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RESEARCH ARTICLE

Effect of hypoxic exercise on glucose tolerance in healthy and prediabetic adults

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Abstract

This study aimed to investigate the mechanisms known to regulate glucose homeostasis in human skeletal muscle of healthy and prediabetic subjects exercising in normobaric hypoxia. Seventeen healthy (H; 28.8 ± 2.4 yr; maximal oxygen consumption ($\dot{V}O_{2max}$): 45.1 ± 1.8 mL·kg^{-1}·min^{-1}) and 15 prediabetic (P; 44.6 ± 3.9 yr; $\dot{V}O_{2max}$: 30.8 ± 2.5 mL·kg^{-1}·min^{-1}) men were randomly assigned to two groups performing an acute exercise bout (heart rate corresponding to 55% $\dot{V}O_{2max}$) either in normoxic (NE) or in hypoxic (HE; fraction of inspired oxygen $F_{I_{O_2}}$ 14.0%) conditions. An oral glucose tolerance test (OGTT) was performed in a basal state and after an acute exercise bout. Muscle biopsies from m. vastus lateralis were taken before and after exercise. Venous blood samples were taken at regular intervals before, during, and after exercise. The two groups exercising in hypoxia had a larger area under the curve of blood glucose levels during the OGTT after exercise compared with baseline (H: +11%; P: +4%). Compared with pre-exercise, an increase in p-TBC1D1 Ser237 and in p-AMPK Thr172 was observed postexercise in P NE (+95%; +55%, respectively) and H HE (+91%; +43%, respectively). An increase in p-ACC Ser212 was measured after exercise in all groups (H NE: +228%; P NE: +252%; H HE: +252%; P HE: +208%). Our results show that an acute bout of exercise in hypoxia reduces glucose tolerance in healthy and prediabetic subjects. At a molecular level, some adaptations regulating glucose transport in muscle were found in all groups without associations with glucose tolerance after exercise. The results suggest that hypoxia negatively affects glucose tolerance postexercise through unidentified mechanisms.

NEW & NOTEWORTHY The molecular mechanisms involved in glucose tolerance after acute exercise in hypoxia have not yet been elucidated in human. Due to the reversible character of their status, prediabetic individuals are of particular interest for preventing the development of type 2 diabetes. The present study is the first to investigate muscle molecular mechanisms during exercise and glucose metabolism after exercise in prediabetic and healthy subjects exercising in normoxia and normobaric hypoxia.

AMPK; cycling; diabetes; GLUT4; OGTT

INTRODUCTION

The prevalence of metabolic disorders has increased during the past decades and is one of the most significant health concerns in our society. Among those disorders, type 2 diabetes (T2D) is commonly associated with an increase in patient hospitalization and mortality (1, 2). The development of T2D is a progressive, multistage, and long-lasting process that can be reversed up to a certain point (3). Prediabetic individuals are of particular interest as they constitute an intermediate category between healthy and T2D individuals, displaying either increased fasting glucose levels or impaired glucose tolerance (4). In contrast to the advanced stages of T2D, prediabetes is still reversible, thus it is crucial to develop effective strategies in subjects presenting with that condition to avoid T2D development.

Exercise is an efficient strategy to prevent T2D and to improve insulin sensitivity (5, 6). Interestingly, exercising in hypoxic (HE) conditions seems to have an additive effect on glucose tolerance and insulin sensitivity compared with exercising in normoxic (NE) conditions in healthy and T2D subjects (7, 8). The molecular mechanisms involved in hypoxic exercise-dependent improvement of glucose tolerance and insulin sensitivity has not yet been evaluated in skeletal muscle. However, based on the mechanisms triggered by exercise and hypoxia separately, some candidates are of particular interest. Exercise and insulin are known to activate distinct signaling pathways that converge on the proteins Tre-2/BUB2/

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cdc 1 domain family 1 (TBC1D1) and 4 (TBC1D4), and Rac1 resulting in glucose transporter 4 (Glut4) translocation to the plasma membrane (9–11). Although insulin increases Glut4 translocation via the Akt pathway, exercise acts via several pathways that will be described below. Hypoxia stimulates glucose uptake partially but not exclusively via the same signaling pathways as exercise (12–14), however, the mechanisms still need to be deepened. Hypoxia and exercise are two energetic stressors which increase the AMP/ATP ratio and activate AMP-activated protein kinase (AMPK), resulting in the stimulation of glucose transport via the TBC1D1/TBC1D4/Glut4 pathway (13, 15, 16). TBC1D1 and TBC1D4 are two GTPase-activating proteins (GAP), which promote the GTPase enzymatic function of the Rab protein family, resulting in the conversion of Rab-GTP to Rab-GDP. Rab-GTP is responsible for Glut4 translocation to the plasma membrane (17). AMPK phosphorylates TBC1D1 and TBC1D4 that inhibits their Rab-GAP activity, which in turn results in increased Rab-GTP and Glut4 translocation (18, 19). In addition to activation of AMPK, hypoxia and exercise increase intracellular free calcium (13), thereby triggering calmodulin and its downstream kinase calcium/calmodulin-dependent protein kinase (CaMK) to facilitate Glut4 translocation in skeletal muscle (20). Moreover, exercise and hypoxia can activate p38 mitogen-activated protein kinase (MAPK) (21, 22), an important kinase in response to cellular stress, that might also be involved in the regulation of glucose transport (23, 24). Finally, hypoxia induces the stabilization of hypoxia-inducible factor 1 alpha (HIF1α) and the expression of its target genes that are involved, among others, in oxygen transport, glycolysis, and glucose transport, such as Rab21 and phosphofructokinase (PFK) (25).

In summary, in certain conditions, acute hypoxia seems to improve glucose tolerance and insulin sensitivity on a whole body level (9). However, the molecular mechanisms have not yet been elucidated in human, certainly not in prediabetic individuals. Based on the literature, we hypothesized that normobaric hypoxia would potentiate the effects of acute exercise on glucose tolerance in prediabetic adults, by enhanced exercise signaling that would carry over and improve postexercise glucose tolerance. The aim of the present study was, therefore, to investigate muscle molecular signaling during exercise and glucose metabolism after exercise in prediabetic and healthy subjects exercising in normoxia and normobaric hypoxia.

