AURORA CORRÊA RODRIGUES

EFEITOS DO EXERCÍCIO AERÓBICO SOBRE A COMPOSIÇÃO CORPORAL, A EXPRESSÃO DE GENES LIPOLÍTICOS E TERMOGÊNICOS E DE CITOCINAS INFLAMATÓRIAS NO TECIDO ADIPOSO DE CAMUNDONGOS *KNOCKOUT* PARA RECEPTOR β1 ADRENÉRGICO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae*.

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À minha família.

Aos animais experimentais.

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“O assunto mais importante do mundo pode ser simplificado até ao ponto em que todos possam apreciá-lo e compreendê-lo. Isso é - ou deveria ser - a mais elevada forma de arte.”

(Charles Chaplin)

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LISTA DE ABREVIATURAS E SIGLAS

AC: adenilato ciclase

Adrb2: gene do receptor β2 adrenérgico

Adrb3: gene do receptor β3 adrenérgico

Akt/PKB: proteína quinase B

AMP'5: adenosina monofosfato'5

AMPc/cAMP: adenosina monofosfato cíclico

AMPK: proteína quinase ativada por adenosina monofosfato

ANP: peptídeo natriurético atrial

aP2: proteína adaptadora 2

ATGL: lipase de triglicerídeos de tecido adiposo

ATP: adenosina trifosfato

BAT: tecido adiposo marrom

BW: peso corporal

cDNA: ácido desoxirribonucleico complementar

Ct: ciclos

DAG: diacilglicerol

DCt: delta Ct

DM2: diabetes melitos tipo 2

ERK: quinase regulada por sinal extracelular

eWAT: tecido adiposo branco epididimal

FA: ácido graxo

FABP: proteínas de ligação ao ácido graxo

FFA: ácido graxo livre

FGF-21: fator de crescimento de fibroblasto 21

FNDC5: fibronectina de tipo III, contendo o domínio de proteína 5

GC: guanilato ciclase

GDP: difosfato de guanidina

Gi: proteína G inibitória

GMPc/cGMP: guanosina monofosfato cíclico

GPCR: receptores acoplados a proteína G

Gs: proteína G estimulatória

HSL: lipase hormônio sensível

Hprt: hipoxantina-guanina fosforibosiltransferase

IFNγ: interferon gama

IGF-1: fator de crescimento como insulina 1

IL: interleucina

IL-1ra: antagonista do receptor de IL-1

IRS1: substrato 1 do receptor de insulina

IRS1: substrato 2 do receptor de insulina

iWAT: tecido adiposo branco inguinal

JNK: quinase terminal N Jun-c

Lipe: gene da lipase hormônio sensível

MCAE: treinamento aeróbico de intensidade moderada

MAG: monoacilglicerol

MAPK: Proteína-quinase ativada por mitógenos

MCP-1: proteína quimiotática de monócitos

MGL: lipase monoacilglicerol

mWAT: tecido adiposo mesentérico

NAFLD: doença hepática gordurosa não alcoólica

NFκB: fator nuclear *kappa* B

Npr1: gene do receptor peptídeo natriurético tipo 1

NPR-A: receptor de peptídeo natriurético

PAI-1: Inibidor do ativador de plasminogênio tipo 1

PBS+EDTA: Tampão fosfato-salino e ácido etilenodiamino tetra-acético

PDE-3B: fosfodiesterase 3B

PGC-1α: gene do co-ativador-1 alfa do receptor ativado por proliferador de peroxissoma

PI3K: fosfatidilinositol 3-quinase

PKA: proteína quinase A

PKG: proteína quinase G

PLN: perilipina A

Prdm16: proteína 16 contendo o domínio PR

qRT-PCR: reação em cadeia de polimerase em tempo real

RNA: ácido ribonucleico

SE: erro padrão

SNS: sistema nervoso simpático

TAG: triacilglicerol

TLR4: receptor do tipo Toll 4

TNF-α: fator de necrose tumoral α

Treg: células T regulatórias

UCP-1: proteína desacopladora 1

Ucp-1: gene da proteína desacopladora 1

VAT: tecido adiposo visceral

WAT: tecido adiposo branco

WT: selvagem

WTc: selvagem controle

WTt: selvagem treinado

α-AR: receptor α adrenérgico

β-AR: receptor β adrenérgico

β_1-AR$^{-/-}$: *knockout* para receptor β1 adrenérgico

β_1-AR$^{-/-}$c: grupo β_1-AR$^{-/-}$ controle

β_1-AR$^{-/-}$t: grupo β_1-AR$^{-/-}$ treinado

RESUMO

RODRIGUES, Aurora Corrêa, D.Sc., Universidade Federal de Viçosa, abril de 2019. **Efeitos do exercício aeróbico sobre a composição corporal, expressão de genes lipolíticos e termogênicos e de citocinas inflamatórias no tecido adiposo de camundongos *knockout* para receptor β_1 adrenérgico.** Orientador: Antônio José Natali. Coorientadores: Helen Hermana Miranda Hermsdorff, Maria do Carmo Gouveia Peluzio e Thales Nicolau Prímola Gomes.

Objetivo: O objetivo deste estudo foi verificar os efeitos do exercício aeróbico regular sobre a composição corporal, a expressão de genes lipolíticos e termogênicos e as concentrações de citocinas inflamatórias no tecido adiposo de camundongos β_1-AR$^{-/-}$. **Material e Métodos:** Camundongos β_1-AR$^{-/-}$ e selvagens machos provenientes da linhagem C57/BL6J com 4 meses de idade foram divididos em quatro grupos de seis animais cada: Selvagem controle; Selvagem treinado; β_1-AR$^{-/-}$ controle e β_1-AR$^{-/-}$ treinado. Os grupos treinados foram submetidos a um programa de exercício aeróbico contínuo de intensidade moderada (EACM) (60 min/dia; 60% da velocidade máxima; 5 dias/semana) durante 8 semanas. Após a eutanásia, os tecidos adiposos foram removidos e usados para o cálculo do índice de adiposidade, análise histomorfométrica, concentrações de citocinas, expressão gênica e atividade de lipases. O fígado foi coletado para análises de citocinas e atividade de lipases. A carcaça eviscerada foi utilizada para a análise de composição corporal (conteúdo de gordura, proteína e água corporal). As comparações entre os quatro grupos foram feitas usando-se ANOVA de duas entradas com *post hoc* de Tukey ou Kruskal-Wallis com *post hoc* de Dunn. Adotou-se o nível de significância de até 5%. **Resultados:** Animais β_1-AR$^{-/-}$ apresentaram maior peso corporal, percentual de gordura e área dos adipócitos no tecido adiposo inguinal e marrom que animais WT ($p<0,05$) e o EACM foi capaz de reduzir o peso corporal, bem como, o peso do tecido adiposo mesentérico, o percentual de gordura, a área de adipócitos do tecido adiposo inguinal e marrom nos animais β_1-AR$^{-/-}$ ($p<0,05$). O EACM aumentou a frequência de pequenos adipócitos e reduziu a frequência de grandes adipócitos do tecido adiposo epididimal dos animais treinados ($p<0,05$). Os animais β_1-AR$^{-/-}$c apresentaram menor frequência de pequenos adipócitos e maior frequência de grandes adipócitos no tecido adiposo inguinal ($p<0,05$), enquanto que os animais β_1-AR$^{-/-}$t apresentaram maior e menor frequência de pequenos e grandes adipócitos ($p<0,05$), respectivamente. Animais β_1-AR$^{-/-}$ apresentaram menor frequência de pequenos adipócitos e maior frequência de grandes adipócitos no tecido adiposo marrom ($p<0,05$). Os animais β_1-AR$^{-/-}$ apresentaram maior e menor atividade de lipases no tecido adiposo mesentérico e no fígado, respectivamente, comparados aos animais WT ($p<0,05$), mas nenhum efeito do EACM foi observado ($p>0,05$). O EACM, no entanto, diminuiu as concentrações de citocinas pró-inflamatórias (IL-12p70, TNF-α, IL-6) e anti-inflamatórias (IL-10) nos tecidos adiposos

(epididimal, inguinal, mesentérico e marrom) e no fígado de animais β_1-AR$^{-/-}$ ($p<0,05$). Além disso, o EACM e a deleção gênica de β_1-AR não alterou a expressão de genes lipolíticos (Lipe e Npr1) e termogênicos (Ucp-1 e Pgc-1α) nos tecidos adiposos de animais β_1-AR$^{-/-}$ ($p>0,05$). Enquanto que animais β_1-AR$^{-/-}$ apresentaram menor expressão do gene Adrb2 ($p<0,05$) e animais treinados maior expressão do gene Adrb3 ($p<0,05$). **Conclusão:** A deleção gênica de β_1-AR aumenta o peso corporal, o percentual de gordura, a área dos adipócitos no tecido adiposo inguinal e marrom, a frequência de pequenos adipócitos no tecido adiposo epididimal e de grandes adipócitos no tecido adiposo inguinal e marrom; reduz a frequência de grandes adipócitos do tecido adiposo epididimal e de pequenos adipócitos no tecido adiposo inguinal e marrom e não altera o índice de adiposidade. O EACM reduz o peso corporal, o peso do tecido adiposo mesentérico, o índice de adiposidade, o percentual de gordura, a área e frequência de grandes adipócitos no tecido adiposo epididimal e inguinal; aumenta a frequência de pequenos adipócitos no tecido adiposo epididimal e inguinal; e não altera a frequência de pequenos e grandes adipócitos no tecido adiposo marrom. A deleção gênica e o EACM não alteram a expressão dos genes lipolíticos (Lipe e Npr1) e termogênicos (Ucp-1 e Pgc-1α) no tecido adiposo. Entretanto, a deleção gênica de β_1-AR reduz a expressão do gene Adrb2 e o EACM aumenta a expressão do gene Adrb3. Nas concentrações de citocinas, a deleção gênica de β_1-AR aumenta as concentrações de TNF-α no tecido adiposo epididimal, inguinal e marrom, IL12p70 no tecido adiposo inguinal, IL-6 e IL-10 no tecido adiposo mesentérico e fígado. Enquanto que o EACM reduz as concentrações de IL12p70 no tecido adiposo inguinal, TNF-α no tecido adiposo epididimal e inguinal, IL-6 no tecido adiposo epididimal, mesentérico e fígado, e IL-10 no tecido adiposo epididimal, marrom, mesentérico e fígado. A deleção gênica e o EACM não alteram as concentrações de IFNγ e MCP-1 nos tecidos adiposos e fígado. A deleção gênica de β_1-AR reduz a atividade de lipases no tecido adiposo mesentérico e aumenta no fígado, enquanto o EACM não afeta a atividade de lipases.

ABSTRACT

RODRIGUES, Aurora Corrêa, D.Sc., Universidade Federal de Viçosa, April, 2019. **Effects of aerobic exercise on the body composition, expression of lipolytic and thermogenic genes and of inflammatory cytokines in adipose tissue of β_1 adrenergic receptor knockout mice.** Adviser: Antônio José Natali. Co-advisers: Helen Hermana Miranda Hermsdorff, Maria do Carmo Gouveia Peluzio and Thales Nicolau Prímola Gomes.

Objective: The aim of this study was to verify the aerobic exercise training on the body composition, expression of lipolytic and thermogenic genes and inflammatory cytokine profile in β_1-AR$^{-/-}$ mice adipose tissue. **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 moderate continuous aerobic exercise (MCAE) regimen (60 min/day; 60% of maximal speed, 5 days/week) on a treadmill, for 8 weeks. After euthanasia, adipose tissues were removed and used to calculate the adiposity index, histomorphometric analysis, cytokines concentrations and quantitative real-time PCR. Liver and mesenteric adipose tissue (mWAT) were used to analyses cytokine concentrations and lipases activity. The empty carcass was used to calculate the body composition (water, fat and protein content). The comparisons between the four groups were made using two-way ANOVA followed by Tukey test or Kruskal-Wallis followed by Dunn test. A statistical significance level of 5% was adopted. **Results:** β_1-AR$^{-/-}$ animals exhibited higher body weight than WT animals ($p < 0.05$), and the MCAE reduced the body weight as well as mWAT weight, fat mass, inguinal adipose tissue (iWAT) and brown adipose tissue (BAT) adipocyte area in β_1-AR$^{-/-}$ animals ($p < 0.05$). β_1-AR$^{-/-}$ groups showed lower lipases activity in mWAT than WT groups ($p < 0.05$), whereas in the liver, β_1-AR$^{-/-}$ animals presented higher lipases activity than WT animals ($p < 0.05$), but no effect of MCAE was found ($p > 0.05$). 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, epididimal (eWAT), BAT or mWAT) and liver of β_1-AR$^{-/-}$ mice ($p < 0.05$). However, MCAE had no effect on the expression lipolytic and thermogenic genes in β_1-AR$^{-/-}$ mice adipose tissue. **Conclusion:** Gene deletion of β_1-AR increase body weight, mass fat, adipocyte area in iWAT and BAT. The MCAE reduce body weight, mWAT weight, adiposity index, mass fat and adipocyte area in eWAT, iWAT or BAT. Also, gene deletion of β_1-AR and MCAE gene do not change expression of lipolytic and thermogenic genes in adipose tissue. However, gene deletion reduces expression of Adrb2 gene and MCAE increases the expression of Adrb3 gene. Gene deletion of β_1-AR increases the concentrations of pro-inflammatory (IL12p70, TNF-α e IL-6) and anti-inflammatory (IL-10) cytokines in adipose tissues and liver, whereas, MCAE reduces the concentrations of pro-inflammatory (IL12p70,

TNF-α e IL-6) cytokines in adipose tissues and liver. The gene deletion of β_1-AR reduces lipases activity in mWAT and increases in liver, while MCAE does not affect lipases activity.

1. INTRODUÇÃO GERAL

O tecido adiposo é primariamente diferenciado em branco e marrom. Enquanto no tecido adiposo branco a lipogênese e a lipólise são atividades metabólicas primárias para manter a homeostase da gordura corporal (1,2), no tecido adiposo marrom a termogênese dissipa energia pela dieta e exercício físico para manter a temperatura corporal (3). Além disso, o tecido adiposo bege pode surgir de um processo chamado *"brownização"*, onde adipócitos com fenótipo de adipócito marrom estão localizados em depósitos de tecido adiposo branco (4).

