have spared the utilization of fatty acids by muscle, thus reducing the lipolysis rate in adipose tissue [29].

Another key regulator factor of lipid metabolism that could be

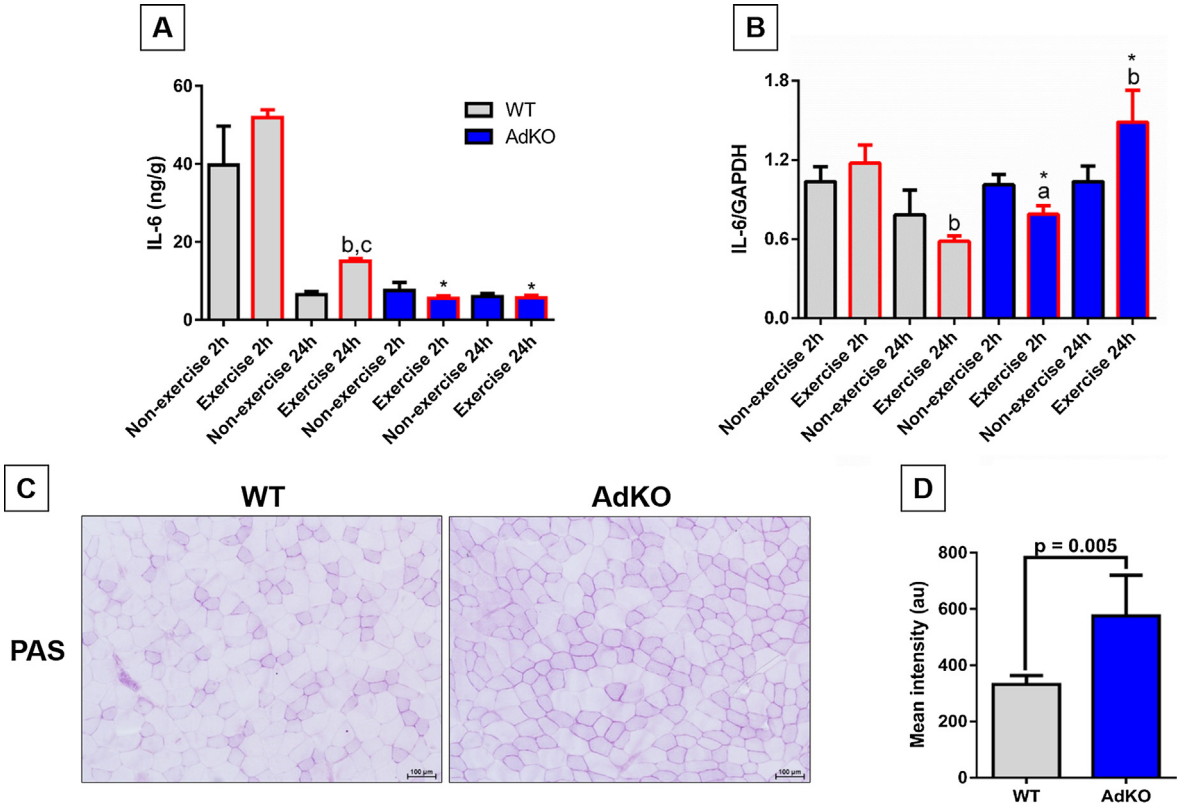

Fig. 6. IL-6 protein expression was suppressed after aerobic exercise, and its gene expression is increased in the gastrocnemius muscle. (A) IL-6 protein expression. (B) IL-6 relative gene expression. (C) Glycogen content measured by Periodic acid–Schiff (PAS) staining from the gastrocnemius muscle. (D) Average PAS intensity. Data were presented as mean ± standard error. a = significantly different from non-exercise 2 h within the genotype. b = significantly different from exercise 2 h within the genotype. c = significantly different from non-exercise 24 h within the genotype. * = significantly different between given genotype/time n = 6–8 mice per group.

involved in the differences in NEFA changes between genotypes is AMPK. AMPK is a heterotrimeric complex constituted by a catalytic α subunit and regulatory β and γ subunits, and it is well described as an energy sensor that is activated by falling cellular energy status, signaled by rising AMP/ATP ratio [16,17]. Here we showed that AdKO mice had increased levels of phospho-AMPK^{Thr172} during basal conditions when compared with WT. Pharmacological and exercise-induced AMPK activation enhance fatty acid oxidation [30–32], fact that could explain lower NEFA levels without muscle triacylglycerol accumulation in our results [33]. In fact AdKO mice paradoxically have an increase of β-oxidation metabolism [29]. Otherwise, Ritchie, et al. [31] produced divergent results, which AdKO mice and WT showed similar AMPK activation immediately after acute exercise. We used controls non-exercise mice for each time and genotype, ensuring better measurement of exercise effects' on metabolism and inflammation, since circadian rhythms influences these parameters [14].