MATERIALS AND METHODS

Participants

Seventeen healthy and 15 prediabetic men participated in this study. The morphological and clinical characteristics of the subjects are presented in Table 1. The study was approved by the Ethical Committee of the Université catholique de Louvain (Belgium) and conducted in accordance with the Declaration of Helsinki. The inclusion criteria was nonsmoking men aged 20–65 yr. Exclusion criteria were as follows: contraindication to intense physical exercise, exposure to an altitude > 1,500 m the month preceding the experiment, physical activity > 3 h/wk, drug treatment for insulin resistance, and sleep apnea syndrome. Healthy group was defined by fasting glucose levels lower than or equal to 100 mg·dL^{-1} and blood glucose levels lower than 140 mg·dL^{-1} at the 120th min during the first oral glucose tolerance test (OGTT). Prediabetic group was defined by fasting glucose levels between 100 and 126 mg·dL^{-1} and/or blood glucose levels between 140 and 200 mg·dL^{-1} at the 120th min during the first oral glucose tolerance test (OGTT) according to the American Diabetes Association. A written consent was obtained from all subjects after explaining all potential risks of the study.

Protocol

The study consisted of two experimental days.

Experimental day 1.

The participants came to the laboratory after an overnight fast. The subjects underwent a medical examination to exclude any contraindication to engage in high-intensity exercise. Percentage of body fat mass was measured by dual-energy X-ray absorptiometry (DEXA). Thereafter, a

Table 1. *Subjects characteristics*

	Healthy	Prediabetic	*P* Value
Age, yr	28.8 ± 2.4	44.6 ± 3.9	0.001
Body mass, kg	73.8 ± 1.2	90.0 ± 4.5	0.001
Waist circumference, cm	82.4 ± 1.5	97.4 ± 3.6	0.0003
BMI, kg·m^{-2}	23.5 ± 0.5	27.3 ± 1.2	0.005
$\dot{V}O_{2max}$, mL·kg^{-1}·min^{-1}	45.1 ± 1.8	30.8 ± 2.5	<0.0001
Body fat mass, %	16.3 ± 1.1	24.4 ± 1.8	0.0005
HbA1C, %	5.0 ± 0.06	5.3 ± 0.07	0.01
Fasting plasma glucose, mg·dL^{-1}	94.3 ± 1.1	107.3 ± 1.8	<0.0001
Oral glucose tolerance test 120 min, mg·dL^{-1}	102.9 ± 6.9	137.6 ± 10.4	0.007
HOMA-IR	1.2 ± 0.1	1.9 ± 0.3	0.04
HOMA-β, %	65.5 ± 7.4	63.6 ± 9.7	ns
QUICKI	0.37 ± 0.005	0.35 ± 0.01	ns
Triglycerides, mmol·L^{-1}	1.1 ± 0.09	1.2 ± 0.06	ns
CRP, mg·L^{-1}	0.5 ± 0.06	1.4 ± 0.3	0.008
LDL, mmol·L^{-1}	2.5 ± 0.2	3.1 ± 0.2	ns
HLD, mmol·L^{-1}	1.4 ± 0.06	1.3 ± 0.06	ns

Values are means ± SE. BMI: body mass index; CRP: C-reactive protein; HbA_{1C}: glycated hemoglobin; HLD: high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β: homeostatic model assessment of β cell function; LDL: low-density lipoprotein; QUICKI: quantitative insulin sensitivity check index. $P < 0.05$; $P < 0.01$; $P < 0.001$ vs. healthy group. ns, Not significant.

catheter was inserted into an antecubital vein of the forearm and a first blood sample was taken to measure glycated hemoglobin (HbA_{1c}), insulin, glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride, and C-reactive protein (CRP) levels, which was followed by a 3-h OGTT (Fig. 1). Morphological (height, body weight, waist circumference) and physiological parameters [blood pressure (Omron)] were determined.

After the OGTT, a maximal incremental exercise test was performed on a cycle ergometer to determine maximal oxygen uptake (maximal oxygen consumption, $\dot{V}O_{2max}$, Ergocard, Medisoft) and maximal power output [watt $(W)_{max}$]. Heart rate (HR, Polar Team System 2; Polar Electro, Kempele, Finland) and respiratory exchanges were continuously monitored during the test. The starting load was 70 W with an increment of 30 W every 2 min until exhaustion. A capillary blood sample (5 μL) was collected from the earlobe to determine blood lactate levels at the beginning, at 190 W, and at the end of the test (Lactate Pro 2, Arkray, Japan). At the end of the first visit, the healthy (H) and prediabetic (P) subjects were divided into two groups (NE, normoxic exercise; HE, hypoxic exercise) in a semirandom manner to ensure that the level of physical fitness ($\dot{V}O_{2max}$) of the two groups was homogeneous (n = 8 in H NE; n = 8 in P NE; n = 9 in H HE; and n = 7 in P HE).

Experimental day 2.