O sistema nervoso simpático (SNS) está envolvido na lipólise, termogênese e "brownização" do tecido adiposo (5). A noradrenalina liberada pelo SNS liga-se aos receptores β-adrenérgicos (β-AR), que ativam a via de sinalização adenilato ciclase-adenosina monofosfato cíclico-proteína quinase A (AC-AMPc-PKA), responsável por fosforilar proteínas chave no processo de lipólise e termogênese, como a lipase hormônio sensível (HSL) e a proteína desacopladora 1 (UCP-1) (6). Embora o β_3-AR seja predominante no tecido adiposo de roedores, camundongos com deleção gênica de β_3-AR não apresentaram ganho de peso corporal significativo, o que pode ser explicado por um aumento compensatório na expressão de β_1-AR (7). Em contrapartida, camundongos β_1-AR$^{-/-}$ apresentaram obesidade induzida pela dieta (8) e camundongos com superexpressão de β_1-AR apresentaram aumento na atividade lipolítica (9). Além disso, a deleção de β_1-AR em camundongos foi associada a inibição de hiperplasia de adipócitos marrons induzida pelo frio (10), enquanto que a estimulação de β_3-AR não foi capaz de reverter esse cenário (11). Esses achados sugerem que o β_1-AR tem papel fundamental no metabolismo do tecido adiposo e na termogênese do tecido adiposo marrom.

A inatividade física é um importante fator de risco independente para diversas doenças crônicas (12). O aumento excessivo do tecido adiposo branco em condições de baixo nível de atividade física ou inatividade física e alta ingestão calórica leva ao aumento de citocinas pró-inflamatórias - fator de necrose tumoral α (TNF-α), interleucina 6 (IL-6), proteína quimiotática de monócitos (MCP-1) - e a inflamação crônica subclínica (13). Por outro lado, o treinamento físico tem sido proposto como uma estratégia terapêutica não farmacológica (14), que promove diversas adaptações no organismo (15).

As catecolaminas elevadas durante a sessão de exercício aeróbico são responsáveis por ativar a via de sinalização β-adrenérgica nos adipócitos, aumentando a atividade lipolítica por meio da fosforilação de HSL (16). A lipólise também pode ser ativada pelos peptídeos natriuréticos atriais, que estão envolvidos na mobilização de lipídios durante o exercício aeróbico. Para exemplificar, o estudo de Moro e colaboradores demonstrou que,

sob inibição β-adrenérgica, o exercício aeróbico é capaz de aumentar as concentrações circulantes de peptídeo natriurético atrial em humanos (17).

Embora alguns dos efeitos benéficos do exercício aeróbico nas doenças metabólicas sejam decorrentes do aumento no gasto energético e, consequentemente, da redução de gordura corporal, esses efeitos podem ser independentes da perda de peso corporal (18). O treinamento aeróbico pode reduzir a inflamação crônica subclínica por aumentar as concentrações de citocinas anti-inflamatórias - IL-10 e IL-15 (19) - e reduzir a expressão de citocinas pró-inflamatórias - TNF-α e MCP-1 (20). Além disso, o treinamento aeróbico foi capaz de aumentar a termogênese do tecido adiposo marrom e a *"brownização"* do tecido adiposo branco, devido à maior expressão de UCP-1 nos adipócitos de ratos (21). Essa adaptação sugere um aumento na adipogênese, na fosforilação oxidativa e no gasto energético do tecido adiposo marrom e, consequentemente, uma melhora no metabolismo corporal.

Diferentes ferramentas metodológicas, como os modelos de animais geneticamente modificados, são usadas para investigar o papel funcional do tecido adiposo na obesidade. Entre estes modelos estão os camundongos com deleção gênica global dos receptores adrenérgicos beta (β-AR), como β_3-AR$^{-/-}$, β_1-AR$^{-/-}$, $\beta_{1,2,3}$-AR$^{-/-}$ e com superexpressão de β_1-AR (8,9,22–24). O modelo animal β_1-AR$^{-/-}$ foi criado para compreender o papel do receptor β_1-AR na função cardíaca, metabolismo e tônus do musculo liso (25). Um estudo que utilizou este modelo animal observou que os camundongos β_1-AR$^{-/-}$ foram suscetíveis à obesidade induzida pela dieta (8). Embora este camundongo não seja preconizado na literatura com um modelo animal de obesidade, a utilização de camundongos β_1-AR$^{-/-}$ pode auxiliar na elucidação das vias de sinalização metabólicas envolvidas na obesidade, visto que os β-AR são fundamentais na via lipolítica do tecido adiposo.

Embora os estudos demonstrem que o β_1-AR desempenha um papel fundamental na lipólise (7,9), termogênese (8,9,26) e *"brownização"* (27) do tecido adiposo de roedores, o mecanismo de sua ação ainda não está claro. Neste contexto, o papel do β_1-AR no tecido adiposo associado ao treinamento físico ainda é desconhecido. Diante do exposto, compreender os efeitos do exercício aeróbico sobre o tecido adiposo de camundongos β_1-AR$^{-/-}$ pode ajudar a elucidar os mecanismos da influência do exercício físico aeróbico neste tecido.

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2. OBJETIVOS

2.1 Objetivo Geral:

Verificar os efeitos do exercício aeróbico sobre a composição corporal, a expressão de genes lipolíticos e termogênicos e as concentrações de citocinas inflamatórias no tecido adiposo de camundongos β_1-AR$^{-/-}$.

2.2 Objetivos Específicos:

Verificar se o programa de corrida em esteira, de intensidade moderada, altera nos camundongos β_1-AR$^{-/-}$:

- O índice de adiposidade e o percentual de gordura;
- A área dos adipócitos no tecido adiposo (epididimal, inguinal e marrom);
- A expressão dos genes lipolíticos: lipase hormônio sensível (Lipe) e receptor peptídeo natriurético tipo 1 (Npr1); genes termogênicos: proteína desacopladora 1 (Ucp-1) e co-ativador-1 alfa do receptor ativado por proliferador de peroxissoma (Pgc-1α); receptor β_3 adrenérgico (Adrb3) e receptor β_2 adrenérgico (Adrb2) no tecido adiposo (epididimal, inguinal e marrom);
- As concentrações de citocinas pró-inflamatórias: IL-12p70, TNF-α, IFNγ, MCP-1, e IL-6; e anti-inflamatória: IL-10 no tecido adiposo (epididimal, inguinal, marrom, mesentérico e fígado);
- A atividade de lipases no tecido adiposo mesentérico e fígado.

3. ARTIGOS CIENTÍFICOS

3.1 Artigo 1 – Review

Aerobic exercise and lipolysis: a review of the β-adrenergic signaling pathways in adipose tissue

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ABSTRACT

Objectives: This study aimed to show the signaling mechanisms regulating lipolysis, and the key proteins involved in these mechanisms and the influence of aerobic exercise in this scenario.

News: Obesity is a public health problem in the world. Lipolytic signaling pathway plays a key role in the metabolic alteration due to obesity. The sympathetic nervous system (SNS) through beta-adrenergic receptor (β-AR) is responsible for the lipolysis in white adipose tissue (WAT) and thermogenesis in brown adipose tissue (BAT), as well browning in WAT. Aerobic exercise is known to protects against obesity due to several adaptations in the organism. Besides reduce the body weight and chronic subclinical inflammation, aerobic exercise can also increase BAT thermogenesis and WAT browning by activation of beta-adrenergic signaling pathway.

Conclusion: Aerobic exercise training promotes various adaptations in the adipose tissue like increased of the lipolysis and browning in WAT, and thermogenesis in BAT, which help in the management of obesity. Also, exercise training has the potential to combat the chronic subclinical inflammation associated with obesity.

INTRODUTION

Obesity is known as a major public health issue because of their association with a wide range of health complications such as diabetes, hypertension, cardiovascular disease and cancers [1,2]. According the WHO, more than 1.9 billion adults are overweight and 650 million are obese in the world [1]. Its complex pathological process is a consequence of the

interaction of genetic and environmental factors [3]. The environmental factors such as poor diet and physical inactivity also contribute to the occurrence of overweight and obesity.

The lipolytic signaling pathway of white adipose tissue (WAT) plays a key role in the metabolic alteration due to obesity [4]. Thus, the identification of the critical stages of lipolysis is one of the strategies for combating obesity and its comorbidities. There is currently increased research to better understand the role of adipose tissue in epidemiological control of obesity [5], focusing, mainly, on the molecular mechanisms associated with its complications.

The sympathetic nervous system (SNS) is the main responsible for WAT lipolysis and brown adipose tissue (BAT) thermogenesis. Catecholamines, manly adrenaline, activate signaling pathways responsible for the phosphorylation of key proteins in the process of lipolysis and thermogenesis [6]. The adipose tissue presents the three isoforms of beta-adrenergic receptor (β-AR). Although β_3-AR is predominant in adipose tissue of rodents, studies have shown that the β_1-AR plays a key role in adipose tissue metabolism and obesity control [6–9]. Exercise training has been proposed as a non-pharmacological therapeutic strategy, which promotes various adaptations in the body and protects against obesity [10]. Although some of the beneficial effects of aerobic exercise on metabolic diseases are due to increased energy expenditure and consequently reduced body fat, these effects may be independent of body weight loss [11]. Aerobic exercise training may reduce chronic subclinical inflammation by increasing the concentrations of anti-inflammatory cytokines - interleukin (IL)-10 and IL-15 [12] - and reducing the expression of proinflammatory cytokines - tumor necrosis factor α (TNF-α) and monocyte chemotactic protein (MCP-1) [13]. In addition, aerobic exercise training increases BAT thermogenesis and WAT browning, due to the greater expression of uncoupling protein 1 (UCP-1) in adipocytes [14,15]. This adaptation suggests an increase in BAT adipogenesis, oxidative phosphorylation and energetic expenditure and, consequently, an improvement in body metabolism.

Considering the above mentioned, understanding the relationship between the aerobic exercise training and β-adrenergic signaling pathway of adipose tissue may have important implications for the development of new therapeutic strategies for obesity and associated comorbidities. In this review, we have shown the signaling mechanisms regulating lipolysis, and the key proteins involved in these mechanisms and the influence of aerobic exercise in this scenario.

White adipose tissue

The excess energy obtained in the diet is stored in WAT as triacylglycerol (TAG), from which the fatty acids (FA) are mobilized to attend the systemic energy demand [16]. The WAT is composed of unilocular white adipocytes with few mitochondria, which have *Myf5*$^{-}$ adipoblast as the percussion [17]. This tissue is located throughout the body, being divided into visceral (mesenteric, perigonadal and omental) and subcutaneous (inguinal) deposits [18]. The adipocytes of visceral and subcutaneous adipose tissue are different in size and metabolic activity, which influences the ability to respond to the insulin antilipolytic and catecholamines lipolytic effects [19].

In visceral adipose tissue there is a lower and higher lipogenic and lipolytic effect, respectively. Since it is more lipolytic, this tissue releases more free FA (FFA) that can be stored in the liver and muscle. This ectopic storage is associated with insulin resistance, which makes this tissue more pathogenic [20]. In addition, the proximity of visceral fat deposit with the organs, as well the drainage of FFA and inflammatory cytokines through the portal vein can contribute to the development of pathologies such as DM2, cardiovascular diseases and others [21].

The WAT has two primary metabolic activities, lipogenesis - synthesis and storage of FA in the form of triglyceride (TAG) - and lipolysis (hydrolysis of TAG in glycerol and FA) [22], being the this a therapeutic target for the obesity treatment. These primary metabolic activities are regulated by endocrine and neural mechanisms, aiming to maintain body fat homeostasis [5]. Catecholamines, mainly adrenaline, are lipolytic agents in WAT in humans and rodents [23]. Lipolysis occurs in response to the β-adrenergic stimulation by catecholamines. β-ARs belong to the large family of G protein-coupled receptors (GPCRs). In rodent WAT, β_3-AR is the predominant receptor subtype and represents the primary signaling pathway for lipolysis [24].

Catecholamines activate β-ARs coupled to stimulatory G protein (Gs), which activate adenylate cyclase (AC). When activated, AC catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (FIGURE 1). This second messenger binds and activates protein kinase A (PKA), which phosphorylates the hydroxyl groups on the serine residue of the hormone-sensitive lipase (HSL). When activated, HSL translocate from the cytosol to the interface of the lipid droplet where it binds to the FA binding proteins (FABP), as FABP4 and aP2, and initiates the hydrolysis of TAG, diacylglycerol and monoacylglycerol [25]. The FA of this hydrolysis will be released and transported in plasma to supply the energy demands of others tissues and organs [26]. The perilipin A protein, which suppresses lipolysis by blocking the access of HSL to the lipid droplet, when it undergoes PKA phosphorylation, moves from the surface of the lipid droplet

to the cytosol, which allows the binding between the HSL and substrate at the droplet interface lipid [27].

Natriuretic peptides are also lipolytic agents that activate the natriuretic peptide A receptor (NPR-A) and, consequently, increase guanylate cyclase (GC) activity and the production of cyclic guanosine monophosphate (cGMP), which activates the protein kinase G (PKG), responsible for phosphorylating HSL in adipose tissue [28] (FIGURE 1). Lipolysis induced by natriuretic peptides does not undergo insulin action, which is associated with the inhibition of lipolysis [23]. Other lipolytic agents are adipose triglyceride lipase (ATGL), responsible for hydrolyzing TAG; lipase monoacylglycerol (MGL), growth hormone; adrenocorticotropin; glucocorticoids and TNF-α.