AMPK is rapidly phosphorylated in response to exercise-induced muscle contraction [34,35]. In this study we demonstrated that activation of AMPK by exercise was disrupt in AdKO mice. These mice showed a different pattern of AMPK phosphorylation after exercise, consisting of a decrease in phospho-AMPK^{Thr172} after exercise, besides higher levels when compared with WT, mainly 24-hours post-exercise. Therefore, we believed that genetic background of AdKO mice could be upregulating AMPK activation during basal conditions, since adiponectin is a well-known AMPK activator in muscle [30,36], and therefore, could decrease its sensitivity to exercise-mediated phosphorylation.

Another well described function of activated AMPK is the inhibition of ACC activity, through its phosphorylation at Ser^{79} site [17,37,38], upregulating FA oxidation [17]. We found that p-ACC:Total ratio was increased after 2-hours of exercise only in WT mice, but in both genotypes the values returned to basal after 24-hours. Therefore, AdKO mice seem to be less sensitivity to AMPK-induced ACC inactivation. Studies have shown similar outcomes by which activation of AMPK through pharmacological or exercise-induced indeed suppress ACC activity, therefore reducing malonyl-CoA levels relieving CPT-I inhibition which allows FA to be subsequently oxidized [39–41].

IL-6 muscle concentrations were also completely blunted after exercise in AdKO mice, whereas upregulated 2 and 24 h post-exercise in WT mice. The difference in IL-6 response among genotypes may be related to glycogen concentrations in basal conditions, since there is a relationship between muscle glycogen stores and the IL-6 response by exercise [6]. Low muscle glycogen content stimulates IL-6 production, thus favoring the pattern observed in wild type animals.

Pedersen and Febbraio [5] showed that exercise-induced muscle contraction stimulate IL-6 release from muscle fibers, which can influences on liver glycogen depletion, promoting an increase levels of glucose on the blood, as well as, muscle glucose uptake and fat oxidation, through respectively, Akt and STAT3-AMPK signaling [6,42]. We did not found an increase in STAT3 gene expression two hours after exercise. Another pathway that could be related to the AMPK-IL-6 pathway is the calcium signaling. The IL-6 treatment in myotubes induces the Ca^{2+} dependent pathway, and the calcium/calmodulin-dependent protein kinase II, the kinase sensible to Ca^{2+} is an upstream activator kinase of AMPK. This pathway is related to p38-mitogen-activated protein kinase (MAPK) and we did not investigated this via [43,44]. Knudsen, et al. [45] showed that the absence of IL-6 in skeletal muscle decrease AMPK phosphorylation. In fact, in WT mice the kinetic of IL-6 protein and AMPK phosphorylation is very similar, pattern that was disrupted in AdKO. We also observed in WT mice that after 24-