At least 4 days after the first visit and after an overnight fast, the participants came to the laboratory and a catheter was inserted into an antecubital vein of the forearm. A first blood sample was taken to measure insulin and glucose levels. Afterward, a first biopsy sample was taken from the right m. vastus lateralis under local anesthesia (1–2 mL lidocaine) through a 5-mm incision in the skin. Muscles samples were immediately frozen in liquid nitrogen and stored at −80°C. Immediately after, a second blood sample was taken. After those first measurements in normoxia, the subjects were transferred to the exercise room, which was either maintained at 20.9% O_2 or set at 14.0% O_2 corresponding to an altitude of ~3,000 m. This F_{IO_2} was chosen based on previous reports showing a positive effect on glucose metabolism with a F_{IO_2} of 14.0% (8, 26, 27). The exercise bout consisted in cycling for 60 min at a HR corresponding to 55% $\dot{V}O_{2max}$. This workload was chosen to ensure that the relative training intensity was comparable between normoxia and hypoxia. The Borg scale, measuring the rate of perceived exertion (RPE), was collected after exercise to determine whether the perception of exercise intensity was equal between the hypoxic and normoxic groups. Moreover, the subject completed the Lake Louise Questionnaire after the exercise session to assess the symptoms of acute mountain sickness (28). During exercise, saturation of

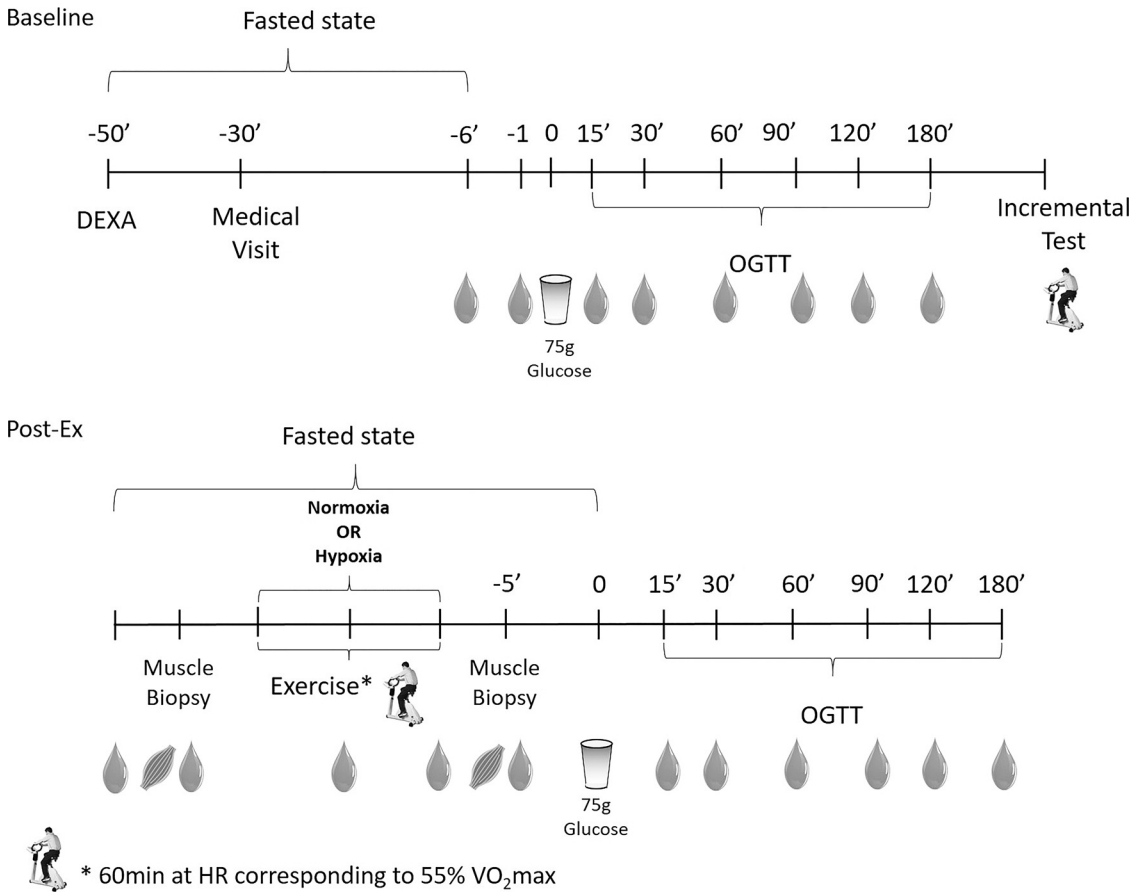

Figure 1. Schematic view of the experimental protocol. Body composition, medical visits, oral glucose tolerance test (OGTT), blood analyses, and maximal incremental exercise test were performed at *visit 1*. One week later (or at least 4 days later), blood samples and muscle biopsies were taken before and after acute exercise followed by an OGTT at *visit 2*. DEXA, dual-energy X-ray absorptiometry; HR, heart rate; $\dot{V}O_{2max}$, maximal oxygen consumption.

peripheral oxygen (S_pO_2; fingertip oximetry device; Trismed; OXY500, Korea) and blood lactate levels were measured every 30 min (T0; T30; T60 min). Venous blood samples were drawn at 30 min and 60 min. Directly after exercise, a second biopsy sample was taken in normoxic conditions and a blood sample was taken followed by an OGTT.

Analysis of Blood Samples

Blood samples were taken from an antecubital vein in 5-mL tubes containing EDTA for HbA_{1C} and in 5- (glucose/insulin) or 10-mL tubes containing heparin lithium for the rest of the analyses (see *Insulin, glucose, HDL, LDL, triglyceride, and CRP levels* details). Heparin lithium tubes were immediately centrifuged at 4°C for 10 min at 3,000 *g*, plasma was transferred to microtubes and stored at −80°C. EDTA tubes were stored at 4°C for one night and HbA_{1C} analyses were performed by chromatography (ADAMS; Menarini; Ha-8180V).

OGTT, homeostatic model assessment, and quantitative insulin sensitivity check index.

Plasma glucose and insulin levels were determined in the fasted state (T0) and 15 (T15), 30 (T30), 60 (T60), 90 (T90), 120 (T120), and 180 min (T180) after glucose ingestion (75 g; Glucomedics; LAMBRA). Insulin sensitivity was estimated by the homeostatic model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI). Those indexes were calculated from fasting blood samples. HOMA of insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were calculated with the following formula: HOMA-IR = [fasting insulin ($mUI{\cdot}L^{-1}$) × fasting glucose ($mg{\cdot}dL^{-1}$)/405] and HOMA-β = [360 × fasting insulin ($mUI{\cdot}L^{-1}$)/fasting glucose ($mg{\cdot}dL^{-1}$) − 63] (29). QUICKI was calculated with the following formula: 1/[log(fasting insulin) + log(fasting glucose)] (30).

