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Effects of aerobic exercise on the inflammatory cytokine profile and expression of lipolytic and thermogenic genes in β_1-AR$^{-/-}$ mice adipose tissue

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ABSTRACT

Aim: Investigate the effects of moderate continuous aerobic exercise (MCAE) on the inflammatory cytokine profile and expression of lipolytic and thermogenic genes in β_1-AR$^{-/-}$ mice adipose tissue.
Main methods: Four- to five-month-old male wild type (WT) and β_1-AR$^{-/-}$ mice were divided into groups: WT control (WTc) and trained (WTt); and β_1-AR$^{-/-}$ control (β_1-AR$^{-/-}$c) and trained (β_1-AR$^{-/-}$t). Animals from trained groups were submitted to a MCAE regimen (60 min/day; 60% of maximal speed, 5 days/week) on a treadmill, for 8 weeks. After euthanasia, white epididymal (eWAT) and inguinal (iWAT) and brown (BAT) adipose tissues were dissected and used to determine: adiposity index; adipocyte histomorphometry; cytokine concentration; and gene expression. The content of fat, protein and water of the empty carcass was determined.
Key findings: MCAE reduced body weight, fat mass as well as iWAT and BAT adipocyte area in β_1-AR$^{-/-}$ animals. Aerobic exercise also diminished the concentrations of pro-inflammatory (IL-12p70, TNF-α, IL-6) and anti-inflammatory (IL-10) cytokines in adipose tissue (iWAT, eWAT or BAT) of β_1-AR$^{-/-}$ mice. However, MCAE had no effect on the expression lipolytic and thermogenic genes in β_1-AR$^{-/-}$ mice adipose tissue.
Significance: Alongside reductions in body weight, fat mass and adipocyte area eight weeks of MCAE improves the profile of inflammatory cytokines in β_1-AR$^{-/-}$ mice adipose tissue, despite no change in Lipolytic and thermogenic gene expression.

1. Introduction

Adipose tissue is primarily differentiated into white and brown. While in white adipose tissue (WAT) lipogenesis and lipolysis are primary metabolic activities to maintain body fat homeostasis [1], in brown adipose tissue (BAT) thermogenesis dissipates energy from diet and exercise to maintain body temperature [2]. In this framework, beige adipocytes can surge from a process called “browning”, where adipocytes with brown adipocyte phenotype are located in WAT [3].

The lipolysis, thermogenesis and browning in adipose tissue are regulated by endocrine and neural mechanisms [4]. The noradrenaline released by sympathetic nervous system binds to β-adrenergic receptor (β-AR) which activates the signaling pathway AC-cAMP-PKA, responsible for phosphorylating key proteins during lipolysis and thermogenesis, such as hormone-sensitive lipase (HSL) and uncoupling protein 1 (UCP-1) [5]. Although β_3-AR is predominant in adipose tissue of rodents, both β_1- and β_3-AR receptors activates the same intracellular signaling pathway [6]. In this sense, β_3-AR$^{-/-}$ mice do not show significant body weight gain, which can be explained by a compensatory increase in β_1-AR expression [7]. Nevertheless, β_1-AR$^{-/-}$ mice showed diet-induced obesity and hypothermia in response to cold [8], while the overexpression of β_1-AR showed increase in the lipolytic activity [9].

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Moreover, deletion of β_1-AR was also associated with inhibition of cold-induced BAT hyperplasia [10], whereas β_3-AR stimulation was not able to reverse this scenario [11]. These findings suggest an important role of β_1-AR in adipose tissue metabolism and BAT thermogenesis.

In a condition of low physical activity or physical inactivity and high food intake, the excessive WAT in the body leads to augments in proinflammatory adipokines [(i.e. tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6)] and a chronic subclinical inflammation is present [12]. Therefore, increased adipocyte size and lipid content by impaired lipolysis activation is observed. On the other hand, in a condition of physical exercise, elevated catecholamines activates the β-adrenergic signaling pathway in adipocytes, increasing the lipolytic activity by phosphorylating HSL. Lipolysis may also be activated by atrial natriuretic peptides (ANP), which are involved in lipid mobilization during exercise [13]. Hence, exercise training may decrease adipocyte size and lipid content by repeated lipolysis activation [14,15]. Exercise training can also reduce chronic subclinical inflammation by increasing and reducing anti-inflammatory and pro-inflammatory cytokines concentrations, respectively [16,17]. Moreover, exercise training can increase BAT thermogenesis and browning by increasing UCP-1 expression in adipocytes [18,19]. Although studies have demonstrated that β_1-AR is critical in adipose tissue lipolysis [7,9], thermogenesis [8,10,20] and browning [6], the role of the β_1-AR associated with exercise training is not known.

Therefore, understanding the effects of aerobic exercise training on adipose tissue in a condition of absence of β_1-AR may help to elucidate the mechanisms of the beneficial influence of regular aerobic exercise on this tissue. Thus, this study was designed to investigate the effects of a moderate continuous aerobic exercise (MCAE) program on the inflammatory cytokine profile and expression of lipolytic and thermogenic genes in adipose tissue of β_1-AR$^{-/-}$ mice.

2. Methods

2.1. Experimental animals

A cohort of 4- to 5-month-old male wild-type (WT) and congenic β_1-AR$^{-/-}$ mice in the C57Bl6/J genetic background were randomly assigned to one of the following groups by using the simple random sampling: WT control (WTc; n = 6), WT trained (WTt; n = 6), β_1-AR$^{-/-}$ control (β_1-AR$^{-/-}$c; n = 6) and β_1-AR$^{-/-}$ trained (β_1-AR$^{-/-}$t; n = 6). Mice were maintained in cages under a 12:12-h light-dark cycle in a temperature-controlled room (22 °C), with free access to water and standard rodent diet. Body weight (BW) was measured every week. This study was conducted in accordance with the ethical principles in animal research adopted by the EU Directive 2010/63/EU for animal experiments. The experimental protocols were approved by the Ethics Committee for Animal Use at the Viçosa Federal University (protocol #53/2017).