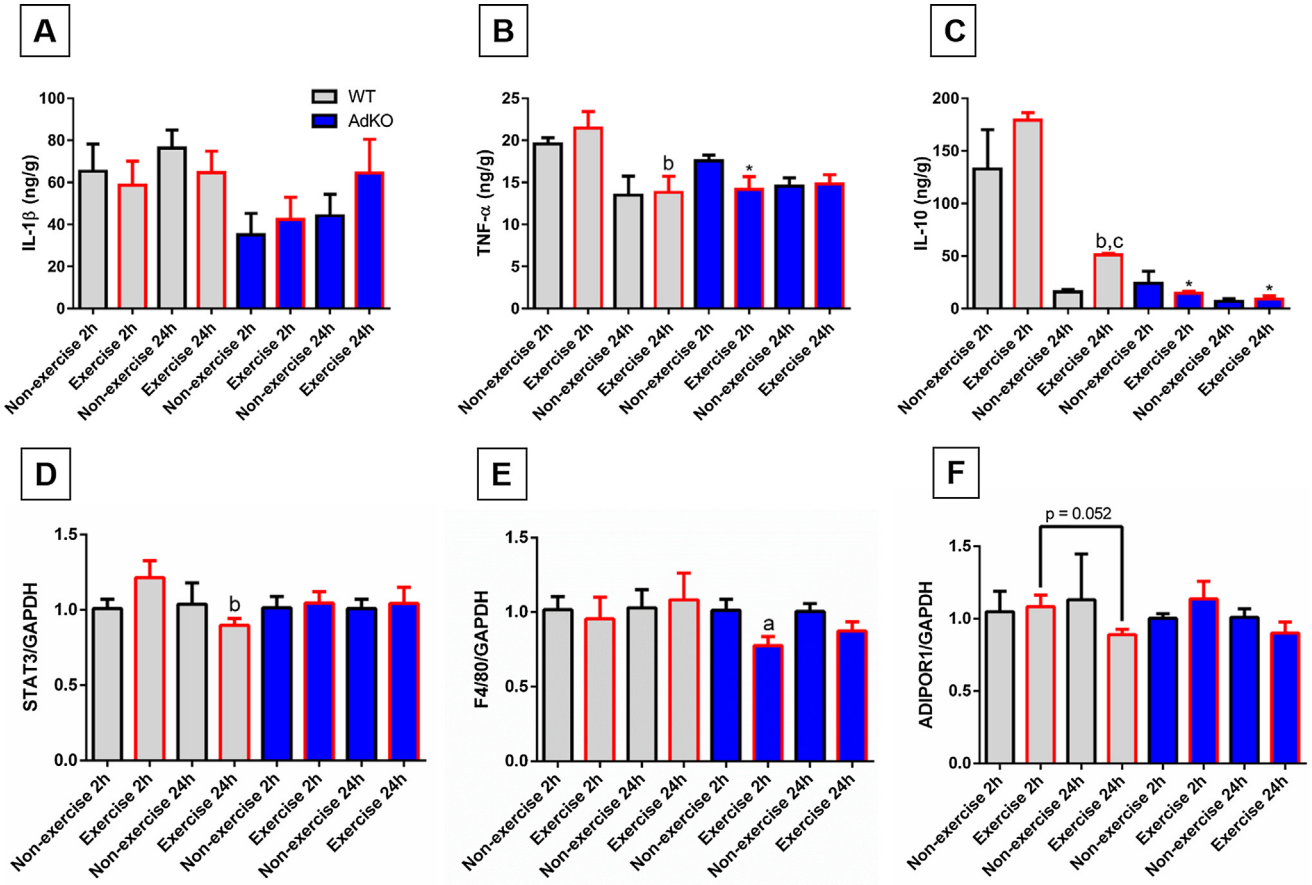

Fig. 7. Acute exercise promotes different muscle inflammatory phenotype between WT and AdKO mice. (A–C) Cytokine content of gastrocnemius muscle. (D–F) Gene expression of genes related to inflammation. Data were presented as mean ± standard error. a = significantly different from non-exercise 2 h within the genotype. a = significantly different from non-exercise 2 h within the genotype. b = significantly different from exercise 2 h within the genotype. c = significantly different from non-exercise 24 h within the genotype.* = significantly different between given genotype/time n = 6–8 mice per group.

hours of exercise IL-6 levels still upregulated. Keller, et al. [6] showed that the peak of IL-6 gene expression occurs post 3-hours of exercise, meaning that it could be translated later. It is interesting that the IL-6, at least in C2C12 and human myotubes, induces the hyper-expression of IL-6 mRNA, leading to a positive feedback [43].

Muscle contraction-induced IL-6 production can be regulated by increase in cytosolic Ca^{2+}, which in turn activated kinases leading to activation of NFAT transcription factor that induces IL-6 transcription [5]. Iwabu, et al. [30] points out the importance of adiponectin-AdipoR1 signaling in the increase of cytosolic Ca^{2+} concentrations. Therefore, exercise-induced IL-6 increase in skeletal muscle can be impaired by the decrease in cytosolic Ca^{2+} concentrations in AdKO mice. Moreover, myocyte enhancer factor-2 (MEF-2) seems to regulates IL-6 expression in muscle [46], which is impaired in the absence of adiponectin [47], a plausible mechanism by with IL-6 was blunted in AdKO mice. Interestingly, IL-6 gene expression, at least in part, was upregulated in AdKO mice, possible suggesting that post-translational modification could be affecting IL-6 protein expression in AdKO mice. However, more studies are needed to conclude that.