Insulin, glucose, HDL, LDL, triglyceride, and CRP levels.

The plasma levels of blood glucose, HDL, LDL, triglyceride, and CRP were assessed using an automatic analyzer (PENTRA C200, HORIBA Medical, Japan). The plasma levels of insulin were determined by ELISA with the ultra-sensitive insulin kit from Mercodia (Winston Salem, NC). The plasma levels of cortisol were determined by ELISA with the cortisol ELISA kit from Abnova (KA3382, Taiwan).

Muscle Analysis

Muscle homogenization.

A portion (~20 mg) of muscle biopsies was freeze-dried for 48 h and dissected free of visible fat, blood, and connective tissue. Muscle tissue was homogenized with stainless steel beads 2 × 30 s at 30 Hz using a TissueLyser II (Qiagen) in 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1% NP-40, 10% glycerol, 2 mM phenylmethanesulfonyl fluoride, 10 $\mu g{\cdot}mL^{-1}$ leupeptin, 10 $\mu g{\cdot}mL^{-1}$ aprotinin, and 3 mM benzamidine. After rotation end-over-end for 30 min, lysate supernatants were collected by centrifugation (10,000 *g*) for 20 min at 4°C.

Immunoblotting.

Lysate protein concentrations were measured using the bicinchoninic acid (BCA) method using bovine serum albumin standards and BCA assay reagents (Pierce Biotechnology, Rockford, IL). Total protein and phosphorylation levels of relevant proteins were determined by standard immunoblotting techniques by loading equal amounts of protein. The primary antibodies used were Glut4 (Thermo, Cat. No. PA1-1065), p-ACC Ser^{212} [Cell Signaling Technologies (CST), Cat. No. 3661], p-AMPK Thr^{172} (CST, Cat. No. 2531L), AMPKα2 tot (Abcam, Cat. No. ab3760), p-TBC1D4 Thr^{642} (CST, Cat. No. 88815), p-CAMKIIα Thr^{287} (CST Cat. No. 3361L), p-TBC1D4 Thr^{588} (CST, Cat. No. 87305), p-TBC1D1 Ser^{237} (Millipore, Cat No. 07-2268), p-AKT Thr^{308} (CST, Cat. No. 9275S), p-AKT Ser^{473} (CST, Cat. No. 92715), CamKII tot (Santa Cruz, Cat. No. SC-13082), TBC1D1 tot (Abcam, Cat. No. ab229504), TBC1D4 tot (Abcam, Cat. No. ab189890), peroxidase-conjugated streptavidin (Jackson Immuno Research, Cat. No. 016-030-084), AKT2 tot (CST, Cat. No. 30635), p-p38 Thr^{180}/Tyr^{182} (CST, Cat. No. 9211), p38 tot (CST, Cat. No. 9212), Redd1 (Proteintech, Cat. No. 10638-1-AP), HIF1α (CST, Cat. No. 14179S), and actin (Sigma Aldrich, Cat. No. A2066). Polyvinylidene difluoride membranes (Immobilon Transfer Membrane, Millipore) were blocked in TBS-Tween containing 5% skim milk or 5% BSA protein for 15 min at room temperature. Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Bands were visualized using the Bio-Rad ChemiDocTM MP Imaging System and enhanced chemiluminescence (ECL+; Amersham Biosciences). The results are presented as the ratio of phosphorylated/total amount of the proteins, except for p-CAMKIIα, Glut4, p-TBC1D1, and Redd1, which were reported to actin, which was found not to be modified by any conditions.

Real-time quantitative PCR.

Approximately, 15 mg of muscle biopsy sample was homogenized in 1 mL of Trizol reagent (Thermo Fisher Scientific) with a TissueLyser II (Qiagen). RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop (Thermo Fisher Scientific) spectrophotometry. Reverse transcription was performed from 1 μg of RNA using the iScriptcDNA Synthesis Kit (Bio-Rad Laboratories), following the manufacturer's instructions. The following primers were used: PFK, forward sequence ATTTGACGAAGCCCTGAAG and reverse sequence GTGCGAACCACTCTTAGATAC; vascular endothelial growth factor (VEGFa), forward sequence TTTCTGCTGTCTTGGGTGCATTGG and reverse sequence ACCACTTCGTGATGATTCTGCCCT; and ribosomal protein L19 (RPL19), forward sequence CGCTGTGGCAAGAAGAAGGTC and reverse sequence GGAATGGACCGTCACAGGC. The 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 using the CFX Connect Real-Time System (Bio-Rad Laboratories). All samples were run in duplicate, and each reaction was processed in a 10-μL volume containing 4.8 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.1 μL of each primer (100 nM final), and 5 μL of cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. Relative mRNA expression levels were normalized to ribosomal protein

L19, the expression of which was unaffected by conditions or exercise.

Muscle glycogen.

Muscle glycogen content was measured in homogenates (150 μg of protein) as glycosyl units after acid hydrolysis (2 h in 2 N HCl at 100°C) determined by a fluorometric method in an automatic analyzer (PENTRA C400, HORIBA Medical, Japan).

Statistics.