TNF-α can promote lipolysis in the adipocyte by reducing the expression and/or function of perilipin via the mitogen-activated protein kinase family (MAPK) and inhibition of insulin receptor (IR) signaling [29] (FIGURE 1). In a study with adipose cells derived from 3T3-L1 fibroblasts, it was observed that in the presence of TNF-α the perilipin expression was reduced and lipolysis increased [30]. A possible mechanism for lipolytic action of TNF-α is its ability to activate the MAPK cascade, mainly extracellular signal-regulated kinase (ERK). Regarding the IR inhibition pathway, insulin binding to IR is known to trigger tyrosine phosphorylation of this receptor and insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), which leads to activation of the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB signaling cascade and, subsequently, phosphodiesterase 3B (PDE-3B) in the cytosol [5,29]. Activation of PDE-3B triggers cAMP hydrolysis in adenosine monophosphate'5 (AMP'5) [28]. In turn, TNF-α has the role of inactivating and reducing IRS-1 in adipocytes, due to its ability to neutralize tyrosine phosphorylation through IRS-1 serine phosphorylation [29].

IL-6 is another cytokine that can act on lipid metabolism (FIGURE 1). This finding is supported by the study which demonstrated that IL-$6^{-/-}$ mice developed obesity when compared to wild animals [31]. Although the regulation of lipid metabolism in adipose tissue via IL-6 has not yet been elucidated, human, rat and in vitro studies suggest that IL-6 can trigger lipolysis by increasing cAMP concentrations and, consequently, adenosine monophosphate-activated protein kinase activation (AMPK) [32]. In turn, AMPK would increase lipolysis via phosphorylation of ATGL [33]. To illustrate, it has been observed that AMPK gene deletion reduces ATGL phosphorylation in adipose tissue of mice, suggesting that AMPK activates lipolysis through the phosphorylation of ATGL and maintains an adequate level of lipolysis in adipose tissue [33].

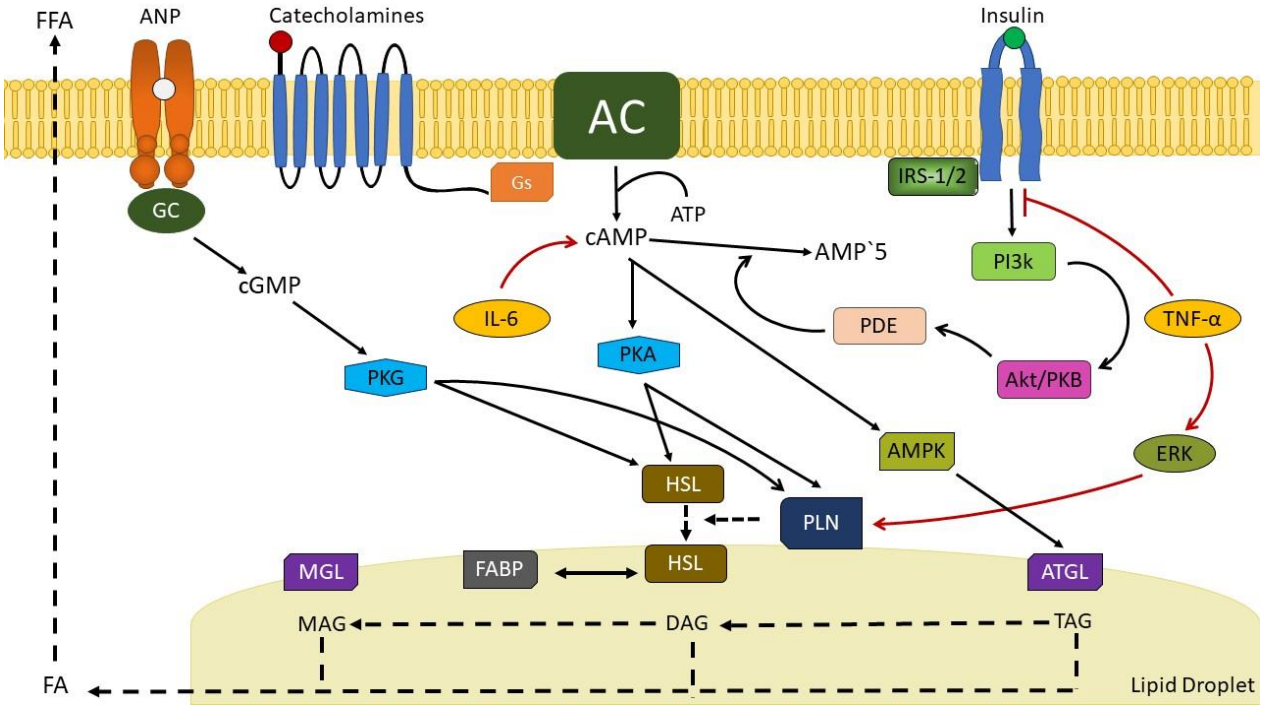

FIGURE 1: Signaling pathway of lipolysis in adipose tissue. AC: adenylate cyclase; Akt/PKB: protein kinase B; AMP'5: adenosine monophosphate'5; cAMP: cyclic adenosine monophosphate; AMPK: adenosine monophosphate-activated protein kinase; ANP: atrial natriuretic peptide; ATGL: adipose triglyceride lipase; DAG: diacylglycerol; ERK: extracellular signal-regulated kinase; FA: fatty acid; FABP: fatty acid binding proteins; FFA: free fatty acid; GC: guanylate cyclase; cGMP: cyclic guanosine monophosphate; Gs: stimulatory G protein; HSL: hormone-sensitive lipase; IL-6: interleukin 6; IR: insulin receptor; IRS-1/2: substrates of insulin receptor 1 and 2; MAG: monoacylglycerol; MGL: monoacylglycerol lipase; NPR-A: natriuretic peptide receptor; PDE-3B: phosphodiesterase 3B; PI3K: 3-kinase phosphatidylinositol; PKA: protein kinase A; PKG: protein kinase G; PLN: perilipin A; TAG: triacylglycerol; TNF-α: tumor necrosis factor α; βARs: beta adrenergic receptors. Continuous black arrow: lipolytic signaling pathways; continuous red arrow: action of inflammatory cytokines; dashed black arrow: lipolysis of fatty acids; red arrow with bar: blocking the signaling pathway.

In addition to TAG storage, WAT also plays an endocrine function by producing adipokines, such as leptin, adiponectin, resistin, visfatin, TNF-α, MCP-1, plasminogen activator inhibitor (PAI-1), and interleukins (IL-6, IL-8, IL-10, IL-1) [34]. In obese individuals, the increase in adipose tissue, mainly visceral, results in a higher production of proinflammatory adipokines, such as TNF-α, IL-6, MCP-1 [35].

WAT expansion leads to necrosis/apoptosis of adipocytes and, consequently, to the release of lipid droplets that are toxic to adipocytes and activate macrophage recruitment [3]. Activation of macrophages can be divided into two states of polarization, M1 and M2. M1 macrophages are induced by inflammatory mediators, such as interferon gamma (IFNγ), and produce proinflammatory cytokines such as TNF-α and IL-6, whereas M2 macrophages produce high concentrations of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist [36]

In macrophages and adipocytes, receptors like Toll (TLR) 2 and 4, which participate in pathogenic and immunological recognition, can be activated by saturated FA [3] and stimulate nuclear factor kappa B (NFκB), leading to production of pro-cytokines inflammatory agents, such as TNF-α and IL-6 [37]. Proinflammatory cytokines associated with the activation of N-terminal kinase Jun-c (JNK) and, subsequently, phosphorylation at the serine residue of IRS1, may impair insulin receptor signaling [3]. Thus, in obesity, these macrophage infiltrates in WAT assume a pro-inflammatory phenotype, because they secrete cytokines that aggravate insulin resistance [38]. This causes a chronic subclinical inflammation state and triggers metabolic disorders such as DM2.

Brown adipose tissue

The adipocytes of BAT have as precursor the *Myf5*$^{+}$ myogenic factor, it is multilocular and present a large number of mitochondria and UCP-1 [17]. The BAT locations in rodents are the interscapular, subscapular, axillary, perirenal and periaortic regions [18]. In humans, BAT is found in the cervical, supraclavicular, paravertebral, mediastinal and perirenal regions [18]. BAT is able to dissipate energy through thermogenesis to maintain body temperature. Thermogenesis without tremor has multiple components, such as diet-induced thermogenesis, exercise-associated thermogenesis, non-exercise activity thermogenesis [17].

UCP-1 belongs to a family of mitochondrial anion-carrying proteins present in the inner membrane of the mitochondria. In BAT, UCP-1 uncouples oxidative phosphorylation, dissipating the electrochemical proton gradient across the inner membrane of the mitochondria, which produces heat and maintains body temperature [39]. Thus, UCP-1 is a

crucial molecule in metabolic thermogenesis because it produces heat induced by cold and diet, being a fundamental component in energy expenditure. In addition, due to its energy expenditure due to thermogenesis, UCP-1 present in BAT has been a therapeutic target for the fight against obesity and the metabolic syndrome [17].

BAT thermogenesis is regulated by the SNS, mainly under cold conditions (FIGURE 2). The noradrenaline released by SNS binds to the β-ARs that activate the AC-cAMP-PKA signaling pathway [40]. PKA phosphorylates intracellular lipases, such as HSL, which hydrolyzes TAGs in FA [40]. FA activate UCP-1 decoupling by displacing the guanidine nucleoside diphosphate (GDP) from UCP-1, and are oxidized by mitochondria as an energy source for heat production [41]. In addition to intracellular lipids, adipocytes can also use as energy source glucose and FFA [42]. The WAT reserves appear to provide the main energy source to sustain thermogenesis by lipolysis, but the lipid stores in the BAT are fundamental for thermogenesis [27].

The three isoforms of β-AR are present in the BAT, with the β_3 isoform being more prevalent [39]. Studies with a genetically modified animal model have shown that mice with the deletion of the three receptor subtypes ($\beta_1^{-/-}$, $\beta_2^{-/-}$, $\beta_3^{-/-}$) present hypothermia in response to cold [43]. However, even β_3-AR being predominant in BAT, β_3-$AR^{-/-}$ mice exhibit cold-induced adaptations, such as increased UCP-1 content in BAT [8]. Contrary to these findings, β_1-$AR^{-/-}$ mice present hypothermia in response to cold [6]. Deletion of β_1-AR was also associated with the inhibition of cold-induced BAT hyperplasia [44]. These findings highlight the importance of β_1-ARs in BAT thermogenesis.

Even sympathetic stimulation directly modulating BAT activity, other factors such as hormones, cytokines, adipokines, myokines and growth factors may interact with the sympathetic pathway or even modulate this signaling pathway in the regulation of BAT activity [17]. As an example, elevated TNF-α concentration suppressed UCP-1 expression in BAT from genetically obese (ob/ob) mice [45]. It is also known that the peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) is responsible for co-activating multiple signals for biogenesis and mitochondrial activity in BAT [46].

Like WAT, BAT plays an endocrine function, secreting factors such as IL-6, IL-1, fibroblast growth factor 21 (FGF-21), and insulin-like growth factor 1 (IGF-1). They act in organs and tissues such as liver, pancreas, brain and WAT, modulating sympathetic tone, FA oxidation, insulin secretion and glucose uptake [17,39].

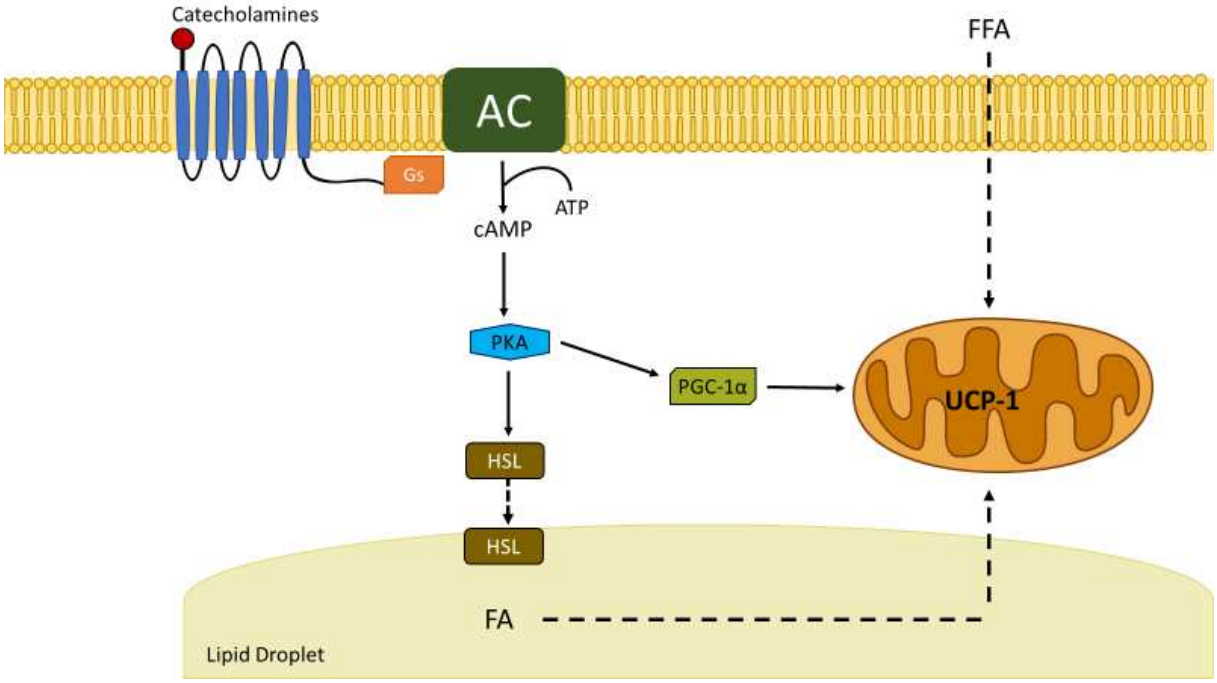

FIGURE 2: β-adrenergic signaling pathway in brown adipose tissue. AC: adenylate cyclase; cAMP: cyclic adenosine monophosphate; FA: fatty acid; FFA: free fatty acid; Gs: stimulatory G protein; HSL: hormone-sensitive lipase; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator 1 α; PKA: protein kinase A; UCP-1: decoupling protein 1; βARs: beta adrenergic receptors. Continuous black arrow: β-adrenergic signaling pathway; black arrow dashed: lipolysis fatty acids and free fatty acids.