2.2. Exercise training protocol and graded treadmill exercise test

The MCAE program was performed on a motor treadmill (Insight Equipamentos Científicos, Brazil) 5 days/week (Monday to Friday), 60 min/day, for 8 weeks. Over the first week, the duration and running speed of exercise were progressively increased from 10 min and 10% of the maximal speed until 60 min and 60% of the maximal speed, achieved during a graded treadmill exercise test. At the end of the fourth week of aerobic exercise training, graded treadmill exercise test was repeated to readjust the running speed. The intensity, duration and treadmill grade were maintained during the rest of the training period. During the training period, animals from the untrained groups were handled every day and subjected to a short period of mild exercise (5 min, 0% grade, 5 m/min, 3 days/week). The running capacity estimated by total distance run was evaluated using a graded treadmill exercise protocol for mice (Panlab/Harvard Apparatus, Spain), as described previously [21]. Briefly, after being adapted to the treadmill for 1 week (10 min/day, 0% grade, 0.3 km/h), mice were placed in the exercise streak and allowed to acclimatize for at least 30 min. The graded treadmill exercise test began at 6 m/min with no grade and increased by 3 m/min every 3 min until fatigue, which was defined as when the test was interrupted because the animals could no longer keep pace with the treadmill speed. The graded treadmill exercise test was performed in WT and β_1-AR$^{-/-}$ untrained and exercise-trained groups before and after the exercise training period.

Table 1
qRT-PCR primer sequences.

	Forward	Reverse
Hprt	CAGTCCCAGCGTCGTGATT	GCAAGTCTTTCAGTCCTGTCCAT
UCP-1	CCGAAACTGTACAGCGGTCT	CCGAGAGAGGCAGGTGTTTC
Pgc-1α	ACAATGAATGCAGCGGTCTT	AGGGTCATCGTTTGTGGTCAG
Lipe	GATTGAGGTGCTGTCGTCTC	AGGTCATAGGAGATGAGCCTG
Nrp1	TTGATGTGTACAAGGTAGAGACC	GATGCGGAAGGAGCGTACA
Adrb3	ACAGGAATGCCACTCCAATC	TTAGCCACAACGAACACTCG
Adrb2	TGCCAAGTTCGAGCGACTAC	CACACGCCAAGGAGATTATGA

2.3. Tissue collection

Forty eight hours after the last exercise training session, mice were weighed and killed by decapitation. Epididymal (eWAT), inguinal (iWAT) and BAT were surgically removed and immediately frozen at −80 °C for further analyses. Fragments of such adipose tissues were stored in Carlsson's formalin (10%) for histomorphometric analyses.

2.4. Body composition

After the euthanasia, the viscera were discarded, leaving only bones, muscles and skin (empty carcass) for the quantitative analysis of water, fat and protein. The percentage of water (% water) was determined by the gravimetric method using heat based on the evaporation of the water in an oven at 105 °C for 24 h. The percentage of fat (% fat) was determined by the gravimetric process using the Soxhlet apparatus using ethyl ether as the solvent in the extraction for 8 h, according to Pitts et al. [22]. The percentage of protein (% protein) was calculated following the methods of Kjeldahl [23], by means of the nitrogen determination using the factor N × 6.258.

2.5. Adiposity index

The eWAT, retroperitoneal and mesenteric adipose tissues were removed and weighted to calculate the adiposity index of each animal. The adiposity index was obtained by the sum of these tissues mass divided by body weight multiplied by 100, expressed as adiposity percent [24].

2.6. Histomorphometric analyses

Fragments of eWAT, iWAT and BAT were fixed in Carson's formalin solution for 48 h and then incubated in 70% ethanol. Samples were then dehydrated and embedded in methacrylate resin (Leica Historesin, Nussloch/Heidelberg, Germany). The samples were sectioned (5 μm thick) with a rotary microtome (Spencer, modelo 19,459, USA) and stained with toluidine blue/sodium borate. The images were scanned using a microscope (200×) (NIKON Eclipse 24 E600), connected to a camera (Feldmann Wild Leitz DigiPro 5.0 M) and software (ACCUSCOPE Micrometrics). The area of 3–5 adipocytes per image (18–20 images per animal) was measured using the software Image Pro-Plus (Media Cybernetics, USA). For analyses purposes, adipocytes from eWAT and iWAT were separated into < and > 1000 μm^2 per animal/group (KAWANISHI et al., 2013b) and those from BAT were into <

Table 2
Running capacity and body composition.

	WTc (n = 6)	WTt (n = 6)	β_1-AR$^{-/-}$c (n = 6)	β_1-AR$^{-/-}$t (n = 6)
Total distance run, m	762 ± 135	957 ± 220*	681 ± 176	1419 ± 234*
Initial BW, g	28.17 ± 0.97	26.17 ± 0.97	34.17 ± 0.97#	33.00 ± 0.97#
Final BW, g	30.67 ± 1.20	28.00 ± 1.20*	37.16 ± 1.20#	33.33 ± 1.09*,#
Fat, %	10.77 ± 1.21	10.26 ± 1.11	16.11 ± 1.21[a]	9.16 ± 1.11[b]
Water, %	1.99 ± 0.22	1.897 ± 0.22	3.89 ± 0.22[a]	2.02 ± 0.22[b]
Protein, %	39.17 ± 0.89	37.42 ± 0.89*	40.07 ± 0.89	37.30 ± 0.89*
Adiposity index, %	2.26 ± 0.25	2.04 ± 0.25*	2.60 ± 0.25	1.65 ± 0.22*

Data are means ± SE of 6 mice per group. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; BW: body weight; two-way ANOVA followed by Tukey test.

* Denotes aerobic exercise factor effect vs. controls ($p < 0.05$).

# Denotes gene deletion factor effect vs. WT ($p < 0.05$).

[a] $p < 0.05$ vs. WTc group.

[b] $p < 0.05$ vs. β_1-AR$^{-/-}$C group.