Based on previous data on glucose levels during an intravenous glucose tolerance test after hypoxic exercise (7), a statistical power analysis has been performed to determine the optimal number of subjects needed to find a difference in the mean of 30% with an SD corresponding to 20% of the means and a power of 80% (n = 7 per group). Statistical analyses were performed using GraphPad Prism 7.00, and data are reported as means ± SE. Figure 2, *A, B, D* and *E*: a two-way ANOVA (mix model) was used at each time point of the OGTT with the subjects as a random variable and groups (healthy and prediabetic) and exercise (baseline and postexercise) as fixed independent variables. Data from Fig. 2, *A* and *B*, and from Fig. 2, *D* and *E*, were analyzed separately. Figure 2, *C* and *F*: the area under the curve (AUC) was calculated using the trapezoid method from the basal value from each individual. Figures 2, *C* and *F*, 3, *B–F*, 4–6: a two-way ANOVA (mix model) within each environmental condition (normoxia and hypoxia) was used with the subjects as a random variable and groups (healthy and prediabetic) and exercise (pre- and postexercise) as fixed independent variables. Figure 4*A* (S_pO_2): a two-way ANOVA (mix model) was used with conditions (normoxia and hypoxia) and exercise (pre- and postexercise) as fixed independent variables. Figure 4*G* (RPE): a one-way ANOVA was used to test differences between the four groups. When the ANOVA test was significant ($P < 0.05$) or tended to be significant ($P < 0.1$) for the groups, environmental conditions, exercise and/or interaction main effects, least significant difference Fisher post hoc tests were performed to compare mean values. Table 1: a Student's t test was used to determine differences in subject's characteristics between the healthy and prediabetic group. Statistical significance was set at $P < 0.05$. Differences ($P < 0.05$) between pre- and postexercise values are indicated by asterisks (*), differences between healthy and prediabetics are indicated by hash signs (#), differences between normoxia and hypoxia are indicated by (ǂ), differences versus T0 are indicated by (£), and differences versus T30 are indicated by (+). When a value exceeded twice the standard deviation, it was considered statistically abnormal and was not taken into account.

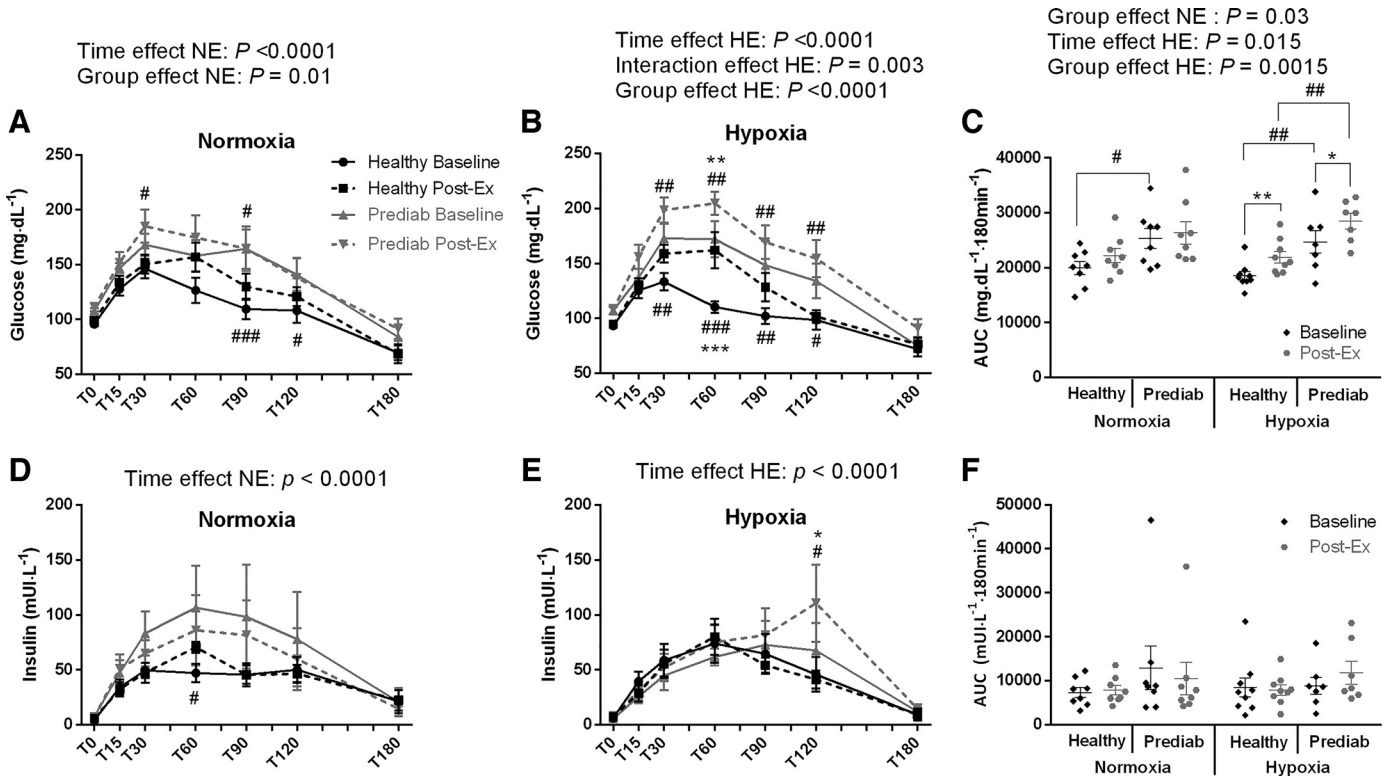

Figure 2. Effect of acute exercise under normoxia or hypoxia on glucose metabolism. Healthy normoxia (H NE, n = 8), prediabetic normoxia (P NE, n = 8), healthy hypoxia (H HE, n = 8), and prediabetic hypoxia (P HE, n = 7). Plasma glucose levels at T0, T15, T30, T60, T90, T120, and T180 in normoxic (*A*) and hypoxic conditions (*B*). *C*: area under the curve (AUC) for plasma glucose in H NE, P NE, H HE, and P HE; plasma insulin levels at T0, T15, T30, T60, T90, T120, and T180 in normoxic (*D*) and hypoxic conditions (*E*). *F:* AUC for insulin plasma levels in H NE, P NE, H HE, and P HE at baseline and postexercise. Values are means ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. baseline. #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ vs. healthy.