Regarding muscle inflammatory profile, we showed that AdKO presented different TNF-α, IL-10 and IL-6 production, and F4/80 gene expression, a well-known marker of macrophages. This type of immune cell play a role in exercise-induced muscle plasticity by two distinct ways, the early phase of muscle repair and posteriorly the resolution phase [48]. In the early stages of muscle repair, M1 polarized-macrophages secrete pro-inflammatory cytokines, such as TNF-α and IL-1β, to stimulate the cleaning of injured tissue [49,50]. In our study, WT mice showed slight increase in TNF-α 2 h post-exercise and its concentration decrease after 24 h of exercise, whereas, AdKO mice showed a decrease of TNF-α 2 h post-exercise, which was in line with lower expression of F4/80 at the same time. IL-1β concentration was not different between genotypes, however, AdKO mice displayed lower muscle amount throughout the time-course. We did not find difference in exercise-induced changes in NF-κB, suggesting that the production of inflammatory mediators were independent of this signaling.

Posteriorly, during the resolution phase, macrophages polarized to M2 phenotype secrete immunomodulatory cytokines, IL-10 and TGF-β, acting improving tissue repair and regeneration [50]. Here we showed that in AdKO mice IL-10 production was totally suppressed after exercise, while upregulated in WT mice. Classic and recent studies have shown an increase in IL-10 post-exercise [51,52], that seems to regulated muscle regeneration [50,53,54]. Interestingly, adiponectin play a role in muscle regeneration through the stimulation of satellites cells [55–57]. Moreover, IL-10 seems to be almost all secreted by muscle-infiltrated macrophages [58], again suggesting that there is less macrophage recruitment in skeletal muscle of AdKO mice after acute exercise. Therefore, our results suggest that the lack of adiponectin can disrupt muscle regeneration after acute exercise by less macrophage-induced cytokines production.

Therefore, we conclude that adiponectin influences muscle metabolism, mainly by fatty acid–glucose shift like skeletal muscle substrate, decrease in exercise-induced AMPK phosphorylation, inflammatory profile and IL-6 muscle production following an acute aerobic exercise.

