american physiological society
JOURNAL OF APPLIED PHYSIOLOGY

J Appl Physiol 134: 569–580, 2023.
First published January 26, 2023; doi:10.1152/japplphysiol.00645.2022

RESEARCH ARTICLE

Regulation of the endocannabinoid system by endurance and resistance exercise in hypoxia in human skeletal muscle

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Abstract

Exercise modulates the circulating levels of the endocannabinoids ligands N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) and possibly the levels of their receptors and downstream signaling in skeletal muscle. The aim of the present study was to investigate the regulation of the endocannabinoid system by several exercise paradigms in human skeletal muscle. A second aim was to compare endocannabinoid regulation in healthy and prediabetic people in response to an acute endurance exercise. Blood and muscle samples were taken before and after resistance and endurance exercise in normoxia and hypoxia to measure plasma endocannabinoid levels as well as muscle protein expression of CB1, CB2, and downstream signaling. We found that: *1*) an acute resistance exercise session decreased plasma 2-AG and N-palmitoylethanolamine (PEA) levels in normoxia; *2*) 4 wk resistance training decreased plasma AEA, PEA, and N-oleoylethanolamine (OEA) levels in both normoxia and hypoxia; *3*) an acute moderate-intensity endurance exercise increased plasma OEA levels in the healthy and prediabetic groups in normoxia and hypoxia, whereas plasma 2-AG levels increased in the healthy group and AEA in the prediabetic group only in normoxia. The expression of the cannabinoid receptors was only marginally regulated by acute exercise, hypoxia, and prediabetes and downstream signaling did not follow the changes detected in the endocannabinoid ligands. Altogether, our results suggest that resistance and endurance exercise regulate the levels of the endocannabinoid ligands and CB1 expression in opposite ways. The physiological impact of the changes observed in the endocannabinoid ligands in human skeletal muscle after exercise needs further investigation.

NEW & NOTEWORTHY We are the first to analyze both endocannabinoids ligands and receptors in response to endurance and resistance exercise. In addition, no study before has compared both exercise paradigms regarding endocannabinoid tone, which is of interest as endocannabinoids regulate energy metabolism, and these are different between endurance and resistance exercise. Furthermore, we investigated whether the endocannabinoid tone was differently regulated in response to acute endurance exercise in prediabetic people. Linking exercise, endocannabinoids and (pre)diabetic people has never been done before.

endocannabinoid; exercise; hypoxia

INTRODUCTION

The endocannabinoid system regulates, among others, energy metabolism, homeostasis, nociception, and inflammation at a central and at a peripheral level in response to stress (1). It is composed of two major ligands, *N*-arachidonoylethanolamine (AEA or anandamide) and 2-arachidonoylglycerol (2-AG), their synthesizing and metabolizing enzymes and two classic cannabinoid receptors (CB), CB1 and CB2 (2). CB1 is highly expressed in the central nervous system, whereas CB2 is almost exclusively expressed in the immune system (1). Nevertheless, CB1 and CB2 expression has been observed in human skeletal muscle and adipose tissue as well (3), regulating insulin-mediated glucose uptake, oxidative phosphorylation, lipogenesis, and inflammation (4).

Plasma endocannabinoids are upregulated by endurance exercise (5), and in particular by moderate-intensity aerobic exercise (6). Furthermore, plasma AEA levels following endurance exercise are increased for a longer duration at high altitude (7). Decreased oxygen availability, or hypoxia, in addition to endurance exercise seems therefore particularly potent to increase the endocannabinoid tone. This may affect energy metabolism management, immune response, protein synthesis, and degradation. Whether the increases in plasma endocannabinoids by endurance exercise and hypoxia translate into modifications of the regulation of the endocannabinoid system in the skeletal muscle in human is currently unknown. Once activated by the endocannabinoids or other agonists, the G protein-coupled receptors CB1 and CB2 inhibit adenylyl cyclase, leading to lower cAMP and

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Submitted 28 October 2022 / Revised 27 December 2022 / Accepted 21 January 2023

consecutive lower AMP-activated protein kinase (AMPK) activation (8), a central cellular energy sensor (9). In turn, AMPK phosphorylates acetyl-Coa-carboxylase (ACC), which inhibits fatty acid oxidation in skeletal muscle (10). CB1 and CB2 activation also induces the phosphorylation of the Akt and the mitogen-activated protein kinase (MAPK) pathway (8), among which the p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) pathways (9). The latter is known to regulate cell proliferation, differentiation, immune response, and apoptosis (11).

It has recently been shown that CB1, but not CB2, expression was higher in skeletal muscle of old compared with young adults and that CB1 and CB2 expression in old people tended to increase after 12-wk resistance training (12). In addition, increases in the expression of CB2 positively correlated with changes in Pax7 and MyoD, two markers for satellite cell activation, as well as forkhead-box O 3a (FOXO3a) phosphorylation, a marker for muscle atrophy. As the endocannabinoid system has been shown to activate molecular pathways involved in cell growth, proliferation, differentiation, apoptosis, and more specifically to regulate myogenesis in vitro (13, 14), it is tempting to propose a role for the endocannabinoid system in muscle plasticity induced by resistance exercise.

Due to its capacity to regulate insulin-mediated glucose uptake, lipogenesis, and inflammation, the endocannabinoid system is of particular interest in obesity and type 2 diabetes. In people with obesity and diabetes, the endocannabinoid system appears to be over-activated (15), resulting in increased food intake and lipogenesis, impaired glucose and lipid metabolism, and induction of insulin resistance (4, 16, 17). Given that the endocannabinoid tone orchestrates metabolic dysregulation in obesity and diabetes (18) and given that exercise regulates the endocannabinoid tone (19), it would be of interest to investigate whether exercise modulates the endocannabinoid system in patients suffering from obesity and/or diabetes. In that perspective, prediabetic people are of particular interest. They show impaired glucose tolerance and higher fasting glucose levels than healthy individuals (20), but their pathological state is still reversible by exercise, contrary to patients suffering from advanced diabetes and a loss of β-cell function.

Altogether, better insight in how the endocannabinoid system in the plasma and skeletal muscle tissue of healthy and prediabetic people responds to exercise is of interest. This might contribute to a better understanding of the therapeutic potential of exercise in metabolic dysregulation and the role of endocannabinoids in the regulation of energy metabolism in response to exercise. As this research area is very recent, the first aim of the study is to determine whether endurance versus resistance exercise as well as acute exercise versus exercise training differently regulate the endocannabinoid system. In addition, as exercise in hypoxic conditions have been shown to enhance plasma endocannabinoid levels (7) as well as physiological and intramuscular adaptations to endurance (21–23) and resistance exercise (24, 25), a second aim of the present study is to test the additional effect of hypoxia on the regulation of the endocannabinoid system in the aforementioned exercise conditions.

MATERIAL AND METHODS

Exercise Protocols

Samples from three independent studies investigating muscle adaptations after acute and chronic hypoxic exercise have been analyzed (25–27). All the three experiments were approved by the Ethical Committee of the Université catholique de Louvain/Saint-Luc Hospital (Belgium) and conducted in accordance with the declaration of Helsinki. A written consent was signed by all subjects after receiving explanations of all potential risks of the study. The trial registration number for the third study, with prediabetic patients is the following: B403201942020. Each of the study obtained a score of 9/10 on Pedro scale, of 7.5/8 on Modified-Jadad scale, and low bias risk for RoB_2.0 (Supplemental Fig. S1; https://doi.org/10.6084/m9.figshare.21777464.v1).