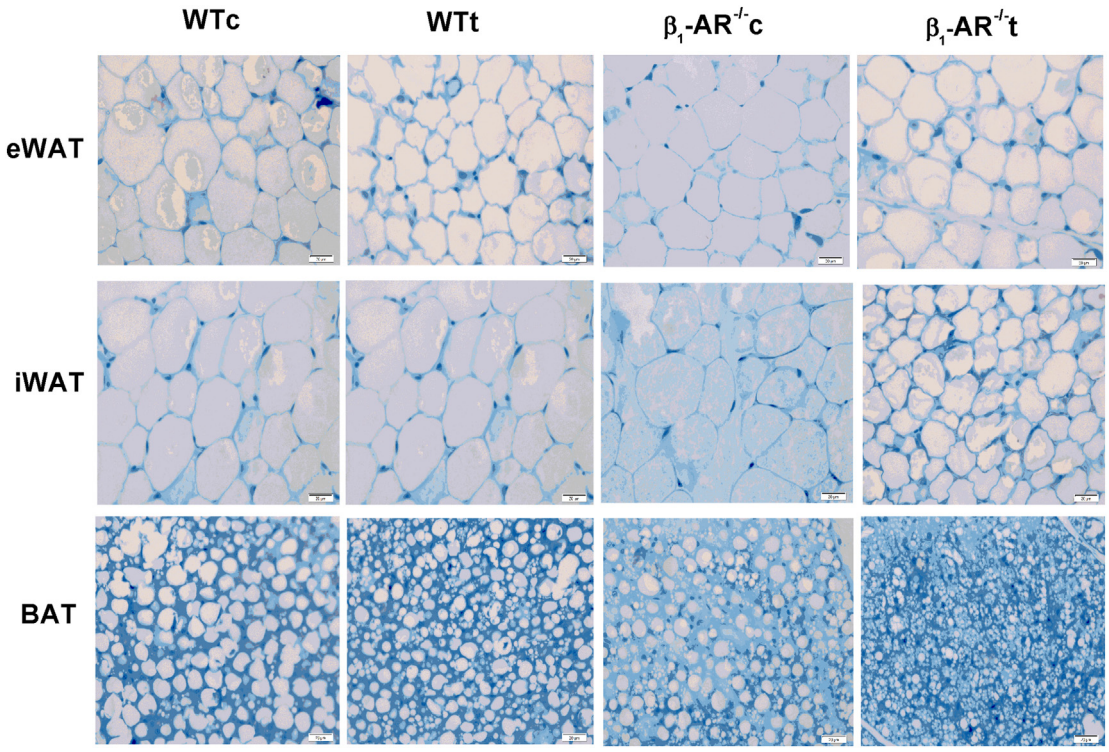

Fig. 1. Representative photomicrographs of adipose tissues. eWAT: epididymal white adipose tissue; iWAT: inguinal white adipose tissue; BAT: brown adipose tissue; WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; Staining: toluidine blue/sodium borate; Bar: 20 μm.

and > 300 μm^2 per animal/group [25].

2.7. Quantitative real-time PCR

Samples of eWAT, iWAT and BAT (30–50 mg) were homogenized to isolate total RNA using TRizol reagent (Invitrogen, Sao Paulo, SP, Brazil) following manufacturer's instruction. RNA purity (260/280 nm ratio) and concentration (ng/mL) were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, Rockford, IL, USA), and RNA integrity was checked electrophoretically by 1% agarose gel stained with Nancy-520 (Sigma-Aldrich, Sao Paulo, SP, Brazil). Messenger RNA (mRNA) levels of the autophagy-related genes: hormone-sensitive lipase (Lipe), uncoupling protein 1 (Ucp-1), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (Pgc-1α), natriuretic peptide receptor 1 (Npr1), beta-3 adrenergic receptor (Adrb3) and beta-2 adrenergic receptor (Adrb2) were assessed by quantitative real-time polymerase chain reaction (qRT-PCR). For this purpose, cDNA was synthetized from 2 μg of total RNA using oligo dT (0.5 μg), RiboLock™ RNAse inhibitor (20 U), 1 mM of dNTP Mix, RevertAid™ Reverse Transcriptase (200 U), totaling a solution with a final volume of 20 μl (Fermentas, Glen Burnie, MD, EUA). After cDNA synthesis, qRT-PCR for target genes and endogenous reference gene Hprt were run separately, and amplifications were performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using Power SYBR Green PCR (*Thermo Fisher Scientific*, EUA). Melting point dissociation curves were used to confirm the purity of the amplification products. Results were expressed using the comparative cycle threshold (Ct) method as described by the manufacturer. The DCt values were calculated in every sample for each gene of interest as Ctgene of interest minus Cthousekeeping, using Hprt as housekeeping. The calculation of the relative changes in the expression level of one specific gene (DDCt) was performed by subtraction of the average DCt from the WTc group to the DCt from each sample, and fold-change determined as $2^{(-DDCt)}$ [26]. For representative purposes, Wtc levels were arbitrarily set to 1. Table 1 shows the primer sequences used.

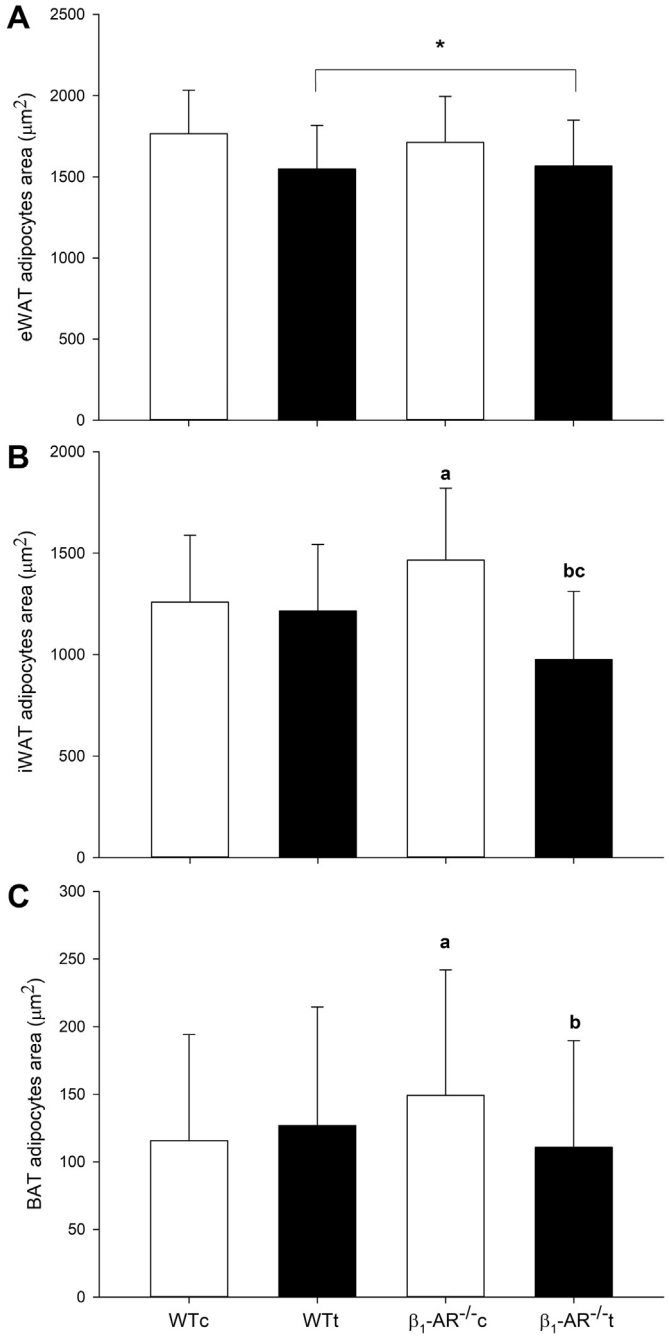

Fig. 2. Adipocytes area in epididymal (eWAT) (A), inguinal (iWAT) (B) and brown (BAT) (C) adipose tissue. Data are means ± SE of 60–100 cells/animal per group. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; (*) denotes moderate continuous aerobic exercise (MCAE) factor effect vs. controls ($p < 0.05$); ([a]) $p < 0.05$ vs. WTc group; ([b]) $p < 0.05$ vs. β_1-AR$^{-/-}$c group; ([c]) $p < 0.05$ vs. WTt group. Two-way ANOVA followed by Tukey test.