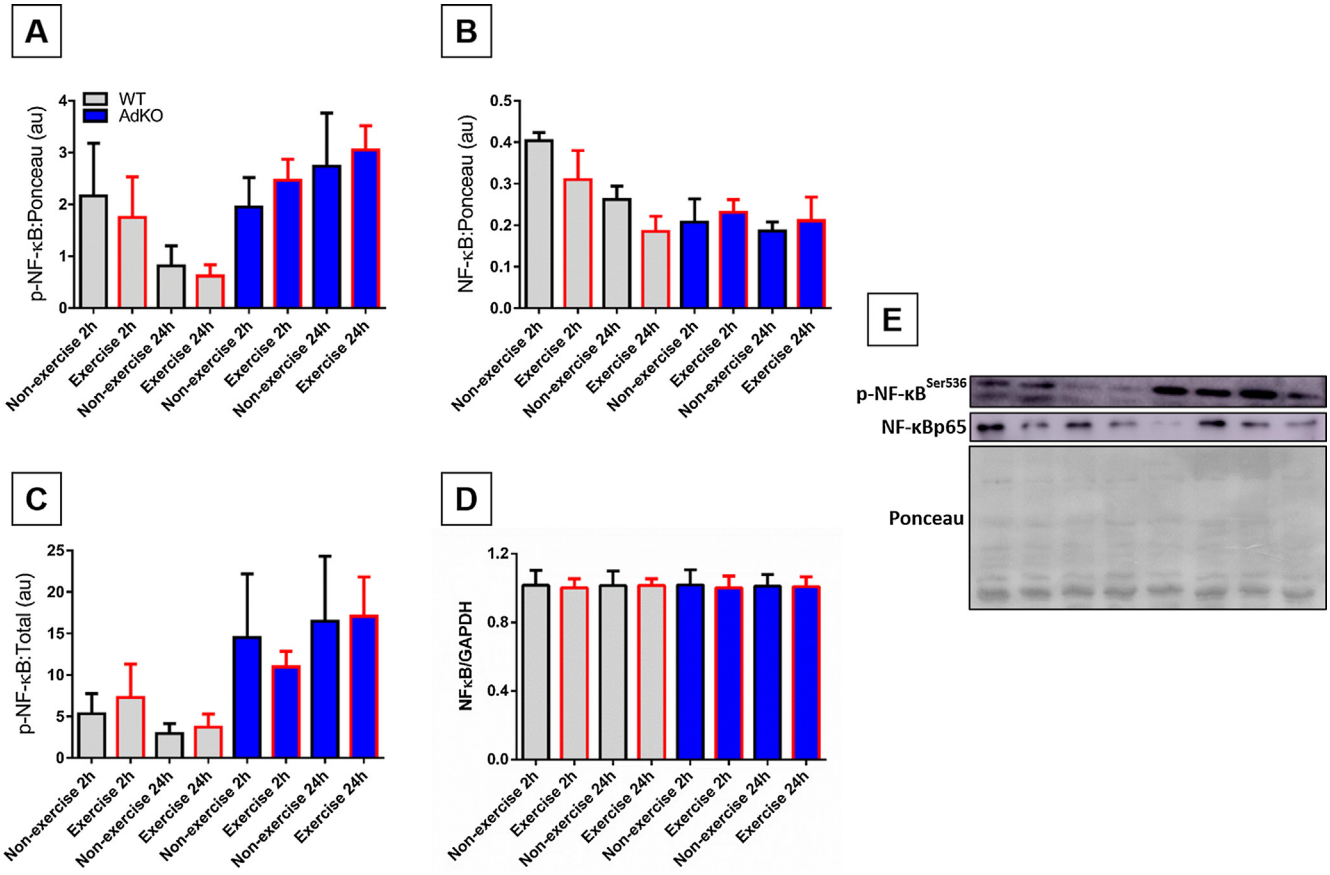

Fig. 8. AdKO mice show different NF-κB protein expression after aerobic exercise in the gastrocnemius muscle. (A–C) Total and phosphorylated NF-κBp65 (Ser536) protein expression. (D) NF-κB relative gene expression. (E) Representative immunoblot images. Data were presented as mean ± standard error. n = 4–6 mice per group.

5. Statement of ethics

The experimental procedures were approved by the Ethics Research Committee on Animal Use – CEUA, University of São Paulo, Institute of Biomedical Sciences (protocol 46/2016).

6. Disclosure statement

The authors have no conflicts of interest to declare.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References