Acute hypoxic resistance exercise in healthy men.

Twenty physically active healthy young men (22.2 ± 0.4 yr; 71.5 ± 1.4 kg; 22.2 ± 0.3 kg·m^{-2}) reported three times to the laboratory (25). One week before the experiment, the 1RM of the exercised leg was determined. The day of the experiment, all subjects received a standardized, meat-free breakfast, complemented with 10 g essential amino acids. One hour after the meal, they performed a single-leg knee extension with the right leg. The exercise session comprised eight sets of eight repetitions at 80% of the 1RM with 120 s recovery between each series (Fig. 1*A*). The left leg stayed at rest. The subjects were either exposed to normoxia (n = 10, $F_{I_{O_2}}$ = 0.21, ∼sea level) or to hypoxia (n = 10, $F_{I_{O_2}}$ = 0.14, ∼3,000 m) during the whole exercise duration, which lasted for ∼30 min. Two muscle biopsies were taken in the vastus lateralis of the exercised and the nonexercised (rest) leg at 15 min and 4 h postexercise. Blood samples were taken from the antecubital vein 24 h before and 2 h after the exercise.

Hypoxic resistance training in healthy men.

Nineteen physically active healthy young men followed a single-leg knee extension resistance training either in normoxia ($F_{I_{O_2}}$ = 0.21, ∼sea level; n = 9; 20.7 ± 0.8 yr; 71.1 ± 3.1 kg; 21.6 ± 0.8 kg·m^{-2}) or in hypoxia ($F_{I_{O_2}}$ = 0.135, ∼3,500 m; n = 10; 21.2 ± 0.5 yr; 73.3 ± 3.0 kg; 21.9 ± 0.6 kg·m^{-2}; 26). The training lasted for 4 wk, with 3 sessions/wk. Each exercise session consisted of six sets of 10 repetitions at 80% of the 1RM with 120 s recovery between each series (Fig. 1*B*). Each exercise session lasted ∼30 min. The 1RM was determined 1 wk before the start of the training. Muscle biopsies in the vastus lateralis of the exercised and the nonexercised leg (rest) and blood samples from the antecubital vein were taken in the fasted state 4 days before and 3 days after the exercise training.

Acute hypoxic endurance exercise in prediabetics and healthy men.

Thirty-two men reported twice to the laboratory and were divided in two groups based on fasting glucose levels and an oral glucose tolerance test (OGTT; 27). The healthy group (n = 17, 28.8 ± 2.5 yr; 73.8 ± 1.2 kg; 23.5 ± 0.6 kg·m^{-2}) was defined by fasting glucose levels ≤100 mg/dL and blood

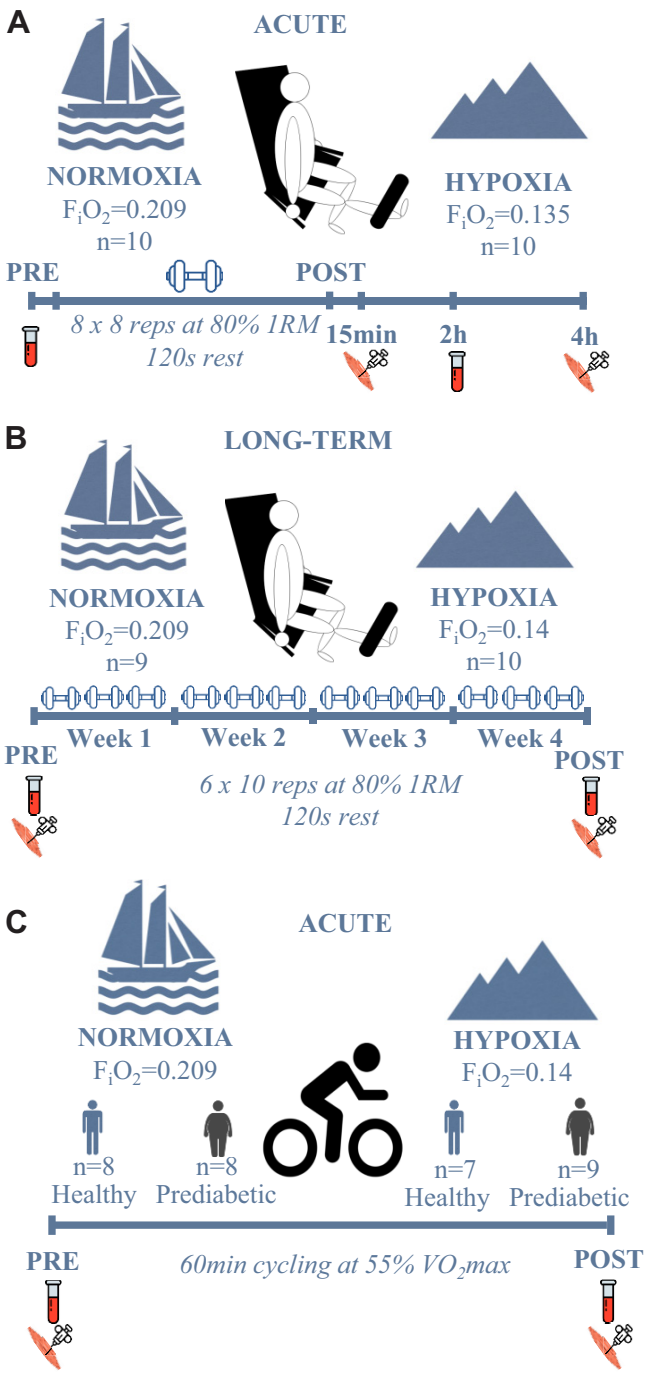

Figure 1. Overview of the exercise protocols. Acute resistance exercise in hypoxia in healthy men (*A*), long-term resistance exercise in hypoxia in healthy men (*B*), acute endurance exercise in hypoxia in healthy and prediabetic men (*C*).

glucose levels <140 mg/dL at 120 min during the OGTT. The prediabetic group (PreT2D, n = 15, 44.7 ± 3.9 yr; 90.0 ± 4.6 kg; 27.3 ± 1.2 kg·m^{-2}) was defined by fasting glucose levels between 100 and 126 mg/dL and blood glucose levels between 140 and 200 mg/dL at 120 min during the OGTT. After the OGTT, a maximal incremental test was performed to determine the maximal oxygen uptake ($\dot{V}O_{2max}$) and the maximal power output in normoxia.

Four days after the tests, all subjects came for a second time to the laboratory. They cycled for 60 min at a heart rate corresponding to 55% $\dot{V}O_{2max}$, either in normoxia ($F_{I_{O_2}}$ = 0.21, ~sea level, healthy: n = 8, PreT2D: n = 8) or in hypoxia ($F_{I_{O_2}}$ = 0.14, ~3,000 m, healthy: n = 7, PreT2D: n = 9). Muscle biopsies in the vastus lateralis and blood samples from the antecubital vein were taken in the fasted state before and directly after exercise (Fig. 1*C*).