Beige adipose tissue

In addition to the BAT found in specific body deposits, it is possible to find adipocytes with characteristics of brown adipocytes in WAT, due to cold environmental conditions and β-adrenergic stimulation [47]. The appearance of adipocytes with BAT phenotype in the WAT deposits is known as browning and is called adipocyte beige or brite (brown and white conjugation) [48]. Beige adipocytes are multilocular and have multiple mitochondria. Like the white adipocytes its precursor is the *Myf5*$^{-}$ adipoblast [17]. These adipocytes can also originate from mature white adipocytes in response to cold or β-adrenergic stimulation [18].

The predominant activation of β_3-ARs in WAT by catecholamines induces lipolysis and UCP-1 synthesis, which is not normally expressed in this tissue [49]. Subcutaneous adipose tissue is more likely to browning compared to visceral adipose tissue, since subcutaneous adipocytes are predominantly smaller and have a greater potential for cell differentiation [50]. In mice, inguinal adipose tissue has been considered to be the deposition of beige adipocytes, due to its greater sympathetic innervation and higher noradrenaline concentration [44].

Although only partially elucidated, the browning process seems to be controlled by factors such as nutritional and metabolic status, physical activity level and environmental condition [39]. Factors such as the gene expression of PGC-1α and PR domain containing 16 (Prdm16) have also been associated with the browning process. Overexpression of the Prdm16 gene is associated with increased energy expenditure, lower body weight gain and improved glucose tolerance in obese mice [51]. Interest in this tissue is due to its capacity to increase energy expenditure, being a potential therapeutic target in the treatment of obesity and associated diseases.

Exercise and Adipose tissue

While physical inactivity is considered to be one of the most important public health problems of the 21st century, regular physical activity promotes important health benefits because it can reduce the risk of chronic diseases, such as cardiovascular, DM2 and some cancers [52]. In this way, exercise training presents not only prophylactic value, but also has therapeutic effect for many diseases, among it the obesity.

Aerobic exercise training may reduce adipocyte size and lipid content by repeated activation of lipolysis, since an exercise session is able to reduce FA synthesis and increase lipolysis in adipose tissue [53]. In addition, aerobic exercise training can lead to adaptations in skeletal muscle, such as increased mitochondrial density and proliferation of capillaries and proteins, leading to greater transport and oxidation of FFA [53].

The increased use of FA during exercise requires the interaction of neural, hormonal, circulatory and muscular factors, which increase energy demand and facilitate the oxidation of intramuscular FFA and TAG in the skeletal muscle [53]. Elevated catecholamines during exercise are responsible for activating the β-adrenergic signaling pathway in adipocytes, increasing lipolytic activity through HSL phosphorylation (FIGURE 3A). Lipolysis may also be active by atrial natriuretic peptides, which are involved in lipid mobilization during exercise (FIGURE 3A). To exemplify, the study by Moro et al. demonstrated that, under β-adrenergic inhibition, aerobic exercise is capable of increasing circulating concentrations of atrial natriuretic peptide in humans [54]. In addition, exercise session may also increase lipolysis in adipose tissue via IL-6 induced by muscle contraction during exercise by means of paracrine mechanisms in the adipose tissue after its secretion in the systemic circulation [55].

It has also been shown that exercise training can reduce visceral adipose tissue, even in the absence of body weight loss [11]. It is suggested that, by limiting the expansion of adipose tissue, exercise training may attenuate the changes resulting from obesity in the immune cells of this tissue [56]. For example, obese mice submitted to moderate aerobic

exercise training had a reduction of infiltrated M1 macrophages and proinflammatory cytokines in visceral adipose tissue compared to control mice [57]. In addition, moderate aerobic exercise training induced phenotypic alteration of M1 macrophage to M2 macrophage in the adipose tissue of obese mice [58].

The greater effect of exercise training, mainly aerobic, on visceral adipose tissue reduction compared to subcutaneous adipose tissue is related to the higher lipolytic response and triglyceride depletion in visceral adipose tissue [20], which presents higher and lower density and sensitivity of β-ARs and α-ARs, respectively [59]. In an animal model, moderate aerobic exercise training has been shown to reduce visceral adipose tissue in obese mice [60]. The visceral fat reduction induced by exercise training may mediate the anti-inflammatory effect, with subsequent reduction in concentrations of pro-inflammatory adipokines [35]. The anti-inflammatory effect of aerobic exercise training is associated with reduced recruitment of M1 macrophages [57] and, consequently, reduction of proinflammatory cytokines - TNF-α and IL-6 - in adipose tissue of mice [7].

Different mechanisms may contribute to this anti-inflammatory effect of exercise training. There is evidence of increased release of cortisol and adrenaline from the adrenal glands [61]; increased myocin production and release; reduction in Toll 4 receptor (TLR4) expression in monocytes and macrophages and, consequently, inhibition of proinflammatory cytokines cascade [62]; inhibition of monocytes and macrophages infiltration into adipose tissue; change in macrophages phenotype present in adipose tissue; reduction in pro-inflammatory monocytes circulation and increase in regulatory T cells (TReg) circulation [35].

Regarding the myokines, their discovery highlights the association between exercise and inflammation. After an exercise session, the release of myokines - IL-6, TNF-α, IL-10 - into the circulation may influence metabolism and modify the production of cytokines in other tissues and organs [63]. IL-6 has pro- and anti-inflammatory effects, and its chronic high level is associated with the development of obesity and DM2. However, the increase in IL-6 due to muscle contractions during aerobic exercise and its return to baseline post-exercise concentrations is an important regulator of the cellular metabolism of other tissues [64], as it increases glucose uptake and intramuscular lipid oxidation [12]. The anti-inflammatory effect of IL-6 may be in part due to its ability to increase IL-10 and IL-1ra production by monocytes and macrophages [65].

In addition to WAT, aerobic exercise can also increase BAT thermogenesis and WAT browning through the SNS activation (FIGURE 3B). The increase of catecholamines induced by exercise activates β-ARs and its signaling pathway that phosphorylates HSL, increasing

lipolysis and, consequently, UCP-1 activity [15]. Recently, moderate aerobic exercise training has been shown to increase UCP-1 protein expression in epididymal WAT of rats [66]. Associated with this mechanism, the increase of natriuretic peptides and myokines in response to exercise activates BAT thermogenesis and WAT browning, by increasing the expression of UCP-1 [15]. The ability to produce and secrete myokines is mainly due to the metabolic changes associated with muscle contractions performed during an aerobic exercise session, which lead to an increase in the release of several myokines - IL-6, IL-15 and irisin - capable of interacting with the adipose tissue [67].

Irisin is a myokines induced by exercise, which leads to subcutaneous WAT browning and activation the thermogenesis of this adipose tissue by increasing the UCP-1 expression [68]. Its release into the bloodstream occurs by the proteolytic cleavage of fibronectin type III domain containing 5 (FNDC5) from skeletal muscle, which is stimulated by the increase in the expression of PGC-1α induced by aerobic exercise [68]. In turn, the increase in PGC-1α expression is due to the catecholamine concentration elevation promoted by exercise, which is responsible for stimulating the mitochondrial proteins expression involved in FA oxidation [69]. In rats submitted to moderate aerobic exercise training there was greater PGC-1α protein expression, compared to control group [66]. Furthermore, aerobic exercise training may increase noradrenergic tone and vascularization in BAT, in addition to increasing the browning of visceral WAT in rats [14]. Additionally, it was demonstrated that mice that exercised in a voluntary wheel-running presented higher UCP-1 gene expression in subcutaneous adipose tissue than their respective controls [70]. These findings suggest a therapeutic potential of aerobic exercise training against metabolic complications related to obesity with visceral fat accumulation.

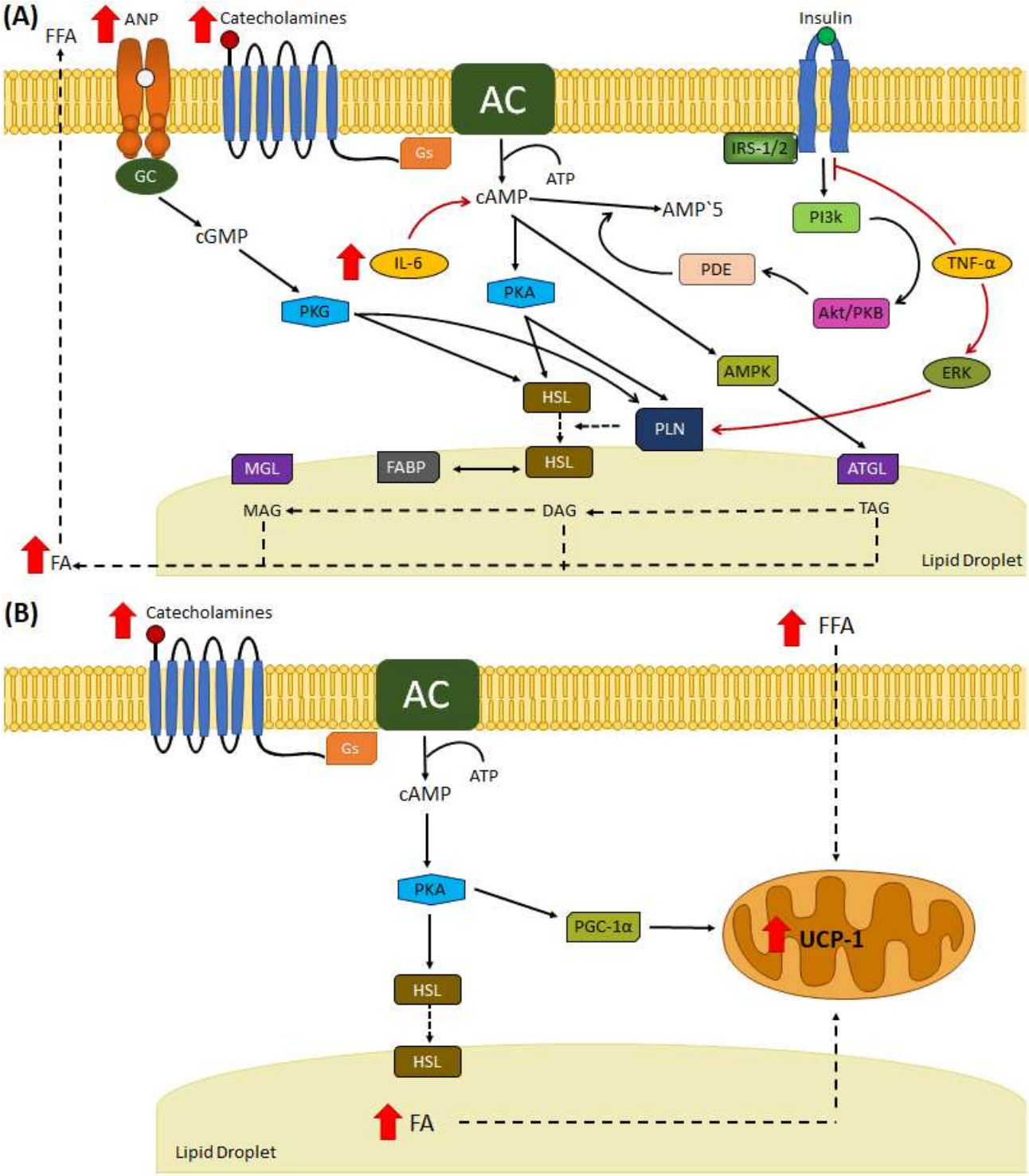

FIGURE 3: Effect of exercise on β-adrenergic signaling pathway in white (A) and brown (B) adipose tissue. AC: adenylate cyclase; Akt/PKB: protein kinase B; AMP'5: adenosine monophosphate'5; cAMP: cyclic adenosine monophosphate; AMPK: adenosine monophosphate-activated protein kinase; ANP: atrial natriuretic peptide; ATGL: adipose triglyceride lipase; DAG: diacylglycerol; ERK: extracellular signal-regulated kinase; FA: fatty acid; FABP: fatty acid binding proteins; FFA: free fatty acid; GC: guanylate cyclase; cGMP: cyclic guanosine monophosphate; Gs: stimulatory G protein; HSL: hormone-sensitive lipase; IL-6: interleukin 6; IR: insulin receptor; IRS-1/2: substrates of insulin

receptor 1 and 2; MAG: monoacylglycerol; MGL: monoacylglycerol lipase; NPR-A: natriuretic peptide receptor; PDE-3B: phosphodiesterase 3B; PGC1α: peroxisome proliferator-activated receptor-γ coactivator 1-α; PI3K: 3-kinase phosphatidylinositol; PKA: protein kinase A; PKG: protein kinase G; PLN: perilipin A; TAG: triacylglycerol; TNF-α: tumor necrosis factor α; UCP-1: decoupling protein 1; βARs: beta adrenergic receptors. Continuous black arrow: signaling lipolytic pathways; continuous red arrow: action of inflammatory cytokines; dashed black arrow: lipolysis of fatty acids and free fatty acids; red arrow with bar: blocking the signaling pathway; red arrow up: effect of exercise.

CONCLUSION

Evidence reported in this review supports the idea that aerobic exercise plays a key role in the management of obesity. The evidence has shown that aerobic exercise training promotes various adaptations in the adipose tissue like increase of the lipolysis and browning in WAT, and thermogenesis in BAT, which help to protects against obesity. Also, aerobic exercise training has the potential to combat the chronic subclinical inflammation associated with obesity. Moreover, it is important to note that further studies are needed to clarify the role of aerobic exercise training in the β-adrenergic signaling pathway of the adipose tissue, so will be possible determine more clearly the therapeutic potential of aerobic exercise training in the treatment of obesity and its complications.

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3.2 Artigo 2 - Original research

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.

Keywords: Moderate exercise; adipocyte; lipolytic genes; inflammatory cytokines.

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]. 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 minutes and 10% of the maximal speed until 60 minutes 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 minutes. The graded treadmill exercise test began at 6 m/min with no grade and increased by 3 m/min

every 3 minutes 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.

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 105ºC for 24 hours. 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 hours, 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 x 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 48h 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 19459, USA) and stained with toluidine blue/sodium borate. The images were scanned using a microscope (200x) (NIKON Eclipse 24 E600), connected to a camera (Feldmann Wild Leitz DigiPro 5.0M) and software (ACCU-SCOPE 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 < 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^{TM} RNAse inhibitor (20 U), 1mM of dNTP Mix, RevertAid^{TM} Reverse Transcriptase (200U), 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.