[1] J. Giudice, J.M. Taylor, Muscle as a paracrine and endocrine organ, Curr. Opin. Pharmacol. 34 (2017) 49–55.
[2] S. Hartwig, S. Raschke, B. Knebel, M. Scheler, M. Irmler, W. Passlack, S. Muller, F.-G. Hanisch, T. Franz, X. Li, Secretome profiling of primary human skeletal muscle cells, Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1844 (2014) 1011–1017.
[3] S. Raschke, K. Eckardt, K.B. Holven, J. Jensen, J. Eckel, Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells, PloS One 8 (2013) e62008.
[4] A.M.W. Petersen, B.K. Pedersen, The anti-inflammatory effect of exercise, J. Appl. Physiol. 98 (2005) 1154–1162.
[5] B.K. Pedersen, M.A. Febbraio, Muscle as an endocrine organ: focus on muscle-derived interleukin-6, Physiol. Rev. 88 (2008) 1379–1406.
[6] C. Keller, A. Steensberg, H. Pilegaard, T. Osada, B. Saltin, B.K. Pedersen, P.D. Neufer, Transcriptional activation of the il-6 gene in human contracting skeletal muscle: influence of muscle glycogen content, FASEB J. 15 (2001) 2748–2750.
[7] P.E. Scherer, S. Williams, M. Fogliano, G. Baldini, H.F. Lodish, A novel serum protein similar to c1q, produced exclusively in adipocytes, J. Biol. Chem. 270 (1995) 26746–26749.
[8] J. Jurimae, P. Purge, T. Jurimae, Adiponectin is altered after maximal exercise in highly trained male rowers, Eur. J. Appl. Physiol. 93 (2005) 502–505.
[9] R.R. Kraemer, K.S. Aboudehen, A.K. Carruth, R.J. Durand, E.O. Acevedo, E.P. Hebert, L.G. Johnson, V.D. Castracane, Adiponectin responses to continuous and progressively intense intermittent exercise, Med. Sci. Sports Exerc. 35 (2003) 1320–1325.
[10] K.A. Simpson, M.A. Singh, Effects of exercise on adiponectin: a systematic review, Obesity 16 (2008) 241–256.
[11] M.P. Krause, Y. Liu, V. Vu, L. Chan, A. Xu, M.C. Riddell, G. Sweeney, T.J. Hawke, Adiponectin is expressed by skeletal muscle fibers and influences muscle phenotype and function, Am. J. Physiol.-Cell Physiol. 295 (2008) C203–C212.
[12] Y. Liu, G. Sweeney, Adiponectin action in skeletal muscle, Best Practice Res. Clin. Endocrinol. Metabol. 28 (2014) 33–41.
[13] M. Liu, F. Liu, Transcriptional and post-translational regulation of adiponectin, Biochem. J. 425 (2010) 41–52.
[14] K.L. Gamble, R. Berry, S.J. Frank, M.E. Young, Circadian clock control of endocrine factors, Nature Rev. Endocrinol. 10 (2014) 466–475.