RNA Isolation and Real-Time PCR

About 20 mg of muscle was homogenized in 1 mL Trizol reagent (Invitrogen, Merelbeke, Belgium) using a Polytron mixer. RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop spectrophotometry. Reverse transcription was performed from 1 μg RNA using the iScriptTM cDNA Synthesis Kit from Bio-Rad Laboratories (Nazareth, Belgium), following the manufacturer's instructions. The primers used are presented in Table 1. PCR was run using the following conditions: 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. All samples were run in duplicate, and each reaction was processed in a 10-μL volume containing 4.8 μL SsoAdvanced Universal SYBR Green SuperMix (Bio-Rad Laboratories), 0.1 μL of each primer (100 nM final), and 5 μL cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. To compensate for variations in input RNA amounts and efficiency of reverse transcription, β-2-microglobulin (B2M) was quantified, and results were normalized to those values. The results are expressed in arbitrary units.

Protein Isolation

Muscle samples (20–30 mg) were homogenized with a Polytron mixer in ice-cold lysis buffer (20 mM Tris-HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM B-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 1% Triton-X 100 and a complete protease inhibitor tablet), centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant aliquoted and stored at −80°C. The DC protein assay kit was used to determine the protein concentration of each sample using bovine serum albumin (BSA, Bio-Rad, Hercules, CA) as a standard.

Western Blotting

Twenty proteins were separated by SDS-PAGE (8%–10% gels) and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk for 1 h and incubated overnight at 4°C with the following antibodies: CB1 (1:400, Ab259323, Abcam), CB2 (1:400, BSA, Ab3561, Abcam), eEF2 (1:1,000, No. 2332, Cell Signaling), phospho-ACC Ser^{212} (1:1,000, No. 3661), ACC (1:1,000, No. 3676, Cell Signaling), phospho-AMPKα Thr172 (No. 2535), AMPKα 23A3 (No. 2603), phospho-Akt Ser473 (No. 4060), Akt (No. 9272), phospho-p38 MAPK Thr180/Tyr182 (No. 9211), p38 MAPK (No. 9212), phospho-p44/42 MAPK Thr202/Tyr204 (No. 4370), and p44/42 MAPK (No. 4696; all Cell Signaling, 1:1,000 excepted p-Akt and p42/44, 1:2,000). Horseradish peroxidase-conjugated anti-mouse (1:10,000, A9044, Sigma) or anti-rabbit (1:5,000, A6154, Sigma) secondary antibodies were used for detection of

Table 1. *Primers sequences*

Gene	Forward	Reverse
B2M	ATG AGT ATG CCT GCC GTG TGA	GGC ATC TTC AAA CCT CCA TG
CNR1	GAT GTA CTT GCC CTG ACC ATG	AAC ATT CTA GGA CTG ATT CAT CAT G
CNR2	ATC CTG AGT GGT CCC CAG AAG	GTG GGA GGA CAG GAT CAG ATA GAG

B2M, β-2-microglobulin; CNR1, gene coding for cannabinoid receptor 1; CNR2, gene coding for cannabinoid receptor 2.

proteins. Membranes were thereafter scanned with GeneSnap, and proteins were quantified with GeneTool (Syngene, Cambridge, UK). The results are presented as the ratio protein of interest/eEF2 or as the ratio of the phosphorylated/total form of the protein. All the results are expressed in arbitrary units.

Immunostaining

Sections of 6 μm from OCT-embedded tissue were cut at −20°C using a cryostat microtome (CryoStar NX70, Thermo Fisher Scientific, Waltham, MA) and put on histological blades. The slides were dried at room temperature for 1 h and stored at −80°C upon immunofluorescence analysis. Tissues sections were washed 2 × 5 min in phosphate-buffered saline (PBS), fixed with cold acetone (−20°C), and blocked for 30 min at room temperature in PBS with 10% BSA, 10% normal goat serum, and 5% Triton. After blocking and washing, sections were incubated overnight at 4°C with the primary antibodies CB1 (1:200, IgG, Ab23703, Abcam) or CB2 (1:200, IgG, Ab3561, Abcam) for cannabinoid receptor identification and BA-D5 (myosin heavy chain I, concentrate, 1:200, 2235587, DSHB, Iowa City, IA) for muscle fiber typing. Of note, unstained muscle fibers were considered as containing myosin heavy chain II. Sections were washed 2 × 5 min and incubated for 1 h at room temperature with the secondary antibody Alexa Fluor 594 IgG (1:300, A-11072, Invitrogen, Carlsbad, CA) for cannabinoid receptor identification and Alexa Fluor 488 anti-rabbit IgG2b (1:500, A-21141, Invitrogen) for muscle fiber typing. Finally, tissue sections were washed 2 × 5 min and covered with an antifade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vectashield, Labconsult, Brussels, Belgium) to visualize the nuclei. Blades were stored for 24 h at 4°C before visualization with the microscope Axio Vert A1 (Zeiss, Oberkochen, Deutschland) equipped with an AxioCam ICm1 camera and ZEN lite 2011 (Zeiss) software. Image processing and analysis were realized with ImageJ.

Plasma Collection

Blood samples were taken from an antecubital vein in 10-mL tubes containing heparin lithium and were immediately put on ice until centrifugation. Soon after collection, the tubes were centrifuged at 4°C for 10 min at 3,000 *g*. Plasma was transferred to microtubes and stored at −80°C upon analysis.

Plasma Endocannabinoid Levels

The endocannabinoid levels of the different plasma samples were analyzed by liquid chromatography-mass spectrometry (UHPLC-MS/MS). Briefly, lipids from plasma were extracted by a liquid/liquid extraction (CH_2Cl_2-MeOH-H_2O) in the presence of the internal standards (d_4-AEA, d_4-PEA, d_4-OEA, and d_5-2-AG). The extracted lipids were purified by solid phase extraction. Then, the endocannabinoid-containing fraction was analyzed with a Xevo-TQS mass spectrometer (Waters, Milford, MA). The UHPLC-MS/MS method used is as follows: a BEH LC-18 column 50 × 2.1 mm, 1.7 μm (Waters) at a temperature of 40°C. The mobile phase consisted in a gradient between A: H_2O 25%-MeOH 75%; B: MeOH 100%, all containing acetic acid (0.1%). An ESI probe operated in positive mode was used for sample ionization. The mass spectrometer parameters are the following: capillary voltage: 2.9 kV; cone voltage: 30 V; desolvation temperature: 550°C; desolvation gas flow: 1,100 L/h: cone gas flow: 170 L/h: nebulizer: 6 bar. The quantification was obtained by establishing the ratio between the area under the curve (AUC) of the lipid of interest and the AUC of the respective internal standard and by plotting these values in the respective calibration curves. Plasma endocannabinoid levels are given in pmol/mL.

Statistics

All values are expressed as the means ± SE. Mixed linear models were applied on the data of the two resistance studies, with groups (normoxia or hypoxia), condition (rest and exercise), and time (pretraining and posttraining) as fixed effects and the subjects as random effects. For the acute endurance study, paired *t* test models were applied with groups (normoxia and hypoxia) and condition (healthy and prediabetics) data not paired, whereas time data (pre and post) were paired for analysis. Statistical analyses were performed in R with nlme library for linear mixed models fit by REML (restricted maximum likelihood) for small sample size. Number of observations, fixed effect estimates and standard errors, random effect's intercept and residual standard deviation, lower (5%) and upper (95%) confidence intervals, and *P* value were extracted for analyses (Supplemental Fig. S2; https://doi.org/10.6084/m9.figshare.21777461.v1). Statistical significance was fixed at $P < 0.05$. R's emmeans library was used for mean comparison. R's effsize library was used to calculate Cohen's d size effect, applying hedge's correction for sample number <50. Graphs were realized with ggplot2 library in R.