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

2.8. 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 8000g 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 (TNF-α) 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, to test the effects of the factors: gene deletion (control vs. knockout); and exercise training (control vs. training); as well as the interactions between factors. For all analyses we used the software SigmaPlot®, 12.5 version (Systat Software, San Jose, CA). Data are presented as means ± SE. A statistical significance level of 5% was adopted.

3. Results

3.1. Running capacity and body composition

Gene deletion, independent of MCAE, did not affect ($p > 0.05$) the animals' running capacity (Table 2). The MCAE increased the total distance run ($p < 0.05$), independent of gene deletion, and such effect can be observed in trained β_1-AR$^{-/-}$ and WT animals. However, there was no interaction between factors ($p > 0.05$).

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, however, reduced ($p < 0.05$) the final body weight, independent of gene deletion (Table 2). Despite that, we found no interaction between factors ($p > 0.05$).

The percentage of body fat and of water in the carcass were not affected by either gene deletion or MCAE factors ($p>0.05$). Nonetheless, there was interaction between factors ($p < 0.05$). The percentages of fat and water were higher 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 these parameters ($p < 0.05$), independent of gene deletion (Table 2). We observed such effects on β_1-AR$^{-/-}$t and WTt groups. Nevertheless, there was neither effect of gene deletion ($p > 0.05$), nor interaction between factors ($p > 0.05$).

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 ± 1.085*$^{\#}$
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; (*) 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. Two-way ANOVA followed by Tukey test.

3.2. Adipocytes area

Figure 1 shows representative photomicrographs analyzed adipose tissues. There was no independent gene deletion effect ($p > 0.05$) on epididymal adipocyte area (Fig. 2A), however, the MCAE affected ($p < 0.05$) the adipocyte area (Fig. 2A), independent of gene deletion. For instance, both trained WT and β_1-AR$^{-/-}$ animals showed lower area than their respective controls. However, there was no interaction between factors ($p > 0.05$).

In the inguinal (Fig. 2B) and brown (Fig. 2C) tissues, neither gene deletion nor MCAE factors affected the adipocyte area ($p > 0.05$). However, there was interaction between the factors ($p < 0.05$), as the area of inguinal adipocytes from β_1-AR$^{-/-}$c group was higher than that of WTc group. In addition, the β_1-AR$^{-/-}$t group exhibited lower inguinal adipocyte area than β_1-AR$^{-/-}$c group. Concerning BAT, the β_1-AR$^{-/-}$c group exhibited higher adipocyte area than WTc group. Moreover, the MCAE reduced the adipocyte area in β_1-AR$^{-/-}$ group only.

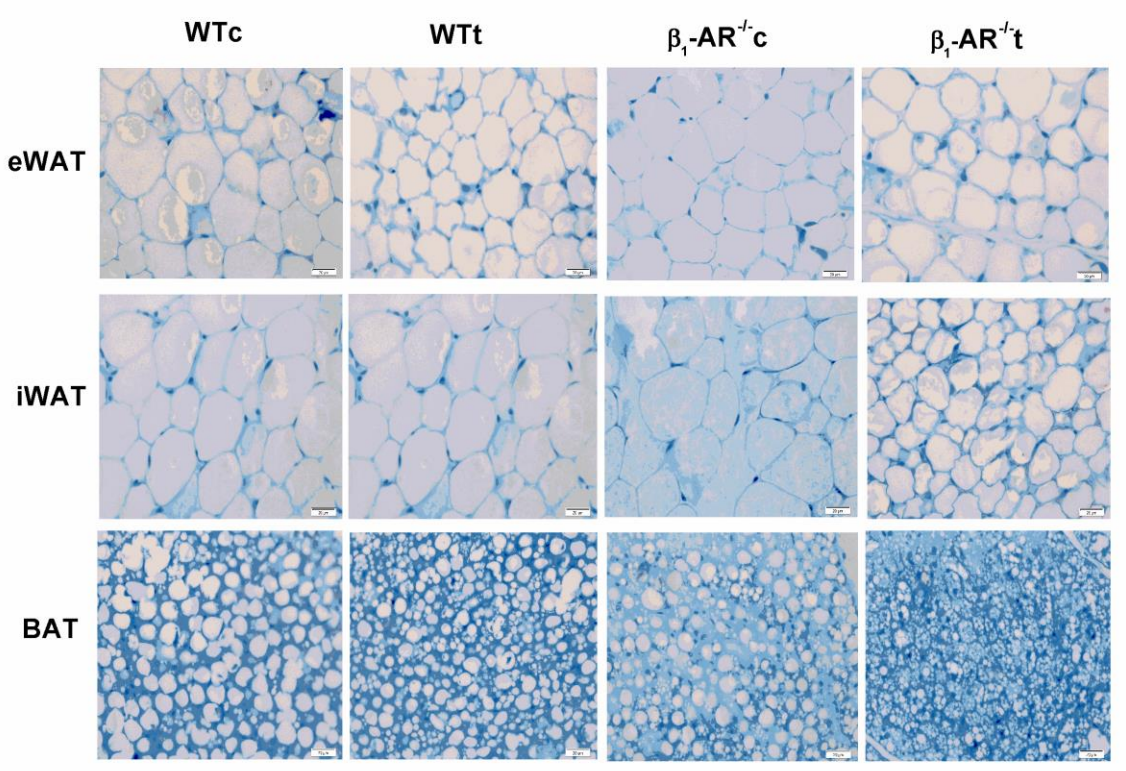

Figure 1. Representative photomicrographs of adipose tissues. eWAT: epididymal white adipose tissue; iWAT: inguinal white adipose tissue; BAT: brown adipose tissue; WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; Staining: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

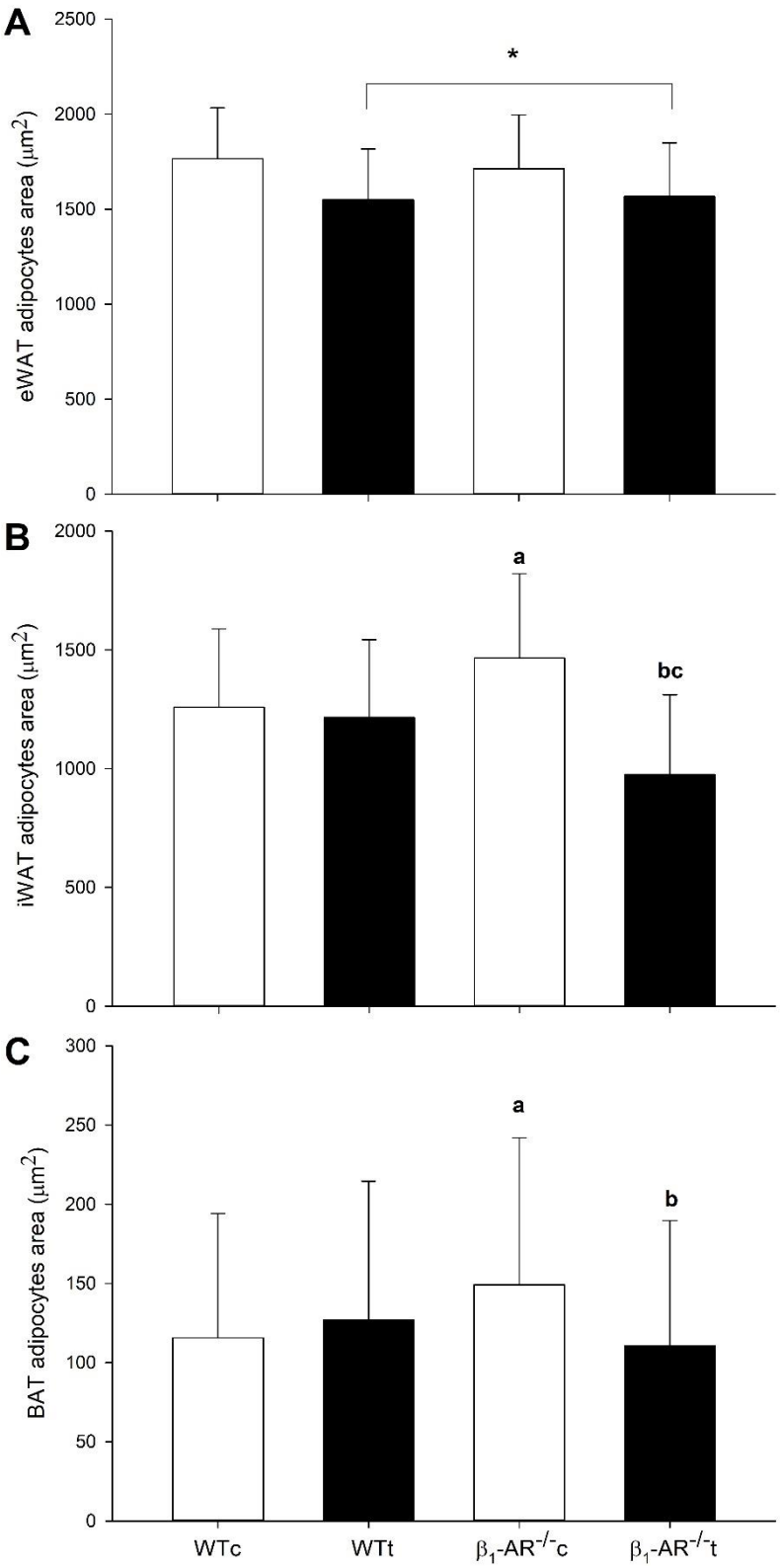

Figure 2. Adipocytes area in epididymal (eWAT) (A), inguinal (iWAT) (B) and brown (BAT) (C) adipose tissue. Data are means ± SE of 60-100 cells/animal per group. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; (*) denotes 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.

3.3. Adipocytes frequency

Figure 3 presents the frequency of adipocytes of different size. In eWAT, the frequency of adipocytes <1000μm^2 and >1000μm^2 (Fig. 3A and B), the independent effects of gene deletion and MCAE factors were observed ($p < 0.05$). The frequency of <1000μm^2 adipocytes was higher in β_1-AR$^{-/-}$ compared to WT animals; and in trained compared to control mice (Fig. 3A). Nonetheless, the adipocytes >1000μm^2 were less frequent in β_1-AR$^{-/-}$ than in WT animals; and in trained than in control animals (Fig. 3B). Despite that, there was no interaction between the factors for the frequency of <1000μm^2 and >1000μm^2 adipocytes ($p > 0.05$).

Respecting the iWAT, the frequency of <1000μm^2 adipocyte (Fig. 3C) was not affected by either gene deletion or MCAE factors ($p > 0.05$). Yet, there was interaction between factors ($p < 0.05$), as the frequency of adipocytes <1000μm^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. In addition, the >1000μm^2 adipocyte frequency (Fig. 3D) had no independent effect of either gene deletion or MCAE ($p > 0.05$). However, we observed no interaction between factors ($p < 0.05$). The frequency of >1000μm^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 ($p < 0.05$).

With reference to BAT, there was an independent effect of gene deletion for the frequency of adipocytes <300μm^2 (Fig. 3E) and >300μm^2 (Fig. 3F). For instance, the <300μm^2 and >300μm^2 adipocytes frequency were higher and lower, respectively, in β_1-AR$^{-/-}$ than in WT animals. Nevertheless, we found no interaction between factors ($p > 0.05$).

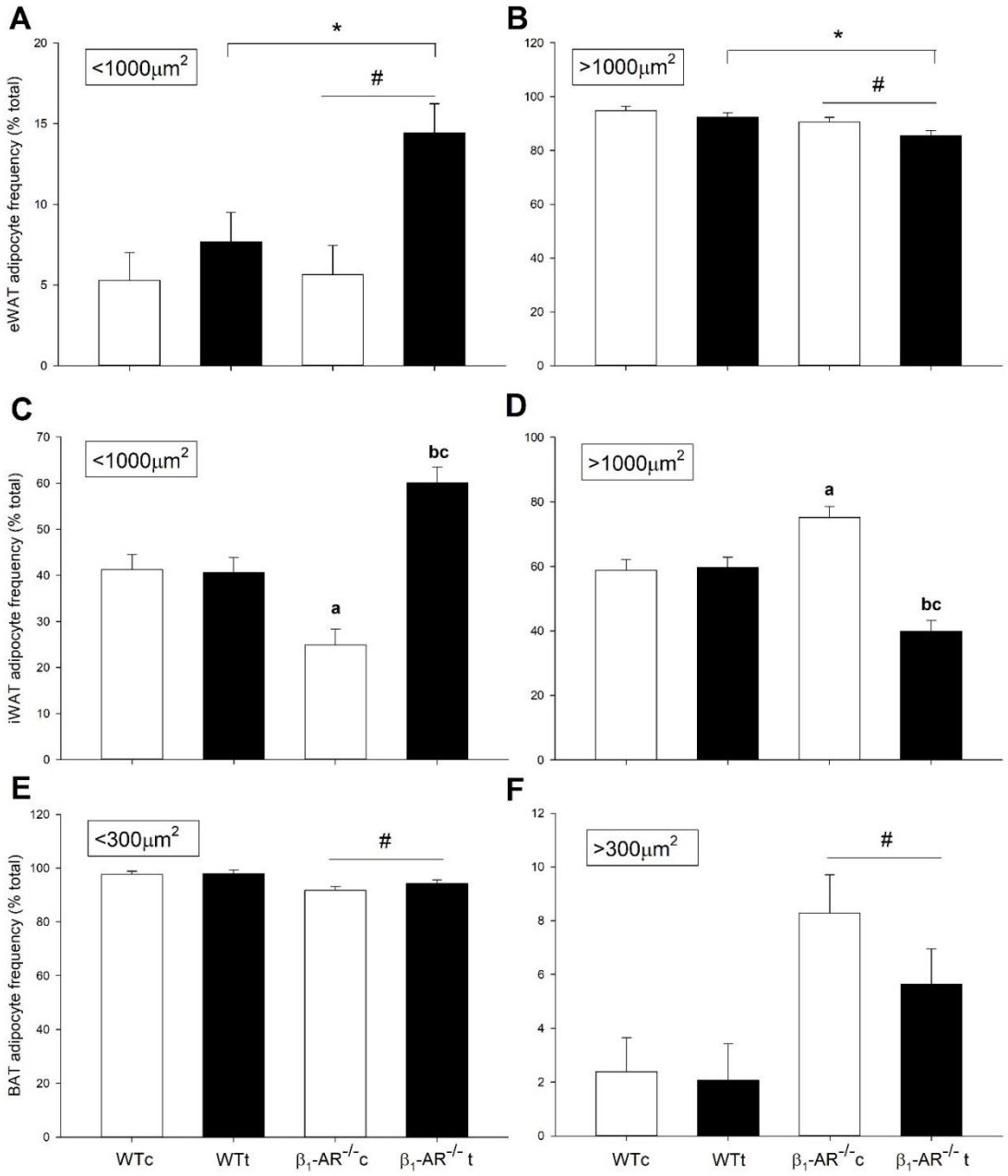

Figure 3. Adipocytes frequency of different sizes in epididymal (eWAT) (A and B), inguinal (iWAT) (C and D) and brown (BAT) (E and F) adipose tissue. Data are means ± SE of 60-100 cells/animal per group. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: 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

Figure 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.