[15] L.S. Silveira, G.D. Pimentel, C.O. Souza, L.A. Biondo, A.A.S. Teixeira, E.A. Lima, H.A. Batatinha, J.C. Rosa Neto, F.S. Lira, Effect of an acute moderate-exercise session on metabolic and inflammatory profile of PPAR-α knockout mice, Cell Biochem. Funct. 35 (2017) 510–517.
[16] S.-C. Lin, D.G. Hardie, AMPK: sensing glucose as well as cellular energy status, Cell Metab. (2017).
[17] H.M. O'Neill, G.P. Holloway, G.R. Steinberg, AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: implications for obesity, Mol. Cell. Endocrinol. 366 (2013) 135–151.
[18] S.M. Jeyakumar, A. Vajreswari, N.V. Giridharan, Chronic dietary vitamin a supplementation regulates obesity in an obese mutant WNIN/Ob rat model, Obesity 14 (2006) 52–59.
[19] M.A.S. Fortes, G.N. Marzuca-Nassr, K.F. Vitzel, C.H. da Justa Pinheiro, P. Newsholme, R. Curi, Housekeeping proteins: how useful are they in skeletal muscle diabetes studies and muscle hypertrophy models? Anal. Biochem. 504 (2016) 38–40.
[20] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[21] R. Higuchi, G. Dollinger, P.S. Walsh, R. Griffith, Simultaneous amplification and detection of specific DNA sequences, Nat. Biotechnol. 10 (1992) 413.
[22] D.J. Mahoney, K. Carey, M.-H. Fu, R. Snow, D. Cameron-Smith, G. Parise, M.A. Tarnopolsky, Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise, Physiol. Genom. 18 (2004) 226–231.
[23] W. Liu, D.A. Saint, Validation of a quantitative method for real time PCR kinetics, Biochem. Biophys. Res. Commun. 294 (2002) 347–353.
[24] J. Folch, J. Sakura, Preparation of the proteolipid apoprotein from bovine heart, liver and kidney, Biochimica et Biophysica Acta (BBA)-Protein Struct. 427 (1976) 410–427.
[25] Y. Liu, S. Chewchuk, C. Lavigne, S. Brûlé, G. Pilon, V. Houde, A. Xu, A. Marette, G. Sweeney, Functional significance of skeletal muscle adiponectin production, changes in animal models of obesity and diabetes, and regulation by rosiglitazone treatment, Am. J. Physiol.-Endocrinol. Metabol. 297 (2009) E657–E664.
[26] R.H. Amin, S.T. Mathews, H.S. Camp, L. Ding, T. Leff, Selective activation of PPARγ in skeletal muscle induces endogenous production of adiponectin and protects mice from diet-induced insulin resistance, Am. J. Physiol.-Endocrinol. Metabol. 298 (2010) E28–E37.
[27] S. Numao, Y. Katayama, Y. Hayashi, T. Matsuo, K. Tanaka, Influence of acute aerobic exercise on adiponectin oligomer concentrations in middle-aged abdominally obese men, Metab. Clin. Exp. 60 (2011) 186–194.
[28] P. Imbeault, I. Depault, F. Haman, Cold exposure increases adiponectin levels in men, Metab. Clin. Exp. 58 (2009) 552–559.
[29] K. Ma, A. Cabrero, P.K. Saha, H. Kojima, L. Li, B.H.-J. Chang, A. Paul, L. Chan, Increased β-oxidation but no insulin resistance or glucose intolerance in mice lacking adiponectin, J. Biol. Chem. 277 (2002) 34658–34661.
[30] M. Iwabu, T. Yamauchi, M. Okada-Iwabu, K. Sato, T. Nakagawa, M. Funata, M. Yamaguchi, S. Namiki, R. Nakayama, M. Tabata, Adiponectin and AdipoR1 regulate PGC-1α and mitochondria by Ca 2+ and AMPK/SIRT1, Nature 464 (2010) 1313.
[31] I.R. Ritchie, T.L. MacDonald, D.C. Wright, D.J. Dyck, Adiponectin is sufficient, but not required, for exercise-induced increases in the expression of skeletal muscle mitochondrial enzymes, J. Physiol. 592 (2014) 2653–2665.
[32] W. Winder, B. Holmes, D. Rubink, E. Jensen, M. Chen, J. Holloszy, Activation of amp-activated protein kinase increases mitochondrial enzymes in skeletal muscle, J. Appl. Physiol. 88 (2000) 2219–2226.
[33] W. Wu, J. Feng, D. Jiang, X. Zhou, Q. Jiang, M. Cai, X. Wang, T. Shan, Y. Wang, AMPK regulates lipid accumulation in skeletal muscle cells through FTO-dependent demethylation of n 6-methyladenosine, Sci. Rep. 7 (2017) 41606.
[34] W. Winder, D. Hardie, Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise, Am. J. Physiol.-Endocrinol. Metabol. 270 (1996) E299–E304.
[35] J.F. Wojtaszewski, P. Nielsen, B.F. Hansen, E.A. Richter, B. Kiens, Isoform-specific and exercise intensity-dependent activation of 5′-AMP-activated protein kinase in human skeletal muscle, J. Physiol. 528 (2000) 221–226.
[36] E. Tomas, T.-S. Tsao, A.K. Saha, H.E. Murrey, C. Cheng Zhang, S.I. Itani, H.F. Lodish, N.B. Ruderman, Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl–CoA carboxylase inhibition and AMP-activated protein kinase activation, Proc. Natl. Acad. Sci. 99 (2002) 16309–16313.