RESULTS

Regulation of the Endocannabinoid System by Acute Hypoxic Resistance Exercise in Healthy Men

The plasma levels of the endocannabinoid ligands AEA and 2-AG and of the endocannabinoid-like ligands *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA) were measured in blood samples taken 24 h before and 2 h after the acute resistance exercise (Table 2). Plasma 2-AG ($P = 0.029$) and PEA ($P = 0.038$) levels decreased from pre- to

Table 2. *Plasma endocannabinoid levels before and after an acute resistance exercise in hypoxia*

	Normoxia (n = 10)		Hypoxia (n = 10)	
pmol/mL	Pre	Post	Pre	Post
AEA	2.9 ± 0.4	2.2 ± 0.4	2.7 ± 0.4	2.4 ± 0.3
2-AG	9.6 ± 0.7	8.3 ± 0.0*	8.8 ± 0.3	8.3 ± 0.1
OEA	2.1 ± 0.2	1.7 ± 0.3	2.1 ± 0.3	1.8 ± 0.2
PEA	20.3 ± 2.2	12.9 ± 3.3*	17.9 ± 3.6	16.7 ± 2.9

Data are presented as the means ± SE. *$P < 0.05$ vs. pre. AEA, N-arachidonoylethanolamine; OEA, N-oleoylethanolamine; PEA, N-palmitoylethanolamine; 2-AG, 2-arachidonoylglycerol.

postexercise in normoxia but not in hypoxia. Plasma AEA and OEA levels were unchanged by hypoxia or by exercise conditions.

In skeletal muscle, mRNA levels of the gene coding for cannabinoid receptor 1 (CNR1) were higher at 15 min postexercise in normoxia, in comparison with hypoxia (P = 0.002), rest (P = 0.008), and 4 h (P = 0.022, Fig. 2*A*). Compared with the rested leg, CB1 protein expression in the exercised leg was lower at 4 h postexercise in hypoxia (P = 0.018; Fig. 2, *B* and *I*). No change in CB2 protein expression in response to exercise and hypoxia was observed (Fig. 2, *C* and *I*). Phospho-Akt was lower at 4 h compared with 15 min postexercise in both the rested (P = 0.017 in normoxia and P = 0.004 in hypoxia) and exercised ($P < 0.001$ in normoxia and hypoxia) legs (Fig. 2, *D* and *J*). Phospho-p38 and phospho-ERK1/2 were unchanged by the exercise and hypoxic conditions (Fig. 2, *E, F,* and *J*). In the rested leg, phospho-AMPK was lower at 15 min in hypoxia compared with normoxia (P = 0.022). In the exercised leg, AMPK phosphorylation was lower in hypoxia compared with normoxia at 4 h postexercise (P = 0.001, Fig. 2, *G* and *J*). Finally, phospho-ACC, a proxy for AMPK activation (10), was higher at 15 min in the exercised leg in comparison with the rested leg (P = 0.009 in normoxia and $P < 0.001$ in hypoxia) and lower at 4 h postexercise compared with 15 min in the exercised leg in both normoxia (P = 0.008) and hypoxia ($P < 0.001$, Fig. 2, *H* and *J*).

In accordance to the observations of Dalle and Koppo (12), immunostaining of cannabinoid receptors CB1 (Fig. 2*J*) and CB2 (Fig. 2*L*) revealed that the cannabinoid receptors were mostly localized at the plasma membrane of human skeletal muscle fibers.

In summary, the acute resistance exercise session induced a decrease in plasma 2-AG and PEA levels exclusively in normoxia, which was accompanied by slight decrease in the expression of the cannabinoid receptor CB1 in the exercise leg but not by changes in downstream signaling.

Regulation of the Endocannabinoid System by Hypoxic Resistance Training in Healthy Men

Plasma levels of AEA, PEA, and OEA were decreased from before to after 4 wk resistance training in both normoxia (AEA: P = 0.006; OEA: P = 0.012; PEA: P = 0.008) and hypoxia (AEA: P = 0.048; OEA: P = 0.037; PEA: P = 0.005; Table 3). Plasma 2-AG levels were unchanged by exercise and hypoxia.

In the skeletal muscle, the mRNA levels of CNR1 increased from before to after 4 wk resistance training with no further difference between and within groups and conditions (time main effect P = 0.042, Fig. 3*A*). The gene coding for cannabinoid receptor 2 (CNR2) was unchanged in our experimental conditions (Fig. 3*B*). CB1 and CB2 protein expression were unchanged by any condition (Fig. 3, *C, D,* and *I*). Phospho-Akt decreased in the exercised leg from before to after resistance training in normoxia (P = 0.030, Fig. 3, *E* and *I*). Furthermore, in comparison with the rested leg, phospho-Akt was lower in the exercised leg after training in hypoxia (P = 0.030). Phospho-p38, -ERK1/2, and -AMPK were unchanged by exercise and hypoxia (Fig. 3, *F–I*).

In summary, plasma AEA, PEA, and OEA levels were decreased after 4 wk resistance training in both normoxia and hypoxia, whereas CNR1 mRNA levels increased in both the rested and exercised legs with no change at the protein levels nor downstream signaling.

Regulation of the Endocannabinoid System by Acute Hypoxic Endurance Exercise in Healthy and Prediabetic Men

We investigated whether there was a difference in the levels of the endocannabinoids and associated N-acylethanolamines (NAEs) between prediabetic and healthy subjects in response to endurance exercise in hypoxia (Table 4). In the rested state, at baseline, no difference in the levels of the endocannabinoid ligands was observed between the healthy and prediabetic groups, excepted for plasma PEA levels (P = 0.019). In the healthy group, a 60-min moderate intensity cycling exercise increased plasma 2-AG (P = 0.024), OEA (P = 0.008), and PEA (P = 0.002) levels in normoxia as well as AEA (P = 0.045), 2-AG (P = 0.008), and OEA levels (P = 0.002) in hypoxia. In the prediabetic group, endurance exercise increased plasma AEA (P = 0.015) and OEA (P = 0.016) levels in normoxia as well as OEA levels in hypoxia (P = 0.026).

CNR1 mRNA levels were lower in response to exercise in prediabetics subjects (PreT2D × Exercise main effect P = 0.04) with no further difference within and between groups and conditions (Fig. 4*A*). CB1 protein expression increased from before to after endurance exercise in prediabetic subjects (PreT2D × exercise main effect P = 0.045) with a within group effect exclusively in the prediabetic group in hypoxia (P = 0.039, Fig. 4, *B* and *I*). CB2 expression and Akt phosphorylation were unchanged by any conditions (Fig. 4, *C, D,* and *I*). p38 phosphorylation increased from before to after exercise in the prediabetic group in normoxia (P = 0.012) and in hypoxia ($P < 0.001$; Fig. 4, *E* and *J*). Phosphorylation of ERK1/2 was unchanged by any conditions (Fig. 4, *F* and *J*). AMPK phosphorylation increased from before to after endurance exercise (exercise main effect, P = 0.027, Fig. 4, *G* and *J*). Finally, ACC phosphorylation increased from before to after exercise in the healthy (P = 0.007) and prediabetic (P = 0.012) groups in normoxia and in hypoxia (healthy: $P < 0.001$, prediabetic: P = 0.039, Fig. 4, *H* and *J*).