RESULTS

Acute Exercise in Hypoxic Conditions Increases Blood Glucose Response to an OGTT

Fasting blood glucose levels were neither modified by exercise nor by the environmental conditions (Fig. 2, *A* and *B*). The AUC for glucose levels was higher during the OGTT at baseline in the prediabetic group compared with the respective healthy group (NE: +26%, $P = 0.02$; HE: +33%, $P = 0.002$; Fig. 2*C*) and was higher during the postexercise OGTT in P HE compared with H HE (+30%, $P = 0.001$; Fig. 2*D*). In the normoxic groups, blood glucose (Fig. 2*A*) and insulin levels (Fig. 2*D*) were similar during the OGTT postexercise compared with the OGTT at baseline. In the hypoxic groups, blood glucose levels were higher at T60 during the OGTT postexercise compared with baseline (H HE: +47%, $P < 0.001$ and P HE: + 19%, $P = 0.03$; Fig. 2*B*). The AUC for glucose levels was higher postexercise compared with baseline (H HE: +11%, $P = 0.008$ and P HE: +4%, $P = 0.03$; Fig. 2*C*). Insulin levels were higher at T120 during the OGTT postexercise compared with baseline in P HE (+68%, $P = 0.03$; Fig. 2*E*). No change was observed for the AUC of plasma insulin in all groups (Fig. 2*F*).

Effect of Hypoxia during Exercise on Blood Parameters

Globally, S_PO_2 during exercise was lower in the hypoxic group (85.5% ± 0.5%) compared with the normoxic group (95.5% ± 0.4%, $P < 0.001$), with no difference between healthy and prediabetic subjects (Fig. 3*A*). Those results indicate that the degree of environmental hypoxia in the present study was able to induce a mild hypoxemia.

During exercise, higher glucose levels were measured in both prediabetic groups ($P < 0.001$) compared with their respective healthy group (Fig. 3*B*). Blood glucose levels

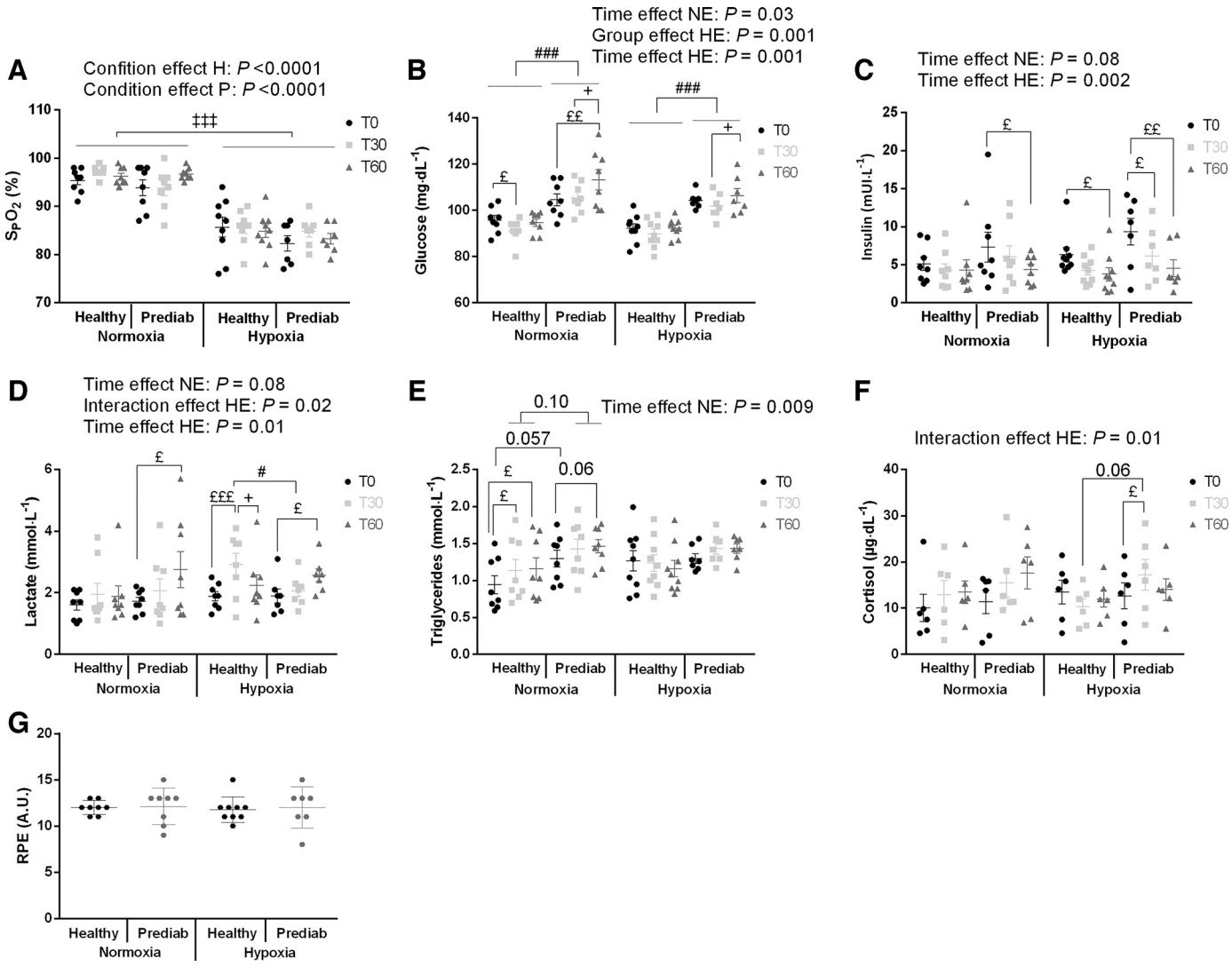

Figure 3. Effect of environmental normoxia or hypoxia during exercise on blood parameters. Healthy normoxia (H NE, $n = 8$), prediabetic normoxia (P NE, $n = 8$), healthy hypoxia (H HE, $n = 8$), and prediabetic hypoxia (P HE, $n = 7$). Saturation of peripheral oxygen (S_PO_2) (*A*), plasma glucose levels (*B*), plasma insulin levels (*C*), blood lactate levels (*D*), plasma triglyceride levels (*E*), plasma cortisol levels (*F*) at T0, T30, and T60 during exercise in H NE, P NE, H HE and P HE. *G*: Borg RPE in H NE, P NE, H HE, and P HE postexercise. Values are means ± SE. #$P < 0.05$; ###$P < 0.001$ vs. healthy. £$P < 0.05$; ££$P < 0.01$; £££$P < 0.001$ vs. T0. +$P < 0.05$ vs. T30. ‡‡‡$P < 0.001$ vs. normoxia. RPE, rate of perceived exertion.