2.8. Inflammatory cytokines analysis

Samples of eWAT, iWAT and BAT (30–50 mg/animal) were homogenized with 500 μL of buffer (10 mM PBS + EDTA, pH = 7.4) and centrifuged at 8000*g* for 10 min at 4 °C. The supernatant was analyzed for the quantification of cytokines. The interleukin-12p70 (IL-12p70), interleukin-10 (IL-10), interleukin-6 (IL-6), interferon gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor alpha (TNFα) concentrations were determined simultaneously using CBA kit (BD Cytometric Bead Array (CBA) Mouse Inflammation Kit) and BD FACSVerse™ flow cytometer.

2.9. Statistical analysis

Data were subjected to Shapiro-Wilk normality tests. The comparisons were made by using the two-way ANOVA followed by Tukey test or Kruskal-Wallis followed by Dunn's test, as appropriate. We used the software SigmaPlot®, 12.5 version (Systat Software, San Jose, CA) to perform all analyses. Data are presented as means ± SE. A statistical significance level of 5% was adopted.

3. Results

3.1. Running capacity and body composition

The gene deletion did not affect ($p > 0.05$) the animals' running capacity (Table 2). The MCAE, however, increased the total distance run ($p < 0.05$), as such effect can be observed in trained β_1-AR$^{-/-}$ and WT animals.

Gene deletion also affected ($p < 0.05$) the initial and final body weights, as β_1-AR$^{-/-}$ animals showed higher body weight than WT (Table 2). The MCAE, on the other hand, reduced ($p < 0.05$) the final body weight (Table 2).

The percentages of body fat and of water in the carcass were higher ($p < 0.05$) in β_1-AR$^{-/-}$c compared to WTc group; and lower in β_1-AR$^{-/-}$t than in β_1-AR$^{-/-}$c group (Table 2). Concerning the percentage of protein and adiposity index, the MCAE reduced ($p < 0.05$) these parameters (Table 2). We observed such effects on β_1-AR$^{-/-}$t and WTt groups. Nevertheless, there was no effect of gene deletion ($p > 0.05$).

3.2. Adipocytes area

Fig. 1 shows representative photomicrographs analyzed adipose tissues. There was no gene deletion effect ($p > 0.05$) on epididymal adipocyte area (Fig. 2A), however, the MCAE reduced ($p < 0.05$) adipocyte area (Fig. 2A). Both trained WT and β_1-AR$^{-/-}$ animals showed lower area than their respective controls.

In the inguinal tissue (Fig. 2B) the area of adipocytes from β_1-AR$^{-/-}$c group was higher than that of WTc group ($p < 0.05$). In addition, the β_1-AR$^{-/-}$t group exhibited lower adipocyte area than β_1-AR$^{-/-}$c group ($p < 0.05$). Concerning BAT (Fig. 2C), the β_1-AR$^{-/-}$c group exhibited higher adipocyte area than WTc group ($p < 0.05$). Moreover, the MCAE reduced ($p < 0.05$) the adipocyte area in β_1-AR$^{-/-}$ group only.

3.3. Adipocytes frequency

Fig. 3 presents the frequency of adipocytes of different size. In eWAT, the frequency of adipocytes $< 1000\ \mu m^2$ and $> 1000\ \mu m^2$ (Fig. 3A and B) was affected by gene deletion and MCAE ($p < 0.05$). The frequency of $< 1000\ \mu m^2$ adipocytes was higher in β_1-AR$^{-/-}$ compared to WT animals; and in trained compared to control mice (Fig. 3A). Nonetheless, the adipocytes $> 1000\ \mu m^2$ were less frequent in β_1-AR$^{-/-}$ than in WT animals; and in trained than in control animals (Fig. 3B).

Respecting the iWAT, the frequency of adipocytes $< 1000\ \mu m^2$ was lower in the β_1-AR$^{-/-}$c than in WTc mice; and higher in β_1-AR$^{-/-}$t group than in WTt and β_1-AR$^{-/-}$c animals ($p < 0.05$) (Fig. 3C). In addition, the frequency of $> 1000\ \mu m^2$ adipocytes was higher in β_1-AR$^{-/-}$c compared to WTc animals ($p < 0.05$); and lower in β_1-AR$^{-/-}$t than in WTt and β_1-AR$^{-/-}$c mice ($p < 0.05$) (Fig. 3D).

With reference to BAT, there was an effect of gene deletion for the frequency of adipocytes $< 300\ \mu m^2$ (Fig. 3E) and $> 300\ \mu m^2$ (Fig. 3F). For instance, the $< 300\ \mu m^2$ and $> 300\ \mu m^2$ adipocytes frequency were higher and lower, respectively, in β_1-AR$^{-/-}$ than in WT animals.