In summary, plasma OEA levels consistently increased after an acute moderate-intensity endurance exercise in the healthy and prediabetic groups in both normoxia and hypoxia. In a group-specific way, exercise increased plasma 2-AG levels in the healthy group and AEA in the prediabetic group only in normoxia. CB1 expression was increased by exercise in the prediabetics group, especially in hypoxia.

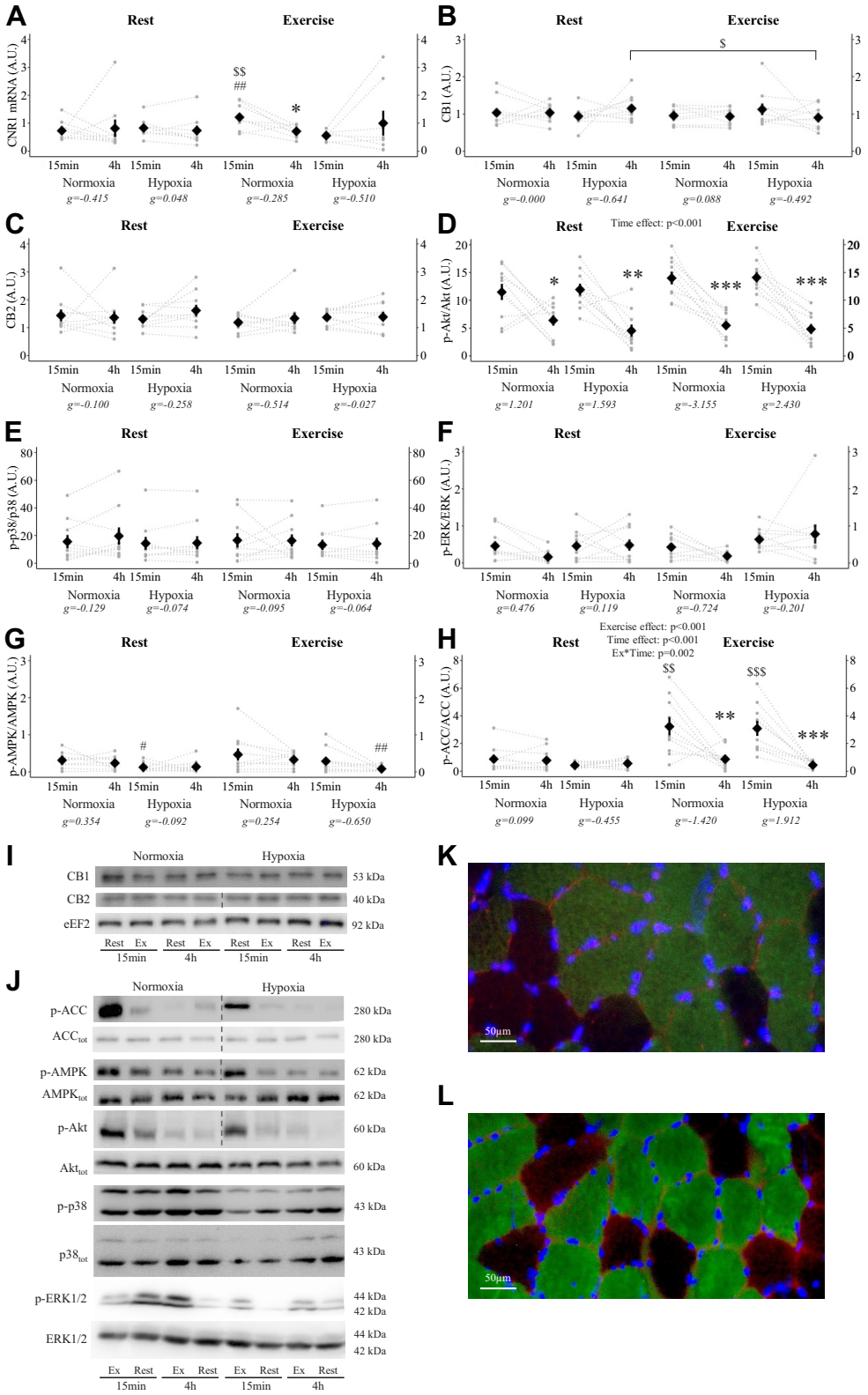

Figure 2. Regulation of the endocannabinoid system and associated downstream pathways by acute resistance exercise in hypoxia in healthy young men (n = 10 for all groups, linear mixed models analysis). CNR1 mRNA levels (*A*) and protein expression of CB1 (*B*), CB2 (*C*), Akt Ser473 (*D*), p38 MAPK Thr180/Tyr182 (*E*), ERK1/2 Thr202/Tyr204 (*F*), AMPK Thr172 (*G*), and ACC Ser79 (*H*) in the rested leg and in the exercised leg at 15 min and 4 h postexercise in normoxia or in hypoxia. *I* and *J* representative Western blots. Representative immunostainings of cannabinoid receptors CB1 (*K*) and CB2 (*L*; red) in human skeletal muscle with myonuclei (blue), MyHCI (green), and MyHCII (black/unmarked). A.U., arbitrary units. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. 15 min; $\#P < 0.05$, $\#\#P < 0.001$ vs. normoxia; $\$P < 0.05$, $\$\$P < 0.01$, $\$\$\$P < 0.001$ vs. rest. Each dot represents a subject's value, each subject over-time evolution is represented by a line linking the two dots associated to a single subject. ACC, acetyl-Coa-carboxylase; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase.

Table 3. *Plasma endocannabinoid levels before and after a resistance training in hypoxia*

	Normoxia (n = 9)		Hypoxia (n = 10)	
pmol/mL	Pre	Post	Pre	Post
AEA	6.0 ± 0.5	4.3 ± 0.4**	7.0 ± 0.6	5.2 ± 0.9*
2-AG	12.5 ± 0.9	12.3 ± 0.6	13.9 ± 0.9	13.7 ± 1.0
OEA	4.3 ± 0.3	3.3 ± 0.2*	4.5 ± 0.2	3.8 ± 0.4*
PEA	63.9 ± 7.0	45.5 ± 4.1**	74.0 ± 7.0	58.1 ± 5.1**

Data are presented as the means ± SE. *$P < 0.05$, **$P < 0.01$ vs. pre. AEA, N-arachidonoylethanolamine; OEA, N-oleoylethanolamine; PEA, N-palmitoylethanolamine; 2-AG, 2-arachidonoylglycerol.

Those increases had a very minimal impact on the regulation of downstream signaling in the skeletal muscle.

DISCUSSION

The aim of the present study was to investigate the modulation of the endocannabinoid system by different exercise paradigms in healthy and prediabetic people. This is the first study to comprehensively analyze the regulation of the endocannabinoid system after both endurance and resistance exercise in human by measuring plasma endocannabinoid levels as well as cannabinoid receptor expression and activation of the Akt, MAPK, and AMPK pathways in the skeletal muscle. We found that: *1*) an acute resistance exercise session decreased plasma 2-AG and PEA levels exclusively in normoxia; *2*) 4 wk of resistance exercise training decreased plasma AEA, PEA, and OEA levels in both normoxia and hypoxia; *3*) an acute moderate-intensity endurance exercise increased plasma OEA levels in the healthy and prediabetic groups in both normoxia and hypoxia, whereas plasma 2-AG levels increased in the healthy group and AEA in the prediabetic group only in normoxia; *4*) CB1 expression was slightly decreased in the exercise leg 4 h after an acute resistance exercise; and *5*) an acute endurance exercise increased CB1 expression in prediabetic subjects, especially in hypoxia. The expression of the cannabinoid receptors was only marginally regulated by hypoxia and prediabetes and downstream signaling did not follow the changes detected in the endocannabinoid ligands. Altogether, the results from our three studies suggest that resistance and endurance exercise regulate the levels of the endocannabinoid ligands and of CB1 expression in opposite ways. Both acute resistance exercise and long-term resistance training decreased plasma endocannabinoid levels whereas an acute moderate-intensity endurance session increased their levels.