increased during exercise in P NE and P HE (P NE: T60 vs. T0: +2%, P = 0.005; T60 vs. T30: +4%, P = 0.01; P HE: T60 vs. T30: + 7%, P = 0.02; Fig. 3*B*) and decreased in H NE (T30 vs. T0: −3%, P = 0.02, Fig. 3*B*). Insulin levels decreased in P NE, H HE, and P HE (P NE: T60 vs. T0: −16%, P = 0.04; H HE: T60 vs. T0: −40%, P = 0.03; P HE: T30 vs. T0: −34%, P = 0.03, T60 vs. T0: −51%, P = 0.002; Fig. 3*C*). Lactate levels increased by 59% at T60 compared with T0 in P NE (P = 0.015; Fig. 3*D*), increased by 80% at T30 in H HE ($P < 0.001$), and by 35% at T60 in P HE (P = 0.03, Fig. 3*D*) compared with T0. In H NE, the triglycerides levels increased by 20% at T30 (P = 0.03) and by 23% at T60 (P = 0.013) compared with T0 (Fig. 3*E*). The levels of plasma triglycerides tended to increase by 13% at T60 compared with T0 in P NE (P = 0.06) and tended to be higher in P NE compared with H NE (P = 0.057 at T0 and P = 0.10 at T30 and T60). In hypoxic conditions, the cortisol levels increased by 35% at T30 (P = 0.01) in the prediabetic group and tended to be higher than in the healthy group at this time point (T30: +65%; P = 0.06).

The RPE at the end of exercise was not different between the normoxic and the hypoxic groups (Fig. 3*G*).

Hypoxia Does Not Modulate Key Regulators of Insulin-Stimulated Glucose Transport in Skeletal Muscle

To determine the mechanisms contributing to the higher glucose levels in response to the OGTT in the groups having exercised in hypoxia but not in normoxia, key regulators of insulin-stimulated glucose metabolism were analyzed in the muscle samples taken before and at the end of the exercise. Muscle glycogen levels decreased by 60% in H NE postexercise compared with pre-exercise (P = 0.001, Fig. 4*A*). Muscle glycogen levels were higher in P HE compared with H HE postexercise (P = 0.014, Fig. 4*A*). Compared with pre-exercise, p-Akt at Ser^{473} decreased after exercise in H NE (−27%, P = 0.015, Fig. 4*B*), whereas p-Akt at Thr^{308} remained unchanged (Fig. 4*C*). Although p-TBC1D4 at Ser^{588} (Fig. 4*D*) and p-TBC1D4 at Thr^{642} (Fig. 4*E*) were not modified by any condition, an increase in p-TBC1D1 at Ser^{237} was observed postexercise in P NE (+95%, P = 0.03) and H HE (+91%, P = 0.02) compared with pre-exercise (Fig. 4*F*). After exercise, whole cell Glut4 expression was increased by 93% compared with pre-exercise only in P NE (P = 0.02; Fig. 4*G*).

Hypoxia Modulates Key Regulators of Exercise-Stimulated Glucose Transport in Skeletal Muscle of Healthy but Not Prediabetic Men

To further determine the mechanisms contributing to the higher glucose levels in response to the OGTT in the groups having exercised in hypoxia but not in normoxia, key regulators of exercise-stimulated glucose metabolism were analyzed. Compared with pre-exercise, p-AMPK at Thr^{172} was increased by 55% in P NE (P = 0.05) and by 43% in H HE (P = 0.02) after exercise (Fig. 5*A*). Its downstream target, p-ACC at Ser^{212} was increased in all groups after exercise compared with pre-exercise (H NE: +228%, P = 0.007; P NE: +252%, $P < 0.001$; H HE: +252%, $P < 0.001$; P HE: +208%, P = 0.012; Fig. 5*B*). The increase in p-ACC was approximately twofold lower in P HE compared with H HE (P = 0.002; Fig. 5*B*). Exercise induced an increase in p-CaMKIIα at Thr^{287} only in H NE (+488%, P = 0.006, Fig. 5*C*). Exercise induced an increase in phospho-p38 at Thr^{180}/Tyr^{182} in all groups except in H HE (H NE: +53%, P = 0.05; P NE: +58%, $P < 0.001$; P HE: +40%, $P < 0.001$; Fig. 5*D*).

The Hypoxia-Inducible Factor Pathway is Not Modified by Any Experimental Condition in Skeletal Muscle

The involvement of the HIF1α pathway in the differential regulation of glucose levels during the OGTT post-hypoxic exercise compared with normoxic exercise was tested by measuring the protein expression of HIF1α as well as different targets thereof. There were no modifications in the protein expression of HIF1α (Fig. 6*A*) or Redd1 (Fig. 6*B*) nor in the mRNA expression of VEGF (Fig. 6*C*) by any condition. Compared with pre-exercise, PFK mRNA expression increased after exercise only in P NE (+30%, P = 0.003; Fig. 6*D*). The mRNA expression of Rab20 tended to increase after exercise in H HE (+61%, P = 0.058; Fig. 6*E*).

DISCUSSION

Here, we hypothesized that exercise performed in hypoxia condition would be more efficient than exercise performed in normoxia condition at improving glucose tolerance in prediabetic and healthy adults and that this additional effect would be due to a greater activation of the exercise-induced pathway in hypoxia.