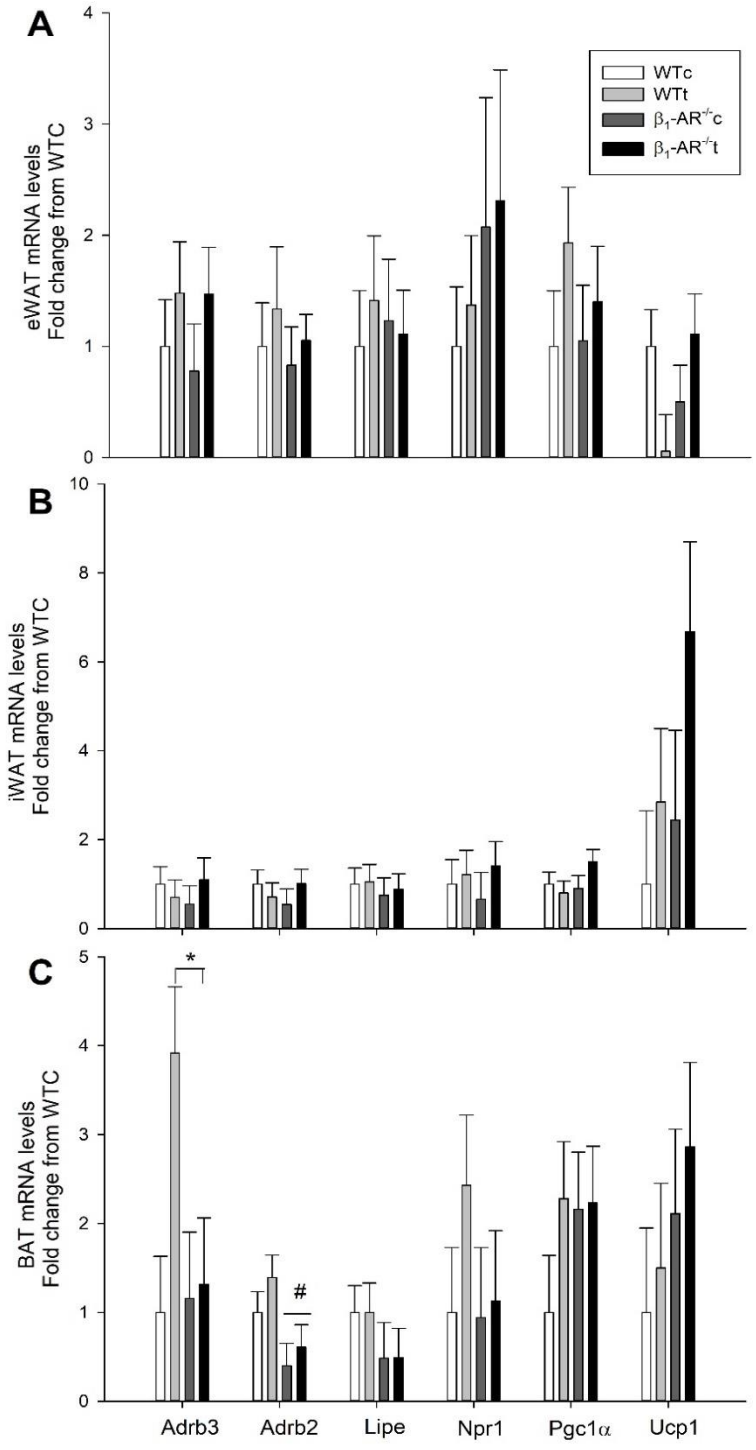

Figure 4. Epididymal (eWAT) (A), inguinal (iWAT) (B) and brown (BAT) (C) adipose tissue gene expression. Data are means ± SE. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1-AR$^{-/-}$t: β_1-AR$^{-/-}$ trained; Lipe: hormone-sensitive lipase; Ucp1: uncoupling 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)

3.5. Inflammatory cytokines concentrations

Figure 5 presents the concentrations of cytokine in eWAT, iWAT and BAT. In all tissues, there was no independent effect of either gene deletion or MCAE ($p > 0.05$) for IL-12p70 (Fig. 5A). However, there was interaction between the factors ($p < 0.05$) in eWAT and iWAT. For example, in eWAT, the concentrations of IL-12p70 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 MCP-1 (Fig. 5C) concentrations, there were no independent effects of either gene deletion or MCAE ($p > 0.05$). Moreover, we found no interactions between factors ($p > 0.05$).

With respect to TNF-α concentrations (Fig. 5D), there was no gene deletion and MCAE independent effects on eWAT ($p > 0.05$). However, in this tissue there was interaction ($p < 0.05$) between gene deletion and MCAE factors. The β_1-AR$^{-/-}$c animals showed higher TNF-α 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, there was an independent effect of the gene deletion factor ($p < 0.05$), since the TNF-α concentrations in β_1-AR$^{-/-}$ animals were higher than those in WT mice. The independent MCAE factor effect was observed in BAT ($p < 0.05$), as trained animals showed lower TNF-α concentrations than control animals. However, there was no interaction between the factors ($p > 0.05$).

The MCAE factor affected ($p < 0.05$) the IL-6 concentrations in eWAT (Fig. 5E), independent of gene deletion. The trained animals showed lower concentrations than control animals ($p < 0.05$). However, there was no interaction between the factors ($p > 0.05$). In iWAT and BAT, neither factor effect of gene deletion and MCAE ($p > 0.05$), nor interaction between factors ($p > 0.05$) were observed.

Regarding the concentrations of IL-10 in eWAT and BAT (Fig. 5F), there was an independent MCAE factor effect ($p> 0.05$). For instance, trained animals showed lower concentrations than controls. However, there was no interaction between these factors ($p> 0.05$). In iWAT, nonetheless, neither factor effect of gene deletion and MCAE ($p > 0.05$), nor interaction between factors ($p > 0.05$) were found.

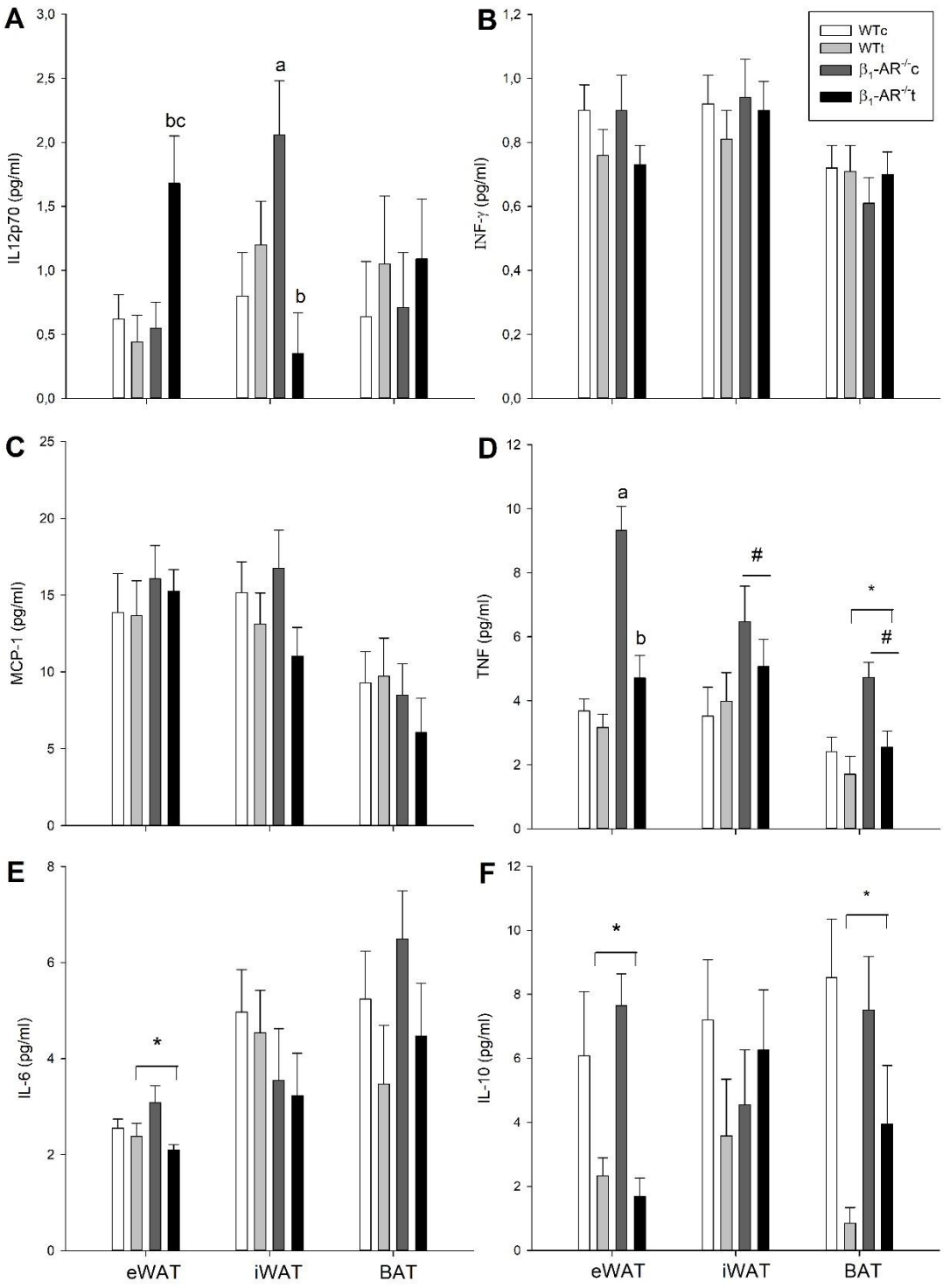

Figure 5. IL-12p70(A), INFγ (B), MCP-1 (C), TNF-α (D), IL-6 (E) and IL-10 (F) inflammatory cytokine concentrations in epididymal (eWAT), inguinal (iWAT) and brown (BAT) adipose tissues; Data are means ± SE of 60-100 cells/animal and 6 animal/group. WTc: wild-type control; WTt: wild-type trained; $\beta_1AR^{-/-}$c: 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)

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. Compensatory mechanisms like increases in β-AR expression or activity may explain such increased expression. There is evidence that exercise may enhance BAT thermogenesis by activating 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 acid 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 has 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 of β_1-AR$^{-/-}$

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 >1000μm^2 and increased that of <1000μm^2 in eWAT. Because β_1-AR$^{-/-}$c mice

showed a frequency of adipocytes <1000μm^2 similar to that of WT mice, our MCAE was important in increasing the frequency of adipocytes <1000μm^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 <1000μm^2 in trained mice.

Concerning iWAT, our β_1-AR$^{-/-}$c mice showed higher and lower >1000μm^2 and <1000μm^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 >1000μm^2 and <1000μm^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 >300μm^2 and <300μm^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.

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3.3 Artigo 3 - Original research

Effects of aerobic exercise on lipases activity and inflammatory cytokine profile in β_1-AR$^{-/-}$ mice mesenteric adipose tissue and liver

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ABSTRACT

Aim: Investigate the effects of a moderate continuous aerobic exercise (MCAE) program on lipases activity and inflammatory cytokine profile in the mesenteric white adipose tissue (mWAT) and liver of β_1-AR$^{-/-}$ mice. **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, mWAT and liver were dissected and used to determine lipases activity and cytokines concentrations. **Results:** β_1-AR$^{-/-}$ animals exhibited higher body weight than WT animals, and it was reduced by the MCAE in both β_1-AR$^{-/-}$ and WT mice. MCAE also reduced mWAT in trained mice (β_1-AR$^{-/-}$t and WTt). β_1-AR$^{-/-}$ groups showed lower lipases activity in mWAT than WT groups, whereas in the liver, β_1-AR$^{-/-}$ animals presented higher lipases activity than WT animals, but no effect of MCAE was found. The higher ($p < 0.05$) concentrations of interleukin (IL)-6 and IL-10 in mWAT and liver found in β_1-AR$^{-/-}$c animals, compared to WTc animals, were also diminished by MCAE in β_1-AR$^{-/-}$t mice. **Conclusion:** Eight weeks of MCAE reduces the body and mWAT weights, and inflammatory cytokines in the mWAT and liver of β_1-AR$^{-/-}$ mice.

Keywords: Moderate exercise; adipocyte; liver; inflammatory cytokines.

1. Introduction

Adipose tissue is located in all body and may be categorized anatomically in visceral or subcutaneous [1]. Visceral adipose tissue (VAT) is subdivided into mesenteric, peri-renal, gonadal, epicardial, retroperitoneal, and omental regions, while SAT is located in inguinal region [2]. The adipocytes of VAT and SAT differ on size and metabolic activity, which influences the ability to respond to anti-lipolytic and lipolytic effects [3]. In VAT there is a lower and higher anti-lipolytic and lipolytic effect, respectively.