Our observations after acute moderate-intensity endurance exercise are in line with previous studies reporting higher plasma endocannabinoid levels after endurance exercise, especially at moderate intensities (5, 6, 28). Thirty-minutes treadmill running at moderate intensity (70% of age-adjusted maximal heart rate), but not at higher intensities, increased AEA levels in recreationally fit runners (6). Progressive increases in plasma AEA, PEA, and OEA, but not 2-AG, levels were found after 60-min cycling at 55% maximal power output (Wmax) followed by a time-trial of 30 min at 75% Wmax and a recovery period of 15 min in trained cyclists, with the highest levels measured after the recovery period (28). Similarly, plasma AEA, but not 2-AG, levels were increased after a 50-min moderate cycling or running exercise at 70%–80% of the maximum heart rate in trained athletes (5). Whereas OEA and PEA share the same synthetic pathway as AEA, composing the NAE family, 2-AG synthesis depends on other enzymes and precursors (29). The different synthetic pathways may contribute to the divergent results found in 2-AG and the other endocannabinoid levels in response to endurance exercise. Divergent results exist as well regarding plasma 2-AG levels as some studies reported no changes (5–7) and others increases (30, 31) in 2-AG levels in response to endurance exercise. Other potential factors contributing to divergent regulations between AEA, 2-AG, OEA, and PEA and between studies are time since the last meal and the composition of the last meal, timing of blood sampling, photic preload, and/or circadian timing of exercise (30). Despite subtle differences between the previous studies, we confirm here that moderate-intensity endurance exercise increases the levels of circulating endocannabinoid ligands.

We did not identify differences in endocannabinoid ligands levels between normoxia and hypoxia. To the best of our knowledge, only one study has measured plasma endocannabinoids levels following exercise in hypoxia (7). Hiking to 3,196 m induced an increase in plasma AEA, but not 2-AG, levels that remained higher after one night at high altitude and a few hours after having returned to lower altitude. Here, we only took blood samples directly or a few hours after exercise. It is thus possible that we missed the long-lasting effects of hypoxia or that the exposure to hypoxia was too short to increase the levels of endocannabinoid ligands. Another parameter to take into consideration is the use of normobaric hypoxia as it was the case in our three studies versus hypobaric hypoxia as used in the aforementioned study (7). Knowing that the physiological adaptations to each of those two types of hypoxia might differ somewhat, this point deserves more investigation (32). Finally, we did not observe differences in basal or postexercise endocannabinoid levels between the healthy and prediabetic subjects. This is the first study to investigate the regulation of the endocannabinoid system by exercise in prediabetic subjects. The absence of modifications in plasma endocannabinoid levels in our prediabetic subjects suggests that the dysregulation of the endocannabinoid system observed in patients with diabetes (16) is likely triggered by higher blood glucose levels and/or a higher inflammatory state than the ones displayed by prediabetic subjects.

Contrary to endurance exercise, the results from our two resistance exercise studies rather indicate a downregulation of the plasma levels of endocannabinoid system mediators. Few studies have analyzed plasma endocannabinoid levels in response to resistance exercise in human. The two following studies determined their levels in the context of exercise-induced analgesia. In the first study, plasma 2-AG, AEA, OEA, and PEA levels increased directly after isometric exercise at 25% of the maximum voluntary contraction for 3 min together with unchanged pain ratings after the administration of an opioid antagonist, suggesting that the role of endocannabinoids could be greater than the role of opioids in exercise-induced analgesia (33). In the second study, although an enhanced pressure pain threshold was observed directly after all four exercise paradigms tested, plasma 2-AG levels were not increased accordingly (34). Even, a decrease