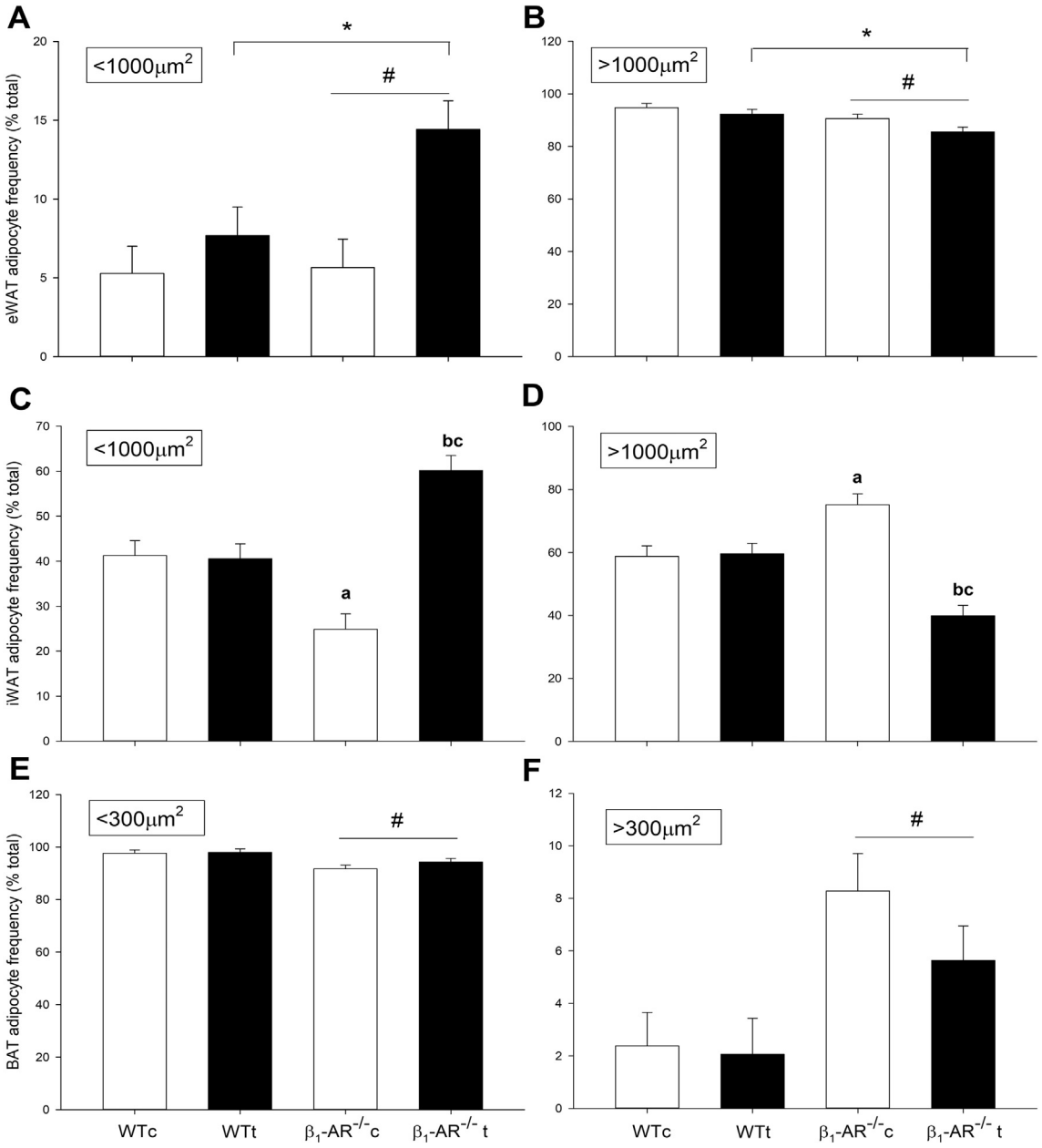

Fig. 3. Adipocytes frequency of different sizes in epididymal (eWAT) (A and B), inguinal (iWAT) (C and D) and brown (BAT) (E and F) adipose tissue. Data are means ± SE of 60–100 cells/animal per group. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; (*) denotes moderate continuous aerobic exercise (MCAE) factor effect vs. controls ($p < 0.05$); ($^{\#}$) denotes gene deletion factor effect vs. WT ($p < 0.05$); (^{a}) $p < 0.05$ vs. WTc group; (^{b}) $p < 0.05$ vs. β_1-AR$^{-/-}$c group; (^{c}) $p < 0.05$ vs. WTt group. Two-way ANOVA followed by Tukey test.

3.4. Gene expression

Fig. 4 shows the results of gene expression. Concerning eWAT (Fig. 4A) and iWAT (Fig. 4B), the expression of Adrb3, Adrb2, lipolytic (Lipe and Npr1) and thermogenic (Pgc-1α and Ucp-1) genes was not affected by either β_1-AR deletion or MCAE ($p > 0.05$).

Concerning BAT (Fig. 4C), the expression of Adrb2 gene was lower ($p < 0.05$) in β_1-AR$^{-/-}$ animals compared to WT mice. The expression of Adrb3 gene was higher in WT trained than in control animals ($p < 0.05$), though the effect on β_1-AR$^{-/-}$ mice was not evident.

3.5. Inflammatory cytokines concentrations

Fig. 5 presents the concentrations of cytokine in eWAT, iWAT and BAT. In eWAT, the concentrations of IL-12p70 (Fig. 5A) were higher in β_1-AR$^{-/-}$t than in WTt and β_1-AR$^{-/-}$c groups. Additionally, in iWAT the concentrations of IL-12p70 were higher in β_1-AR$^{-/-}$c than in WTc animals; and lower in β_1-AR$^{-/-}$t compared to β_1-AR$^{-/-}$c animals.

With regard to INFγ (Fig. 5B) and MCP1 (Fig. 5C) concentrations, there were no effects of either gene deletion or MCAE ($p > 0.05$).

With respect to TNF-α concentrations (Fig. 5D), in eWAT the β_1-AR$^{-/-}$c animals showed higher concentrations than WTc animals; while such concentrations were lower in β_1-AR$^{-/-}$t compared to β_1-AR$^{-/-}$c group. In iWAT and BAT, nevertheless, the TNF-α concentrations in β_1-AR$^{-/-}$ animals were higher than those in WT mice. The MCAE effect was observed in BAT ($p < 0.05$), as trained animals showed lower TNF-α concentrations than control animals.

The IL-6 concentrations in eWAT (Fig. 5E) were lower in rained animals compared to those in control animals ($p < 0.05$). In iWAT and BAT, neither gene deletion nor MCAE ($p > 0.05$) effects was observed.