[37] G. Merrill, E. Kurth, D. Hardie, W. Winder, Aica riboside increases amp-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle, Am. J. Physiol.-Endocrinol. Metabol. 273 (1997) E1107–E1112.
[38] M.R. Munday, D.G. CampbellL, D. Carling, D.G. Hardie, Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-coa carboxylase, FEBS J. 175 (1988) 331–338.
[39] W. Winder, J. Arogyasami, I. Elayan, D. Cartmill, Time course of exercise-induced decline in malonyl-coa in different muscle types, Am. J. Physiol.-Endocrinol. Metabol. 259 (1990) E266–E271.
[40] W.W. Winder, Malonyl-coa–regulator of fatty acid oxidation in muscle during exercise, Exer. Sport Sci. Rev. 26 (1998) 117–132.
[41] S. Herzig, R.J. Shaw, AMPK: Guardian of metabolism and mitochondrial homeostasis, Nat. Rev. Mol. Cell Biol. 19 (2018) 121.
[42] B.K. Pedersen, M.A. Febbraio, Muscles, exercise and obesity: Skeletal muscle as a secretory organ, Nat. Rev. Endocrinol. 8 (2012) 457–465.
[43] C. Weigert, M. Düfer, P. Simon, E. Debre, H. Runge, K. Brodbeck, H.U. Häring, E.D. Schleicher, Upregulation of IL-6 mRNA by IL-6 in skeletal muscle cells: role of IL-6 mRNA stabilization and Ca2+-dependent mechanisms, Am. J. Physiol.-Cell Physiol. 293 (2007) C1139–C1147.
[44] A. Combes, J. Dekerle, N. Webborn, P. Watt, V. Bougault, F.N. Daussin, Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CAMKII phosphorylation in human skeletal muscle, Physiol. Rep. 3 (2015).
[45] S.H. Knudsen, L.S. Hansen, M. Pedersen, T. Dejgaard, J. Hansen, G.V. Hall, C. Thomsen, T.P. Solomon, B.K. Pedersen, R. Krogh-Madsen, Changes in insulin sensitivity precede changes in body composition during 14 days of step reduction combined with overfeeding in healthy young men, J. Appl. Physiol. 113 (2012) 7–15.
[46] E. Allen, P. Gray, A. Kollias-Pearson, E. Oag, K. Pratt, J. Henderson, S.R. Gray, The effect of short-duration sprint interval exercise on plasma postprandial triacylglycerol levels in young men, J. Sports Sci. 32 (2014) 911–916.
[47] K. Dadson, S. Turdi, S. Hashemi, J. Zhao, N. Polidovitch, S. Beca, P.H. Backx, J.C. McDermott, G. Sweeney, Adiponectin is required for cardiac MEF2 activation during pressure overload induced hypertrophy, J. Mol. Cell. Cardiol. 86 (2015) 102–109.
[48] E. Rigamonti, P. Zordan, C. Sciorati, P. Rovere-Querini, S. Brunelli, Macrophage plasticity in skeletal muscle repair, BioMed Res. Int. 2014 (2014).
[49] N. Kawanishi, T. Mizokami, H. Niihara, K. Yada, K. Suzuki, Macrophage depletion by clodronate liposome attenuates muscle injury and inflammation following exhaustive exercise, Biochem. Biophys. Rep. 5 (2016) 146–151.
[50] G.J. Koelwyn, D.F. Quail, X. Zhang, R.M. White, L.W. Jones, Exercise-dependent regulation of the tumour microenvironment, Nat. Rev. Cancer 17 (2017) 620–632.
[51] C. Cabral-Santos, J. Gerosa-Neto, D.S. Inoue, V.L.G. Panissa, L.A. Gobbo, A.M. Zagatto, E.Z. Campos, F.S. Lira, Similar anti-inflammatory acute responses from moderate-intensity continuous and high-intensity intermittent exercise, J. Sports Sci. Med. 14 (2015) 849.
[52] K. Ostrowski, T. Rohde, S. Asp, P. Schjerling, B.K. Pedersen, Pro-and anti-inflammatory cytokine balance in strenuous exercise in humans, J. Physiol. 515 (1999) 287–291.
[53] B. Deng, M. Wehling-Henricks, S.A. Villalta, Y. Wang, J.G. Tidball, Il-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration, J. Immunol. 189 (2012) 3669–3680.
[54] J.G. Tidball, S.A. Villalta, Regulatory interactions between muscle and the immune system during muscle regeneration, Am. J. Physiol.-Regul. Int. Compar. Physiol. 298 (2010) R1173–R1187.
[55] T. Fiaschi, E. Giannoni, M.L. Taddei, P. Chiarugi, Globular adiponectin activates motility and regenerative traits of muscle satellite cells, PloS One 7 (2012) e34782.
[56] T. Fiaschi, F. Magherini, T. Gamberi, P.A. Modesti, A. Modesti, Adiponectin as a tissue regenerating hormone: more than a metabolic function, Cell. Mol. Life Sci. 71 (2014) 1917–1925.
[57] T. Fiaschi, F.S. Tedesco, E. Giannoni, J. Diaz-Manera, M. Parri, G. Cossu, P. Chiarugi, Globular adiponectin as a complete mesoangioblast regulator: Role in proliferation, survival, motility, and skeletal muscle differentiation, Mol. Biol. Cell 21 (2010) 848–859.
[58] R. Furrer, P.S. Eisele, A. Schmidt, M. Beer, C. Handschin, Paracrine cross-talk between skeletal muscle and macrophages in exercise by PGC-1α-controlled BNP, Sci. Rep. 7 (2017) 40789.