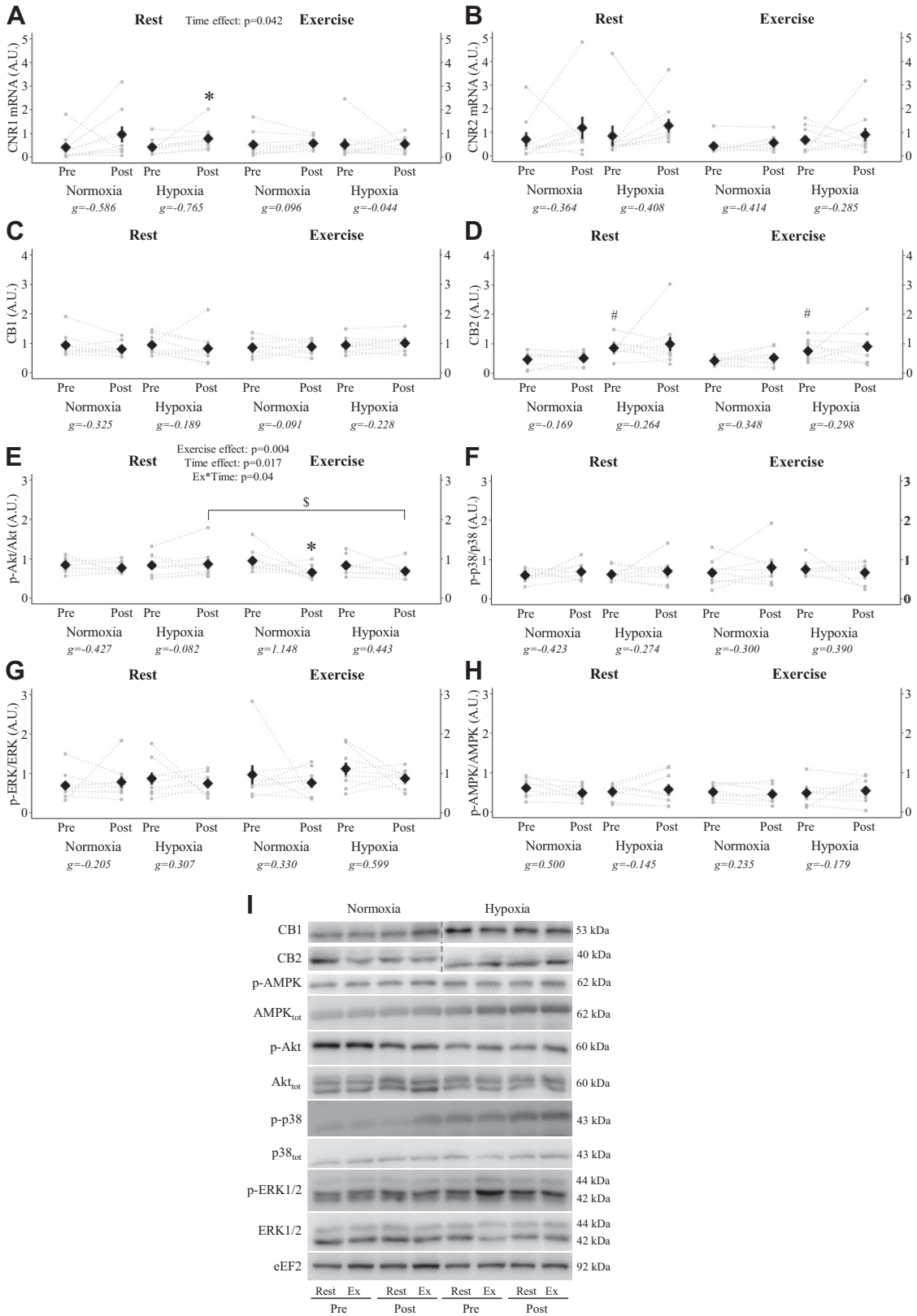

Figure 3. Regulation of the endocannabinoid system and associated downstream pathways by long-term resistance exercise in hypoxia in healthy young men (n = 9 for both normoxic groups, n = 10 for both hypoxic groups, linear mixed models analysis). CNR1 (*A*) and CNR2 (*B*) mRNA levels and protein expression of CB1 (*C*), CB2 (*D*), Akt Ser473 (*E*), p38 MAPK Thr180/Tyr182 (*F*), ERK1/2 Thr202/Tyr204 (*G*), and AMPK Thr172 (*H*) in the rested leg and in the exercised leg before and after 4 wk of resistance training in normoxia or in hypoxia. *I*: representative Western blots. A.U., arbitrary units. #$P < 0.05$ vs. normoxia; \$$P < 0.05$, vs. rest. *$P < 0.05$ vs. pre-exercise. Each dot represents a subject's value, each subject over-time evolution is represented by a line linking the two dots associated to a single subject. AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase.

Table 4. *Plasma endocannabinoid levels before and after an acute endurance exercise in hypoxia*

	Normoxia		Hypoxia	
pmol/mL	Pre	Post	Pre	Post
	(n = 8) *Healthy*		(n = 7)	
AEA	2.8 ± 0.4	3.7 ± 0.5	2.4 ± 0.3	3.4 ± 0.4*
2-AG	9.2 ± 0.4	10.2 ± 0.7*	8.4 ± 0.3	9.2 ± 0.4**
OEA	2.5 ± 0.3	3.2 ± 0.3**	2.3 ± 0.2	2.9 ± 0.2**
PEA	20.0 ± 2.2	26.9 ± 2.3**	22.6 ± 3.9	25.8 ± 2.6
	(n = 8) *PreT2D*		(n = 9)	
AEA	3.7 ± 0.5	5.0 ± 0.8*	2.7 ± 0.2	3.5 ± 0.3
2-AG	8.8 ± 0.4	9.3 ± 0.2	8.4 ± 0.2	8.8 ± 0.2
OEA	2.9 ± 0.3	3.6 ± 0.4*	2.3 ± 0.3	2.9 ± 0.2*
PEA	27.7 ± 2.0$	28.9 ± 2.7	28.1 ± 4.6	26.3 ± 2.6

Data are presented as the means ± SE. *$P < 0.05$, **$P < 0.01$ vs. pre. $$P < 0.05$ vs. healthy. AEA, N-arachidonoylethanolamine; OEA, N-oleoylethanolamine; PEA, N-palmitoylethanolamine; 2-AG, 2-arachidonoylglycerol.

in plasma 2-AG levels was found after 24 h, not after 5 min, and exclusively after a heavy load resistance exercise (70% 1RM), not after light load resistance exercise (30% 1RM) nor after resistance exercise (30% 1RM) coupled to blood flow restriction with low (40% limb occlusion) or high (80%) pressure. As β-endorphin levels were increased 5 min after the end of the two blood flow restriction conditions, it was suggested that β-endorphin, rather than endocannabinoids, would contribute to exercise-induced analgesia. Those two studies are inconclusive regarding the plasma levels of endocannabinoid ligands after resistance exercise and their role in exercise-induced analgesia. The second study reported no change in plasma 2-AG levels after blood flow restriction resistance exercise. The latter may be considered as a hypoxic resistance exercise paradigm, though hypoxia is limited to a limb and physiological adaptations differ somewhat from systemic hypoxic resistance exercise (35). Together with our results, hypoxic resistance training does not seem to regulate endocannabinoid levels differently to normoxic resistance training. More studies are required to determine the minimal intensity and number of repetitions needed to elicit measurable responses in plasma endocannabinoid levels and the kinetics of those responses after resistance exercise.

After having investigated their circulating levels, we identified whether the upregulation of endocannabinoid ligands after endurance exercise and their downregulation after resistance exercise modified the expression of the two cannabinoid receptors, i.e., CB1 and CB2, as well as downstream signaling. To the best of our knowledge, no previous study has measured skeletal muscle CB1 and CB2 protein expression in response to acute endurance exercise, and only two studies have investigated their expression in response to resistance exercise in human (12, 36). In the first study, compared with pre-exercise, CB1 expression was downregulated 48 h but not 1 h after acute resistance exercise in the skeletal muscle of untrained young men (36). In our acute resistance exercise study, we found a lower CB1 expression in the exercised leg compared with the rested leg at 4 h postexercise but not at 15 min. Those results corroborate the downregulation of CB1 expression previously found 48 h after resistance exercise (36) and suggest that this downregulation could be initiated as soon as 4 h postexercise. In the second study, CB1 and CB2 expression tended to increase from before to after 12 wk of resistance training in the skeletal muscle of healthy old people (12). In the present study, a three time shorter protocol of 4 wk did not modify CB1 and CB2 expression in the skeletal muscle of young men. As an increase in CNR1 mRNA levels was observed from before to after the 4-wk training, it is likely that the latter response was initiated by a systemic factor and that a higher expression of CB1 could have become detectable in the following weeks. In addition to the duration of the protocol, another important difference between the two studies was the age of the participants (20 vs. >65 yr). Provided the mild inflammatory status in older people and the key role of the immune system in the regulation of the endocannabinoid system (37), the latter could have contributed to the increase in CB1 and CB2 in Dalle and colleagues (12), although it is not clear yet which factors drive CB1 expression in old skeletal muscle. CB1 can also be expressed by interstitial cells such as fibroblasts and immune cells that infiltrate the old skeletal muscle at a higher rate than young muscle (38). The observation that basal CB1 expression was higher in old compared with young adults in the same study is in favor of this hypothesis (12). Based on the two aforementioned studies and ours, it seems that acute resistance exercise would rather decrease the expression of the cannabinoid receptors, whereas acute endurance exercise and resistance training would induce the opposite regulation. This might be explained by the fact that CB1 is highly expressed in mitochondria (39), and mitochondrial density is higher in type I muscle fibers, whose percentage increases over age and also characterizes the response to endurance training. More studies are needed to confirm those preliminary observations on the regulation of CB1 and CB2 protein expression by endurance and resistance exercise and training.