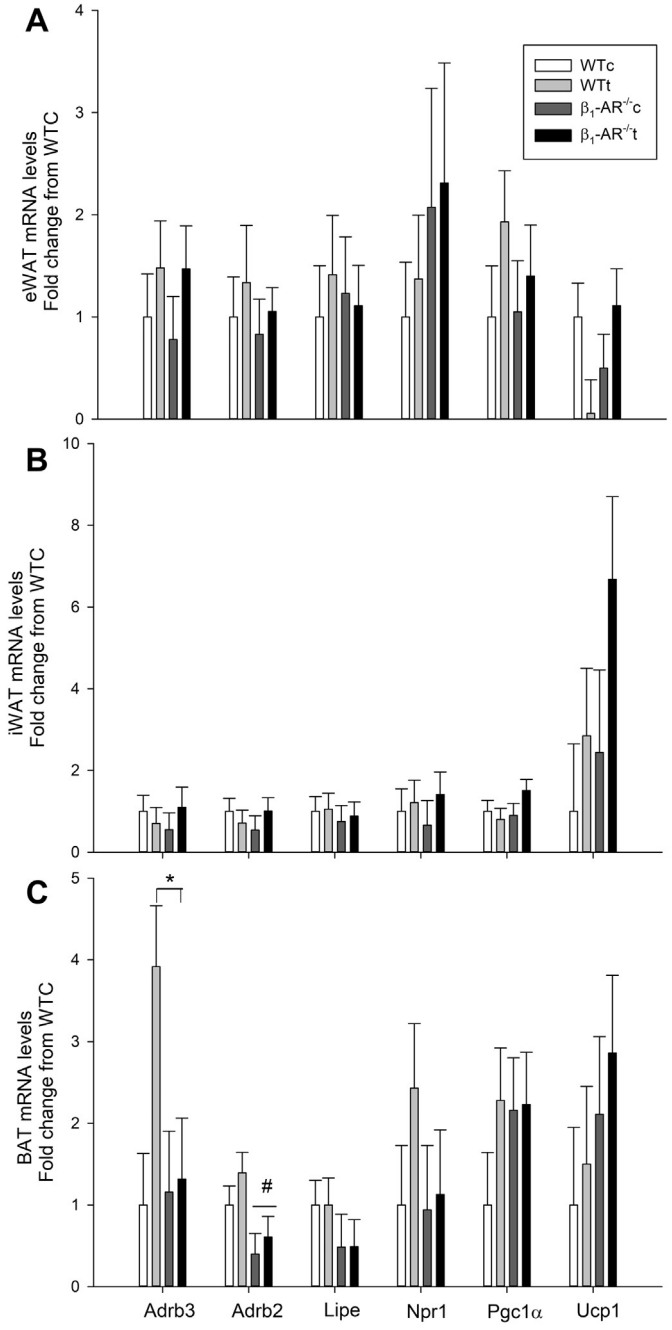

Fig. 4. Epididymal (eWAT) (A), inguinal (iWAT) (B) and brown (BAT) (C) adipose tissue gene expression. Data are means ± SE. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; Lipe: hormone-sensitive lipase; Ucp1: uncoupling protein 1; Pgc1α: peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; Npr1: natriuretic peptide receptor 1; Adrb3: beta-3 adrenergic receptor; Adrb2: beta-2 adrenergic receptor. (*) denotes moderate continuous aerobic exercise (MCAE) factor effect vs. controls ($p < 0.05$); (#) denotes gene deletion factor effect vs. WT ($p < 0.05$). Two-way ANOVA followed by Tukey test (Epididymal: Adrb3; Inguinal: Pgc1α, Lipe; Brown: Adrb3, Adrb2, Pgc1α); Kruskal-Wallis followed by Dunn test (Epididymal: Lipe, Npr1, Adrb2, Ucp-1, Pgc1α; Inguinal: Npr1, Adrb2, Adrb3, Ucp-1; Brown: Lipe, Npr1, Ucp-1).

Regarding IL-10, in eWAT and BAT (Fig. 5F) trained animals showed lower ($p < 0.05$) concentrations than controls. In iWAT, nonetheless, neither gene deletion nor MCAE ($p > 0.05$) affected it.

4. Discussion

In this study, we demonstrated that by the side of reductions in body weight fat mass and adipocyte area, eight weeks of MCAE improved the profile of proinflammatory cytokines in eWAT, iWAT or BAT in β_1-AR$^{-/-}$ mice adipose tissue, in spite of no change in lipolytic and thermogenic gene expression.

The observed increase in the concentrations of IL-12p70, IL-6 and TNF-α in β_1-AR$^{-/-}$ mice's adipose tissue might be due in part to the augmented body fat found in these animals (e.g. increase in BW, % fat, iWAT and BAT area). In fact, the increase in adipose tissue, mainly visceral tissue, results in a higher production of proinflammatory adipokines (i.e. TNF-α, IL-6, MCP-1) [12]. It is known that adipocytes expansion leads to hypoxia and, consequently, necrosis/apoptosis of these cells [27]. Thus, cell death induces adipocytes to release lipid droplets, which are toxic to adipocytes, and to activate macrophage recruitment [28]. Although, we did not observe significant increase in IFNγ and MCP-1 concentrations, these inflammatory mediators recruit M1 macrophages and, consequently, increase proinflammatory cytokines production and recruitment, such as TNF-α and IL-6 [29,30].

We also found high concentrations of TNF-α in the eWAT, iWAT and BAT of β_1-AR$^{-/-}$ mice. TNF-α is an inflammatory cytokine highly expressed in adipose tissue under obesity conditions [31], as observed in WAT and BAT of obese mice [32,33]. Moreover, β_2-AR activation reduces TNF-α gene expression in mice adipose tissue macrophages, which contributes to the anti-inflammatory status [34]. Our observation that β_1-AR$^{-/-}$ mice showed lower β_2-AR gene expression in BAT might explain, in part, the high concentrations of TNF-α in the BAT of β_1-AR$^{-/-}$ mice.

Concerning aerobic exercise, the applied MCAE reduced the concentrations of IL-12p70 in iWAT, IL-6 in eWAT and TNF-α in eWAT, iWAT and BAT. The lower body fat, adipose tissue area and adipocyte frequency > 1000 μm^2 induced by MAEC are in line with the lower proinflammatory cytokines concentrations in trained mice. Although not assessed in the present study, the MCAE anti-inflammatory effect and hence the improvement in body composition might be explained by increases in adrenaline release [35]; myocin production/release; reduction in the expression of monocytes and macrophages Toll-like receptor 4 (TLR4) [36] and in the infiltration of monocytes and macrophages into adipose tissue [12].

Unexpectedly, our trained mice exhibited lower IL-10 concentrations in eWAT and BAT, compared to control mice. IL-10 is known to attenuate the inflammatory response by inhibiting the release of proinflammatory cytokines [37]. In addition, IL-6 may also have anti-inflammatory effects because of its capacity to stimulate IL-10 production [38]. During exercise, IL-6 is released from active skeletal muscle, however, long-term exercise training decreases the circulating concentrations of IL-6 and alters the expression of IL-6 receptor due to its increased sensitivity [39]. Inasmuch as our trained animals also showed lower IL-6 concentrations, such decrease may be associated with the low IL-10 concentrations observed in eWAT and BAT.