The higher lipolytic rate is because of higher β-adrenergic receptor (β-AR) and lower α-adrenergic receptor (α-AR) sensitivity to catecholamine stimulation [4]. Since it is more lipolytic, VAT releases more free fatty acid (FFA) that can be stored in the liver and muscle. The fat ectopic accumulation also known as non-alcoholic hepatic steatosis (NAFLD) is associated with insulin resistance, which makes this tissue more pathogenic [5,6]. Furthermore, the proximity of VAT with the organs and the drainage of FFA and inflammatory cytokines (e.g. TNF-α, MCP-1 and IL-6) through the portal vein to the liver may contribute increased liver fat [7] and to the onset of pathologies such as DM2 and cardiovascular diseases [8].

Exercise training may reduce VAT, even in the absence of body weight loss [9]. By limiting the expansion of adipose tissue, exercise training may improve the resulting excess weight changes in the immune cells of this tissue [10]. A study observed that obese mice submitted to moderate aerobic exercise training showed reduction of proinflammatory cytokines in VAT compared to control group [11]. Thus, the reduction in VAT induced by aerobic exercise training may mediate the anti-inflammatory effect, with subsequent reduction in concentrations of pro-inflammatory adipokines (e.g. TNF α, IL-6 and MCP-1) [12,13].

In addition, the greater effect of aerobic exercise training on the reduction of VAT is related to the higher lipolytic response in VAT [5], which presents higher and lower density and sensitivity of β-AR and α-AR, respectively [14,15]. In animal model, moderate aerobic exercise training was shown to reduce the VAT of obese mice [16], as well as in rats fed a high-fat diet [17]. In humans, combined exercise training (aerobic and resistance) associated with diet was more effective in reducing VAT compared to SAT [18]. Concerning the hepatic adaptations, the aerobic exercise training may reduce liver fat, which is a major risk factor for the complications related to metabolic syndrome [19]. It is known that aerobic exercise training also correlates with the improve on treatment of NAFLD [20]. Furthermore, a study observed that the voluntary running reduced expression of proinflammatory cytokines (e.g. IL-6, IL-1β and MCP-1) in liver of mice [21].

Although β-AR has a key role in VAT lipolysis and exercise training may affect the inflammatory profile in VAT and liver, the role of the β_1-AR associated with aerobic exercise training on lipases activity and cytokines profile of VAT and liver is not known. Therefore, understanding the effects of aerobic exercise training on VAT and liver in a condition of absence of β_1-AR may help to clarify the mechanisms of the beneficial influence of regular aerobic exercise on these organs. Thus, this study aims to investigate the effects of a moderate continuous aerobic exercise (MCAE) program on lipases activity and inflammatory cytokine profile in mWAT and liver 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), WT trained (WTt), β_1-AR$^{-/-}$ control (β_1-AR$^{-/-}$c) and β_1-AR$^{-/-}$ trained (β_1-AR$^{-/-}$t). 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 [22]. 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.

2.3. Tissue collection

Forty-eight hours after the last exercise training session, mice were weighed and killed by decapitation. Mesenteric white adipose tissue (mWAT) and liver were surgically removed and immediately frozen at -80° C for further analyses.

2.4. Lipases activity analysis

Lipases activity was measured in mWAT and liver using tributyrin as substrate. The protein extract of tissue was incubated under orbital shaking at 750 rpm for 1h at 37°C in 950 µl of the reaction medium (10% v/v tributyrin, 0.2% m/v gum arabic) previously emulsified in an ultrasonic bath for 10 min; 250 µl of 0.1 mol/L Tris.HCl buffer, pH 7.5; 50 µL of protein extract and 350 µL of distilled water. After incubation, the samples were conditioned on ice and added 50 µL of orthophosphoric acid (14.5 M), vortexed and centrifuged at 10000g for 10 min. After centrifugation, 500 µL of assay were filtered in 0.45 µm membranes and stored for the quantification of butyryl liberated from tributyrin by the enzymatic action. The whites were made by adding to orthophosphoric acid prior to addition of the enzyme extract. To read the results, a high-performance liquid chromatography (HPLC) (SPD-20A VP, Shimadzu®, Japan) was used, at 210 nm, on a capillary column (VP-ODS/C8/ Phenyl, Shimadzu®, Japan), with 150 by 4.6 mm. The injected sample volume was 20 µl, the flow was 0.6 ml/min, the oven temperature was 25°C and the mobile phase was 0.04% sulfuric acid in water. Identification and quantification of AGCC were done by comparison with the retention time of butyric acid standard (Sigma-Aldrich®, EUA). The calculation of liberated butyrate concentration was performed through a standard curve and the result expressed in mg liberated per mg of protein in the extract.

2.5. Inflammatory cytokines analysis

Samples of mWAT and liver (30-50 mg/animal) were homogenized with 500 µL of buffer (10 mM PBS+EDTA, pH=7.4) and centrifuged at 8000g 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 (TNF-α) concentrations

were determined simultaneously using CBA kit (BD Cytometric Bead Array (CBA) Mouse Inflammation Kit) and BD FACSVerse™ flow cytometer.

2.6. 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 weight

The running capacity and body weight are presented in table 1. The MCAE increased the total distance run ($p < 0.05$), independent of gene deletion, and such effect can be observed in trained β_1-AR$^{-/-}$ and WT animals. However, gene deletion, independent of MCAE, did not affect ($p > 0.05$) the animals' running capacity and no interaction between factors was observed ($p > 0.05$).

The β_1-AR$^{-/-}$ animals showed higher ($p < 0.05$) initial and final body weights than WT. The MCAE, however, reduced ($p < 0.05$) the final body weight, independent of gene deletion. Although, no interaction between factors was found ($p > 0.05$). The MCAE reduced the mWAT weight ($p < 0.05$) in trained β_1-AR$^{-/-}$ and WT animals, independent of gene deletion. No interaction between factors was observed ($p > 0.05$).

Table 1. Running capacity and body weight

	WTc	WTt	β_1-AR$^{-/-}$c	β_1-AR$^{-/-}$t
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 ± 1.085*$^{\#}$
mWAT weight (g)	0.22 ± 0.03	0.18 ± 0.03*	0.32 ± 0.03	0.19 ± 0.03*

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; mWAT: mesenteric white adipose tissue; (*) denotes aerobic exercise factor effect; ($^{\#}$) denotes gene deletion factor effect. Two-way ANOVA followed by Tukey test.

3.2. Lipases Activity

Figure 1 shows the results of lipases activity. Gene deletion, independent of MCAE, affect ($p > 0.05$) the animals' lipase activity (Figure 1). In mWAT, lipases activity was lower ($p < 0.05$) in β_1-AR$^{-/-}$ animals compared to WT mice (Fig. 1A). While, the lipase activity in the liver was higher in β_1-AR$^{-/-}$ animals compared to WT mice (Fig. 1B) ($p < 0.05$). There was no effect of MCAE in mWAT and liver ($p > 0.05$).

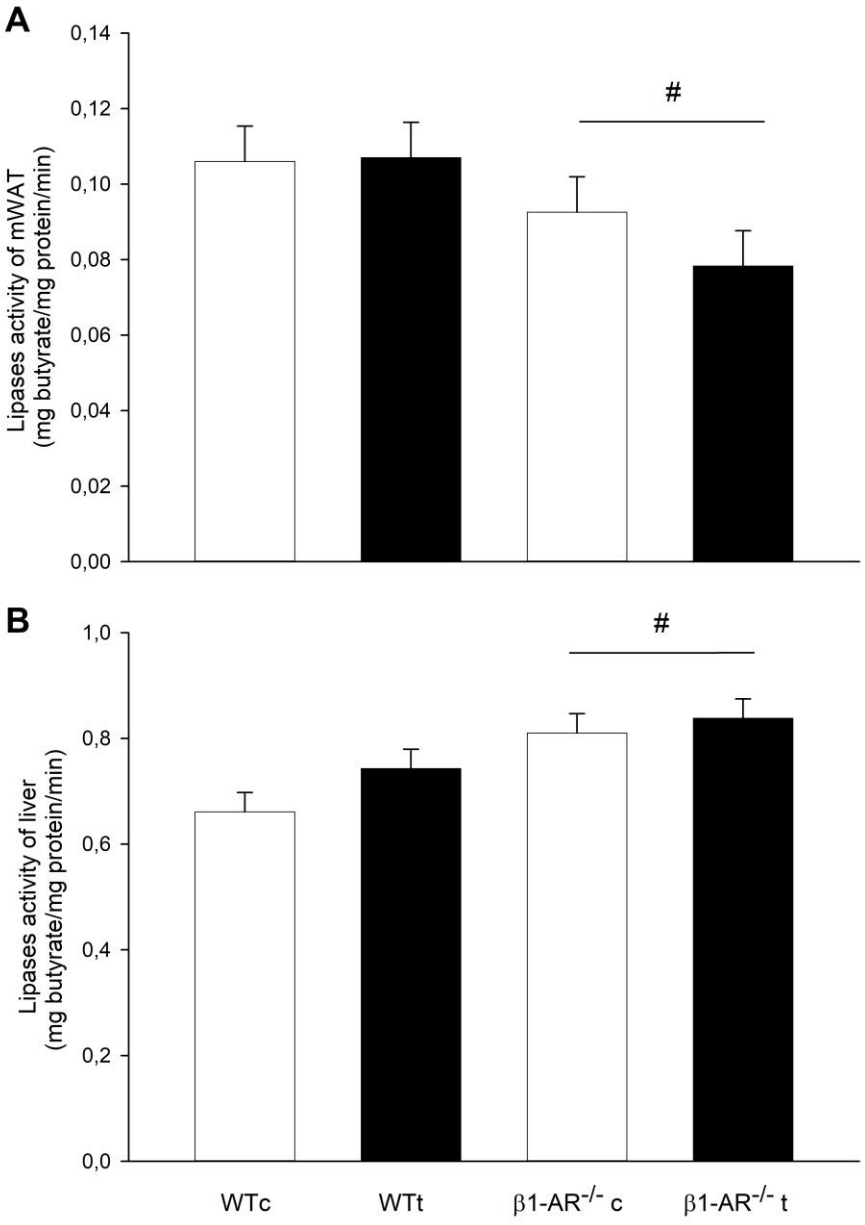

Figure 1. Lipases Activity in mesenteric white adipose tissue (mWAT) and liver. Data are means ± SE of 5 mice per group. WTc: wild-type control; WTt: wild-type trained; β_1-AR$^{-/-}$c: β_1-AR$^{-/-}$ control; β_1AR$^{-/-}$t: β_1-AR$^{-/-}$ trained. ($^{\#}$) denotes gene deletion factor effect. Two-way ANOVA followed by Tukey test.

3.3. Inflammatory cytokines concentrations

The concentrations of cytokine in mWAT and liver are presented in figure 2. With regard to IL-12p70 (Fig. 2A), INFγ (Fig. 2B), MCP-1 (Fig. 2C) and TNF-α (Fig. 2D) concentrations, there were no independent effects of either gene deletion or MCAE ($p > 0.05$). Besides that, no interactions between the factors ($p > 0.05$) were found.

With respect to IL-6 and IL-10 concentrations (Fig. 2E and 2F), no gene deletion and MCAE independent effects were observed in mWAT and liver ($p > 0.05$). However, in this tissue there was interaction ($p < 0.05$) between gene deletion and MCAE factors. The β_1-AR$^{-/-}$c animals showed higher IL-6 and IL-10 concentrations than WTc animals; while such concentrations were lower in β_1-AR$^{-/-}$t compared to β_1-AR$^{-/-}$c group.