Finally, as both resistance and endurance exercise were found to modulate the levels of plasma endocannabinoid ligands, we were interested in investigating downstream signaling pathways known to be regulated by CB1 and CB2. The Akt, MAPK, and AMPK pathways are canonical pathways triggered by CB1 and CB2 activation (4, 40). In addition, all three pathways are greatly involved in the adaptations to exercise. The two aforementioned studies that determined the expression of CB1 and CB2 after acute resistance exercise (36) and training (12) also investigated the activation of the Akt and the MAPK pathways. In the context of resistance exercise, the latter is of particular interest as they regulate protein synthesis and degradation and therefore potentially muscle hypertrophy (41). Forty-eight hours after acute resistance exercise, CB1 expression was downregulated, whereas the phosphorylation of ERK1/2 was increased (36). After 12 wk resistance training, CB1 and CB2 expression tended to increase and the phosphorylation of FOXO3a increased, whereas the phosphorylation of mammalian target of rapamycin (mTOR) was unchanged compared with pretraining in older adults (12). As both FOXO3a and mTOR are downstream targets of Akt (41), it is difficult to conclude to an activation or not of the Akt pathway in that study. In the present study, the changes observed in the Akt, MAPK, and AMPK pathways were not related to the changes in the endocannabinoid ligands nor in the expression of the receptors. One important limitation when investigating the endocannabinoid system

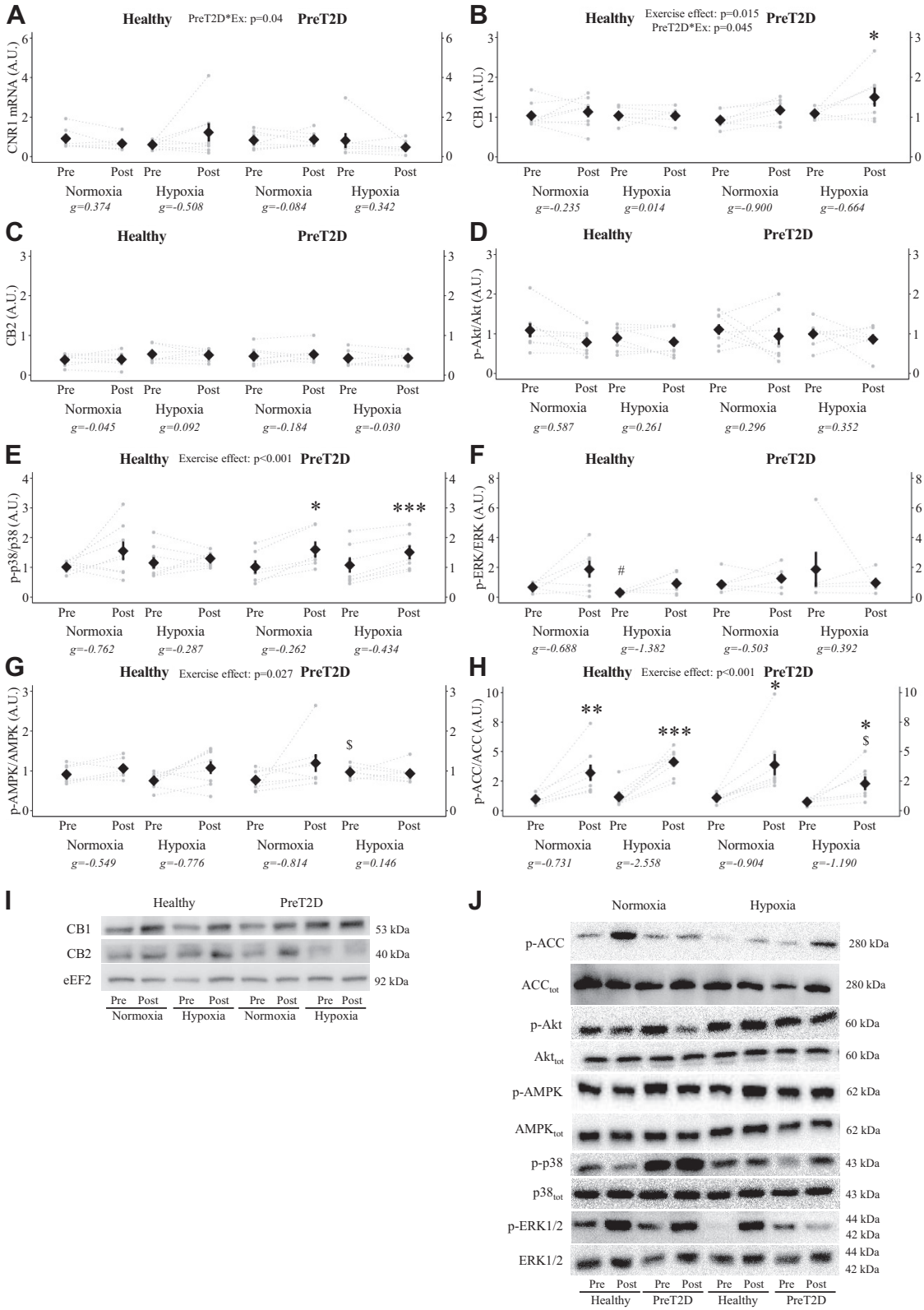

Figure 4. Regulation of the endocannabinoid system and associated downstream pathways by acute endurance exercise in hypoxia in healthy and prediabetics men (n = 8 for both normoxic groups, n = 7 for the healthy hypoxic group, n = 9 for the prediabetic hypoxic group, linear mixed models analysis). CNR1 mRNA levels (*A*) and protein expression of CB1 (*B*), CB2 (*C*), Akt Ser473 (*D*), p38 MAPK Thr180/Tyr182 (*E*), ERK1/2 Thr202/Tyr204 (*F*), AMPK Thr172 (*G*), and ACC Ser79 (*H*) before and after an acute endurance exercise in normoxia or in hypoxia. *I* and *J*: representative Western blots. A.U., arbitrary units. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. pre-exercise; \$$P < 0.05$ vs. healthy men. #$P < 0.05$ vs. normoxia. Each dot represents a subject's value, each subject over-time evolution is represented by a line linking the two dots associated to a single subject. ACC, acetyl-Coa-carboxylase; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase.

and its physiological significance is the lack of a specific signaling pathway triggered by CB1 and CB2. The Akt, MAPK, and AMPK pathways are commonly regulated by endurance and resistance exercise, mechanical and metabolic stress as well as changes in hormones and growth factors by exercise all contribute to their regulation. Therefore, the investigation of the role of endocannabinoid ligands and receptors in the activation of those pathways in vivo, certainly in human skeletal muscle, remains limited. This said, this is the first study to look at the regulation of the whole endocannabinoid system after both endurance and resistance exercise, starting from the ligands in the plasma to the intracellular mechanisms.

In conclusion, resistance exercise decreased plasma endocannabinoid levels after an acute session as well as after resistance training, whereas an acute moderate-intensity endurance session increased their levels. The expression of the cannabinoid receptors was only marginally regulated by acute exercise, hypoxia, and prediabetes, and downstream signaling did not follow the changes detected in the endocannabinoid ligands. The physiological impact of the changes observed in the endocannabinoid ligands in human skeletal muscle after exercise needs further investigation.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Fig S1: https://doi.org/10.6084/m9.figshare.21777464.v1.

Supplemental Fig S2: https://doi.org/10.6084/m9.figshare.21777461.v1.

GRANTS

S. Dalle has received a postdoctoral fellowship (Grant No. 12Z8622N) from Research Foundation Flanders (FWO).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.D. conceived and designed research; S.v.D.d.t.R., R.T., G.G.M., and L.D. performed experiments; S.v.D.d.t.R., R.T., G.G.M., and L.D. analyzed data; S.v.D.d.t.R., R.T., G.G.M., and L.D. interpreted results of experiments; S.v.D.d.t.R. and L.D. prepared figures; S.v.D.d.t.R. and L.D. drafted manuscript; S.v.D.d.t.R., S.D., R.T., K.K., G.G.M., and L.D. edited and revised manuscript; S.v.D.d.t.R., S.D., R.T., K.K., G.G.M., and L.D. approved final version of manuscript.

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