Our β_1-AR$^{-/-}$ mice showed low β_2-AR gene expression in BAT. β-adrenergic pathway activates UCP-1 leading to BAT thermogenesis [5]. Although three β-AR isoforms exist in BAT, β_3 is the most prevalent in this tissue [40]. However, studies have observed that β_1-AR plays a key role in mice BAT thermogenesis [8,10]. Furthermore, low or no β_2-AR expression in isolated brown adipocytes has been suggested by others [41,42]. The BAT β_2-AR detection may be due to the presence of blood vessels, as BAT is highly vascularized and β_2-AR activation increases the blood flow in this tissue [42]. Thus, the low β_2-AR gene expression observed in our β_1-AR$^{-/-}$ mice might be associated with the lower BAT vascularization due to a higher brown adipocyte area observed in these mice.

Regarding MCAE, it increased the expression of β_3-AR (Adrb3) gene in the BAT of WT mice, which was not evident in β_1-AR$^{-/-}$ mice. There is evidence that exercise may enhance BAT thermogenesis by activating

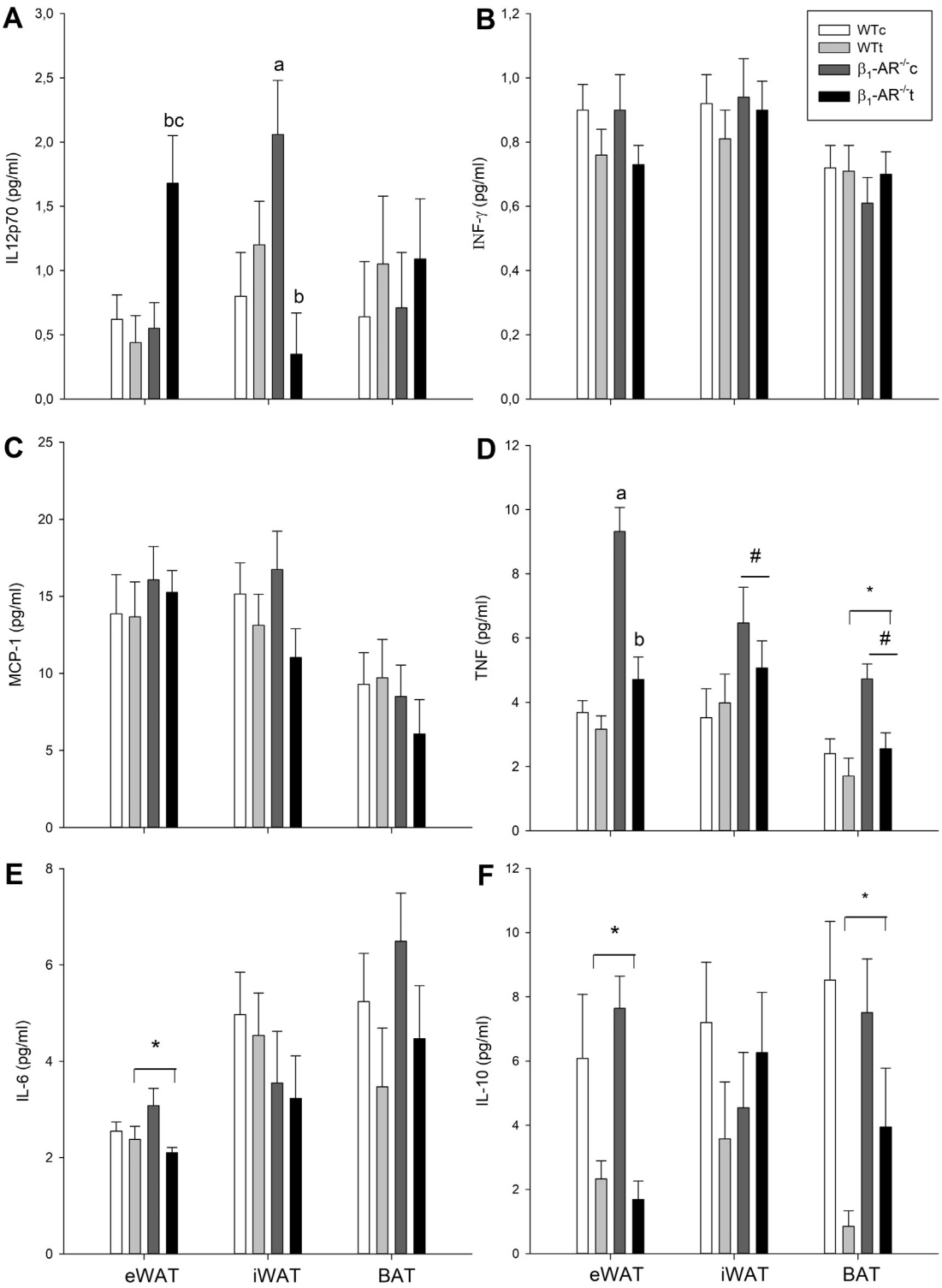

Fig. 5. IL-12p70 (A), INFγ (B), MCP-1 (C), TNFα (D), IL-6 (E) and IL-10 (F) inflammatory cytokine concentrations in epididymal (eWAT), inguinal (iWAT) and brown (BAT) adipose tissues; data are means ± SE of 60–100 cells/animal and 6 animal/group. WTc: wild-type control; WTt: wild-type trained; $\beta_1AR^{-/-}$c: β_1-$AR^{-/-}$ control; β_1-$AR^{-/-}$t: $\beta_1AR^{-/-}$ trained; EPI: epididymal adipose tissue; ING: iWAT; BAT: brown adipose tissue; IL-12P70: interleukin-12p70; TNF: tumor necrosis factor alpha; INFγ: interferon gamma; MCP-1: monocyte chemoattractant protein-1; IL-10: interleukin-10; IL-6: interleukin-6; (*) denotes moderate continuous aerobic exercise (MCAE) factor effect vs. controls ($p < 0.05$); ($^{\#}$) denotes gene deletion factor effect vs. WT ($p < 0.05$); (^{a}) $p < 0.05$ vs. WTc group; (^{b}) $p < 0.05$ vs. β_1-$AR^{-/-}$c group; (^{c}) $p < 0.05$ vs. WTt group. n = 6 animals/group, except BAT (WTt: n = 5; $\beta_1AR^{-/-}$T: n = 4). Two-way ANOVA followed by Tukey test (IL12p70: EPI, ING; INFγ: EPI, ING, BAT; MCP-1: ING, TAM; TNF: EPI, ING; IL-6: BAT; IL-10: EPI, BAT); Kruskal-Wallis followed by Dunn test (IL12p70: BAT; MCP-1: EPI; TNF: BAT; IL-6: EPI, ING; IL-10: ING).