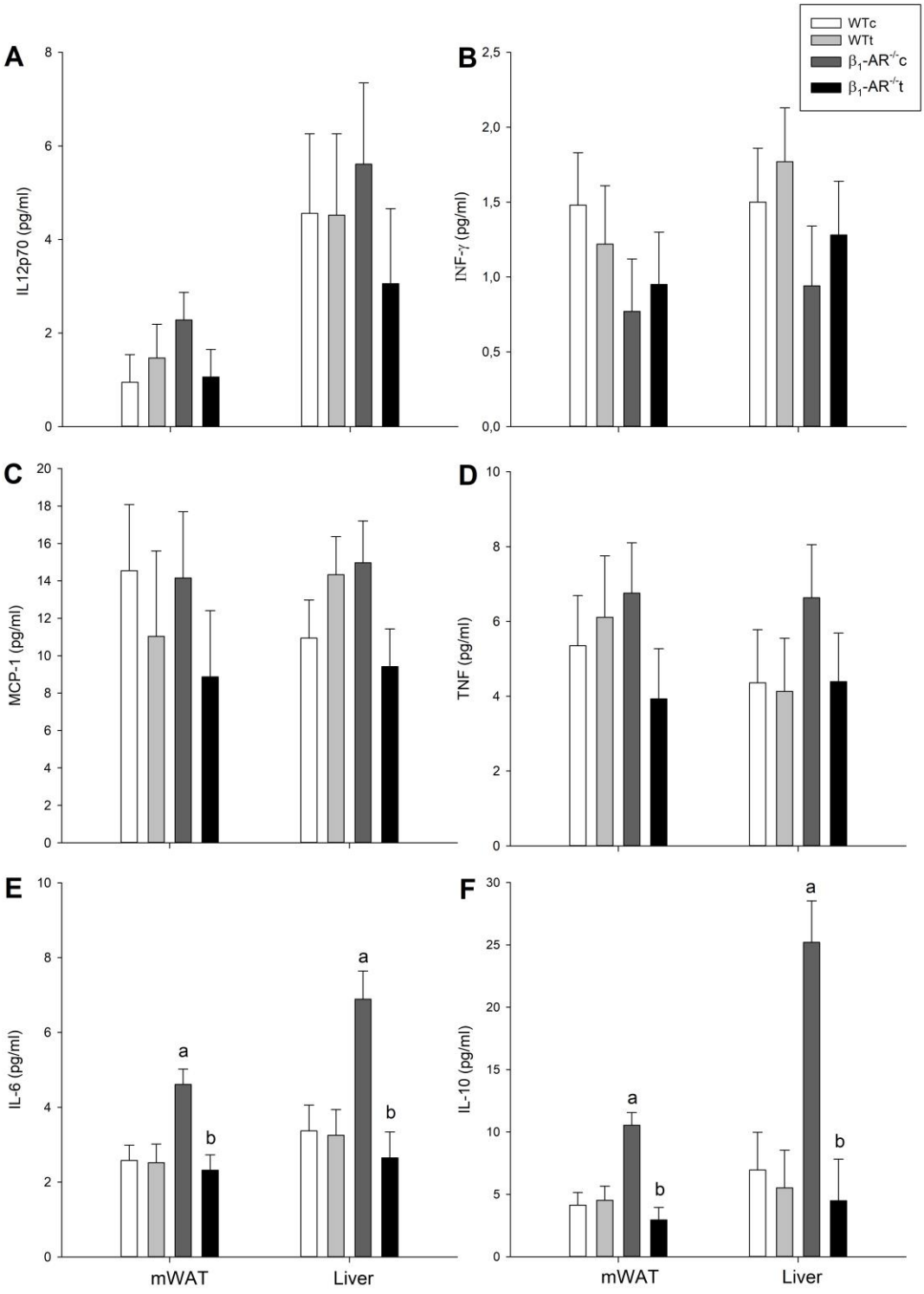

Figure 2. IL-12p70(A), INFγ (B), MCP-1 (C), TNF-α (D), IL-6 (E) and IL-10 (F) inflammatory cytokines concentrations in mesenteric white adipose tissue (mWAT) and liver; Data are means ± SE of 6 animal/group. WTc: wild-type control; WTt: wild-type trained; $\beta_1AR^{-/-}$c: $\beta_1AR^{-/-}$ control; $\beta_1AR^{-/-}$t: $\beta_1AR^{-/-}$ trained; mWAT: mesenteric white adipose tissue; IL-12P70: interleukin-12p70; TNF-α: tumor necrosis factor alpha; INFγ: interferon gamma; MCP-1: monocyte chemoattractant protein-1; IL-10: interleukin-10; IL-6: interleukin-6; ([a]) $p < 0.05$ vs. WTc group; ([b]) $p < 0.05$ vs. β_1-$AR^{-/-}$c group. Two-way ANOVA

followed by Tukey test (mWAT: IL12p70, MCP-1, IL-6 and IL-10; Liver: IL-12p70, INFγ, MCP-1, TNF-α and IL-6); Kruskal-Wallis followed by Dunn test (MAT: INFγ and TNF-α; Liver: IL-10)

4. Discussion

In this study, we tested whether a MCAE program would affect the lipases activity and inflammatory cytokine profile in the mWAT and liver of β_1-AR$^{-/-}$ mice. The main finding was that gene deletion of β_1-AR increased the IL-6 and IL-10 concentrations in mWAT and liver, as well as reduced and increased lipases activity in mWAT and liver, respectively. More important, eight weeks of MCAE reduced the IL-6 and IL-10 concentrations in both mWAT and liver of β_1-AR$^{-/-}$ mice, but no effect of MCAE on lipases activity was observed.

Gene deletion of β_1-AR increased concentrations of IL-6 in mWAT and liver of β_1-AR$^{-/-}$c animals. The higher concentrations of this proinflammatory cytokine in β_1-AR$^{-/-}$ animals can be explained, in part, by the increase in body weight of these animals. It is known that the increase in VAT is related with production of proinflammatory adipokines, such as IL-6 [23]. Moreover, the adipocytes expansion leads to hypoxia and, consequently, necrosis/apoptosis of these cells [24], which causes adipocytes to release lipid droplets that are toxic to adipocytes and activate the recruitment of macrophages and, consequently, proinflammatory cytokines [25,26]. In addition, the proximity of VAT with the organs and the drainage of proinflammatory cytokines through the portal vein to the liver may explain the higher concentrations of IL-6 in liver of β_1-AR$^{-/-}$ animals [7].

The MCAE employed here, on the other hand, reduced IL-6 concentrations in mWAT and liver of β_1-AR$^{-/-}$t animals. The effect of MCAE can be explained by the lower infiltration of macrophages and proinflammatory cytokines into adipose tissue [12], since MCAE also reduced the body and mWAT weight in trained animals (WTt and β_1-AR$^{-/-}$t). In addition, a previous study also showed that reduction in body fat, adipose tissue area and frequency of large adipocytes induced by MCAE are associated with lower concentrations of proinflammatory cytokines in adipose tissue of trained β_1-AR$^{-/-}$ and wild mice [27].

Surprisingly, β_1-AR$^{-/-}$c animals had higher concentrations of IL-10 in mWAT and liver than WTc mice, whereas MCAE was not able to maintain these concentrations in β_1-AR$^{-/-}$t animals. IL-10 is an anti-inflammatory cytokine that attenuates the inflammatory response by inhibiting the release of proinflammatory cytokines [28]. The high concentrations of IL-10 may be a compensatory increase to the elevated concentrations of IL-6 observed in β_1-AR$^{-/-}$c animals. It is known that IL-6 may increase IL-10 production via activation of monocytes and macrophages [29]. However, although IL-6 may present anti-inflammatory effect by increase IL-10 production, its chronic high level is associated with obesity and DM2 [30]. Differently,

β_1-AR$^{-/-}$t animals showed lower concentrations of IL-6, which may not have caused compensatory increase of IL-10 in β_1-AR$^{-/-}$t animals. Despite the finds indicate a compensatory increase of IL-10 in mWAT and liver of β_1-AR$^{-/-}$c animals, more studies are needed to explain the association between IL-10 and β-adrenergic signaling pathway in β_1-AR$^{-/-}$ mice.

Our β_1-AR$^{-/-}$ mice exhibited reduced lipases activity in mWAT and increased body weight. In adipose tissue, lipolysis occurs primarily in response to the β-adrenergic activation by catecholamines [31]. In addition, although β_3-AR is predominant subtype of β-AR in rodent adipose tissue [32], our findings confirm that the β_1-AR is essential in adrenergic signaling pathway and, consequently, lipolysis on the adipose tissue. Previous studies have also demonstrated this relevance of β_1-AR, as increased lipolytic activity in WAT of mice with overexpression of β1-AR [33], as well as increase in body weight [34] and fat percentage, adipose tissue area and frequency of large adipocytes of β_1-AR$^{-/-}$ mice [27].

Otherwise, β_1-AR$^{-/-}$ mice showed increased lipases activity in the liver. Although both β_1- and β_2-ARs are presents in liver, several studies have demonstrated the relevance of β_2-ARs in liver lipid metabolism [35–39]. Like adipocytes, hepatocytes also use lipolytic mechanism to catabolize lipid droplets via cAMP/PKA pathway and requires the hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) [35]. Since, β_1-AR$^{-/-}$ mice present β_2-AR and, in liver, this receptor exhibited greater high-affinity agonist binding compared to β_1-AR [38], the increased hepatic lipases activity in β_1-AR$^{-/-}$ mice may be relative the β_2-ARs signaling pathway, which is preserved in these animals. Thereby, our findings help to confirm the importance of β_2-AR in hepatic lipid homeostasis, nevertheless further studies using β_1-AR$^{-/-}$ mice are necessary to better understand the interface between β_2-AR and lipases activity in the liver.

Our MCAE regimen reduced body and mWAT weight in trained animals. It is known that exercise training may reduce adipocyte by repeated activation of lipolysis, and elevated catecholamines during exercise are responsible for activating the β-adrenergic signaling pathway in adipocytes, increasing lipolytic activity through HSL phosphorylation [40,41]. Since β_1-AR$^{-/-}$ animals do not have β_1-AR and this receptor plays a key role in the lipolytic signaling pathway of adipose tissue, the activation of NPR-A by natriuretic peptides, which activates the GC-cGMP-PKG pathway, responsible for phosphorylating HSL in adipose tissue [42] may be an alternative pathway to explain the reduced body and mWAT weight in trained animals. Confirming that, a previous study on humans reported that under β-adrenergic inhibition, exercise is capable of increasing circulating concentrations of atrial natriuretic peptide [43].

The MCAE used here also increased the total distance run in the trained animals (WTt and β_1-AR$^{-/-}$t) compared to control animals (WTc and β_1-AR$^{-/-}$c). The increase in exercise capacity induced by MCAE is associated with cardiovascular [44] and muscular [40] adaptations. Previous studies using the same exercise protocol also observed increased exercise capacity in trained mice [27,45,46]. In addition, the reduction in body weight of trained animals may have aided in increasing exercise capacity. Studies with obese individuals have observed a correlation between body weight reduction and better exercise capacity [47,48].

5. Conclusion

In conclusion, eight weeks of MCAE reduces the body and mWAT weights, and inflammatory cytokines in mWAT and liver of β_1-AR$^{-/-}$ mice, despite no effect on lipases activity. These findings are of clinical relevance for obesity management since it shows potential benefits of MCAE training in the β-adrenergic signaling pathway, mainly in the reduction of adipose tissue in visceral adipose tissue.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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4. LIMITAÇÕES DO ESTUDO

Este estudo possui uma limitação. Foram utilizados camundongos *knockout* global e alterações sistêmicas desconhecidas podem ter ocorrido, o que pode ter confundido efeitos do treinamento físico aplicado, assim, esses resultados devem ser interpretados com cautela.

5. CONCLUSÕES GERAIS

- O exercício aeróbico contínuo de intensidade moderada reduz o índice de adiposidade nos animais treinados, independente da deleção gênica;
- O percentual de gordura é aumentado pela deleção gênica de β_1-AR e o exercício aeróbico contínuo de intensidade moderada é capaz de reverter esse aumento;
- Enquanto a deleção gênica de β_1-AR aumenta a área dos adipócitos no tecido adiposo inguinal e marrom, o exercício aeróbico contínuo de intensidade moderada reduz a área dos adipócitos no tecido adiposo epididimal, inguinal e marrom;
- O exercício aeróbico contínuo de intensidade moderada e a deleção gênica de β_1-AR aumentam a frequência de pequenos adipócitos e reduzem a frequência de grandes adipócitos no tecido adiposo epididimal. No tecido adiposo inguinal, a deleção gênica de β_1-AR$^{-/-}$ reduz e aumenta a frequência de pequenos e grandes adipócitos, respectivamente, enquanto o exercício aeróbico contínuo de intensidade moderada aumenta e reduz a frequência de pequenos e grandes adipócitos, respectivamente. No tecido adiposo marrom, a deleção gênica de β_1-AR reduz e aumenta a frequência de pequenos e grandes adipócitos, respectivamente; mas o exercício aeróbico contínuo de intensidade moderada não afeta estas frequências;
- Não há alteração na expressão dos genes lipolíticos (Lipe e Npr1) e termogênicos (Ucp-1 e Pgc-1α) no tecido adiposo (epididimal, inguinal e marrom). A deleção gênica de β_1-AR reduz a expressão do gene Adrb2 e o exercício aeróbico contínuo de intensidade moderada aumenta a expressão do gene Adrb3;
- O exercício aeróbico contínuo de intensidade moderada aumenta e reduz as concentrações de IL12p70 no tecido adiposo epididimal e inguinal, respectivamente. A deleção gênica aumenta as concentrações de IL12p70 no tecido adiposo inguinal;
- As concentrações de TNF-α no tecido adiposo epididimal, inguinal e marrom são aumentadas pela deleção gênica de β_1-AR e o exercício aeróbico contínuo de intensidade moderada foi capaz de reverter esses aumentos no tecido adiposo epidimal e marrom;
- O exercício aeróbico contínuo de intensidade moderada reduz as concentrações de IL-6 no tecido adiposo epididimal, mesentérico e fígado. Enquanto que, a deleção gênica de β_1-AR aumenta as concentrações de IL-6 no tecido adiposo mesentérico e no fígado;

- O exercício aeróbico contínuo de intensidade moderada reduz as concentrações de IL-10 no tecido adiposo epididimal, marrom, mesentérico e fígado. A deleção gênica de β_1-AR aumenta as concentrações de IL-10 no tecido adiposo mesentérico e fígado;
- Não há alteração nas concentrações de IFNγ e MCP-1 nos tecidos adiposos e fígado, em função da deleção gênica e do exercício aeróbico contínuo de intensidade moderada;
- A deleção gênica de β_1-AR reduz a atividade de lipases no tecido adiposo mesentérico e aumenta no fígado, enquanto que o exercício aeróbico contínuo de intensidade moderada não afeta a atividade de lipases.

6. ANEXOS

Anexo 1 - Parecer de Aprovação da Comissão de Ética no Uso de Animais UFV

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE VIÇOSA
PRÓ REITORIA DE PESQUISA E PÓS GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
Campus Universitário - Viçosa, MG - 36570-000 - Telefone: (31) 3899-3783

CERTIFICADO

A Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa, CEUA/UFV, certifica que o Processo nº 53/2017, com o Projeto de Pesquisa intitulado, "**Efeitos do treinamento físico sobre expressão gênica e citocinas inflamatórias do tecido adiposo de camundongos *Knockout* para receptor β 1 adrenérgico**" coordenado pelo(a) professor(a) Antônio José Natali do Departamento de Educação Física, está de acordo com a legislação vigente, Lei 11.794, de 08 de outubro de 2008, com as Resoluções Normativas editadas pelo Conselho Nacional de Controle da Experimentação Animal, CONCEA e, apresenta especificidade, caracterizando "*A não utilização de animais vivos*", portanto sendo aprovado por esta comissão em 12 de julho 2017.

CERTIFICATE

The Ethics Committee in Use of Animals of the University of Federal de Viçosa, CEUA-UFV, certify that the 53/2017 Process, with the Research Project titled, "**Effects of physical training on gene expression and inflammatory cytokines of adipose tissue of mice Knockout for β 1 adrenergic receptor**", coordinated by the Antônio José Natali teacher of Department Educação Física, is of according to current legislation, Law No. 11,794, of october 08, 2008, with the Normative Resolutions issued by the National Council for the Control of Animal Experimentation, CONCEA and, presents specificity, characterizing "Non-use of live animals", therefore being approved by this commission in July 12, 2017.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV

Anexo 2 - Primeira página do artigo publicado (artigo 1)

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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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