AC-cAMP-PKA [5]. In addition, elevated catecholamines' concentrations induced by exercise seems to increase the expression of PGC-1α, which is responsible for stimulating the mitochondrial proteins expression involved in fatty acid (FA) oxidation [43]. Fatty acids from lipolysis activate UCP-1 uncoupling and are oxidized in mitochondria, as an energy source for the heat production [4]. Despite the absence of

increases in PGC-1α and UCP-1 expression in the BAT of our trained animals, the increase in β_3-AR expression is possible the first step for the thermogenic adaptations induced by MCAE in this tissue. Increases in UCP-1 and PGC-1α protein expression by MCAE in rats have been reported [18,44]. In addition, aerobic exercise increases noradrenergic tone and vascularization in rats' BAT [18], which indicates high sympathetic activity and, consequently, β-adrenergic activation in this tissue. Moreover, in the present study we observed no alteration in Lipe and Npr-1 gene expression in our mice's BAT. While BAT lipid storage is critical for thermogenesis, WAT reserves appear to provide the main source of energy to support thermogenesis via lipolysis [45].

With reference to eWAT and iWAT, there was no change in lipolytic and thermogenic genes' expression in response to either gene deletion or MCAE. Nevertheless, an increase in the body composition (i.e. BW and % fat) and cell structure (i.e. adipocytes area and size) was observed in β_1-AR$^{-/-}$ mice and our MCAE reduced the body composition in these mice. These findings suggest that changes in signaling pathways are more related to lipolysis in β_1-AR$^{-/-}$ and trained mice than to alterations in the analyzed genes' expression. It is known that β-AR activation by catecholamines stimulates the adenylate cyclase, cyclic AMP and protein kinase A (AC-cAMP-PKA) signaling pathway, which increases lipolysis by HSL phosphorylation [46]. Alternatively, NPR-1 activation by natriuretic peptides stimulates the guanylyl cyclase, cyclic GMP and protein kinase G (GC-cGMP-PKG) signaling pathway, which phosphorylates HSL in adipose tissue [47]. Thus, our results suggest that both β_1-AR gene deletion and MCAE influences the activation of these signaling pathways, inasmuch as there was an increase in BW, % fat, adipocytes area/size in β_1-AR$^{-/-}$ mice; and reduction in BW, % fat, adiposity index and adipocytes area/size in trained mice.

We observed an increase in BW, % fat and inguinal and brown adipocyte's area in response to β_1-AR gene deletion. Although β_3-AR is predominant in rodents' adipose tissue [48], our findings demonstrate that β_1-AR plays a key role in mice's adipose tissue metabolism. Additionally, increased BW in β_1-AR$^{-/-}$ mice [8] and lipolytic activity in mice overexpressing β_1-AR [9] has been reported.

Our MCAE reduced BW, % fat, adiposity index and adipocytes area in the WAT and BAT of β_1-AR$^{-/-}$ and WT mice. One single aerobic exercise session is known to increase adipose tissue lipolysis, thus exercise training may reduce adipocytes' size by the repeated activation of lipolysis [14,15]. The catecholamines elevation during exercise activates the β-adrenergic signaling pathway, which increases the HSL phosphorylation in adipocytes. It is known that MCAE increases resting lipolysis up to 2–5 fold [46]. As previously stated, lipolysis induced by exercise also occurs through the NPR-1 activation by natriuretic peptides [47].

Our trained animals also exhibited lower % protein than that of control mice. This reduction is in line with the aerobic exercise regime applied here. It is known that aerobic exercise is an effective exercise type for BW and % fat reduction, whereas, resistance training is the exercise type indicated to maintain or increase lean body mass [49].

We demonstrate that both gene deletion and MCAE reduced the frequency of adipocytes $> 1000\,\mu m^2$ and increased that of $< 1000\,\mu m^2$ in eWAT. Because β_1-AR$^{-/-}$c mice showed a frequency of adipocytes $< 1000\,\mu m^2$ similar to that of WT mice, our MCAE was important in increasing the frequency of adipocytes $< 1000\,\mu m^2$ in β_1-AR$^{-/-}$ mice. Lipolysis activation and muscle adaptations induced by exercise are associated with reduction of adipocytes and free FA transport and oxidation [14,15]. While there were no significant differences in β_3-AR and NPR-1 gene expression in eWAT, a compensatory increase in these receptors' activity might help to explain the increased frequency of adipocytes $< 1000\,\mu m^2$ in trained mice.

Concerning iWAT, our β_1-AR$^{-/-}$c mice showed higher and lower $> 1000\,\mu m^2$ and $< 1000\,\mu m^2$ adipocyte frequency, respectively, compared to WT animals. Inguinal WAT has greater sympathetic innervation than other adipose tissues [10]. Since the adrenergic signaling pathway is an important regulator of adipose tissue metabolism and β_1-AR plays a key role in lipolytic signaling pathway, these results suggest that β_1-AR gene deletion impairs the adrenergic signaling pathway and, consequently, lipolysis in iWAT.

Regarding the MCAE, β_1-AR$^{-/-}$t mice showed lower and higher frequency of adipocytes $> 1000\,\mu m^2$ and $< 1000\,\mu m^2$ in iWAT than untrained animals, respectively. Such beneficial effect of our exercise regime might be explained by the high sympathetic innervation in iWAT and by the aerobic exercise effectiveness in reducing adipocytes' size via lipolysis activation [14,15].

With reference to BAT, our β_1-AR$^{-/-}$ mice showed higher and lower frequency of adipocytes $> 300\,\mu m^2$ and $< 300\,\mu m^2$ than WT mice, respectively. As observed in iWAT, these results suggest that β_1-AR deletion impairs the adrenergic signaling pathway, which is an important regulatory pathway for adipose tissue lipolysis.

5. Conclusion

In conclusion, alongside reductions in body weight, fat mass and adipocyte area eight weeks of MCAE improves the profile of inflammatory cytokines in β_1-AR$^{-/-}$ mice adipose tissue, despite no change in lipolytic and thermogenic gene expression. These findings highlight the therapeutic potential of MCAE in the treatment of obesity and its complications.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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