In contrast with our hypothesis, we found that exercising in hypoxia increased glucose AUC during a postexercise OGTT in the healthy and prediabetic groups and increased plasma insulin levels at T120 after glucose ingestion in the prediabetic group. These results are also in contrast to other studies showing an improved glycemic regulation after exercising in hypoxia in T2D subjects (7, 8). Based on our results, we expected to observe impaired activation of downstream signaling pathways involved in glucose metabolism in skeletal muscle. The key pathway in the regulation of insulin-stimulated glucose transport, i.e., the Akt/TBC1D1-4/Glut4, was not downregulated by hypoxia. We then tested the activation of the exercise-stimulated glucose transport pathway, i.e., the AMPK pathway. The activation of this pathway and glucose disposal by exercise is largely determined by the absolute exercise intensity (16). Since the absolute exercise intensity was lower in hypoxia than in normoxia, a lower or even a lack of activation of the AMPK pathway was expected in the hypoxic exercise groups compared with the normoxic groups. Nevertheless, an increase in acetyl-CoA carboxylase phosphorylation was measured in the four groups after exercise, with no difference between normoxia and hypoxia. Neither was the case for phospho-AMPK, nor for phospho-TBC1D1/TBC1D4, indicating that the AMPK pathway was not differently activated by hypoxic versus normoxic exercise, despite a lower absolute exercise intensity in hypoxia. In addition to AMPK, hypoxia has been shown to increase free intracellular calcium (13), thereby potentially triggering the CaMKK pathway. Two previous studies reported an involvement of the calcium-calmodulin pathway in the increased glucose transport by hypoxia (13, 31). The increase in phosphorylation of CaMKII was only observed in healthy subjects exercising in normoxia, indicating here as well that, in our

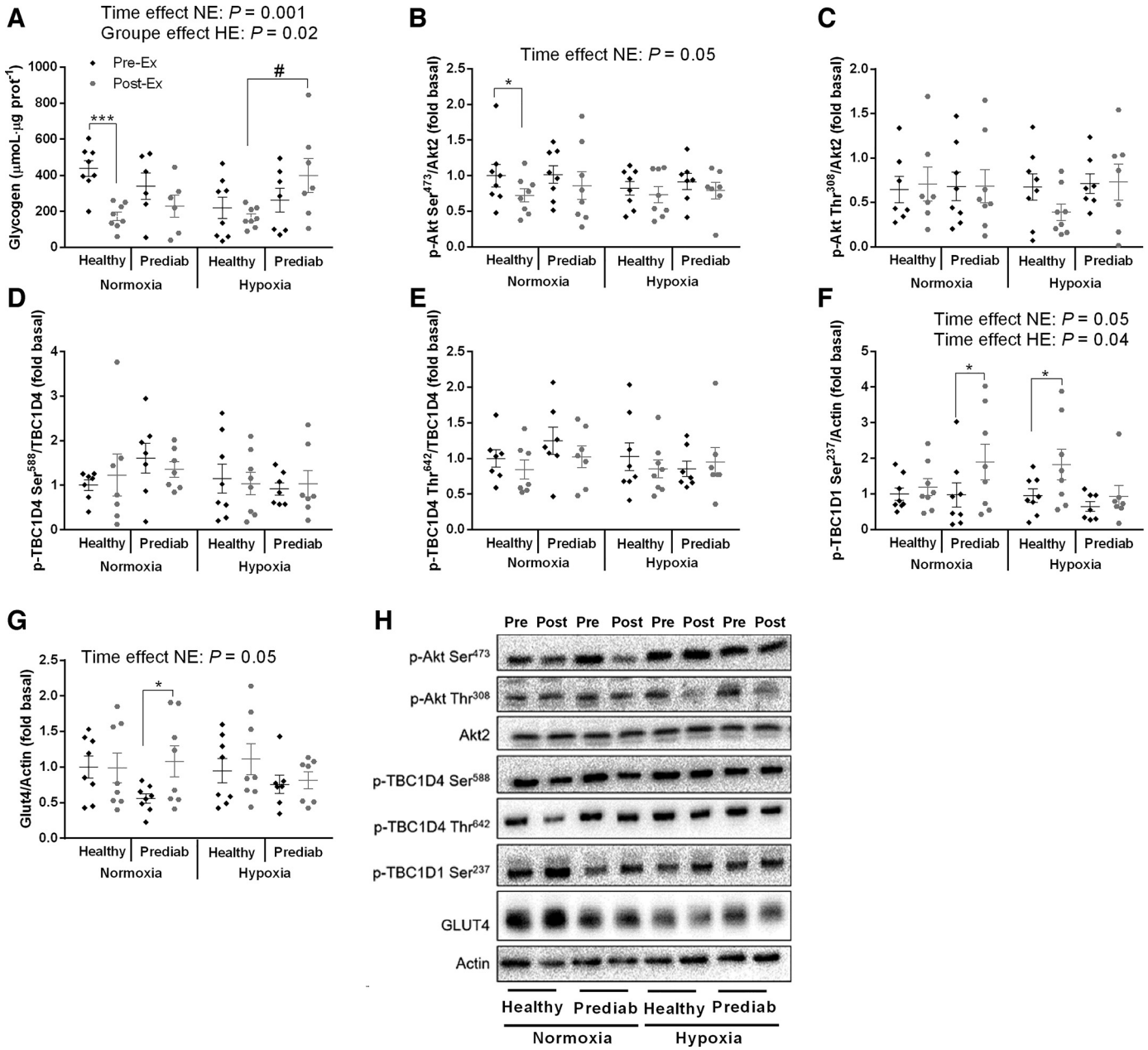

Figure 4. Effect of environmental hypoxia and exercise on key regulators of insulin-stimulated glucose transport. Healthy normoxia (H NE, $n = 8$), prediabetic normoxia (P NE, $n = 8$), healthy hypoxia (H HE, $n = 8$), and prediabetic hypoxia (P HE, $n = 7$) except when specified. Muscle glycogen levels (*A*), Akt phosphorylation at Ser^{473}(*B*), Akt phosphorylation at Thr^{308} (H NE; n: −1) (*C*), Tre-2/BUB2/cdc 1 domain family 4 (TBC1D4) phosphorylation at Ser^{588} (H NE; n: −1 and P NE; n: −1) (*D*), TBC1D4 phosphorylation at Thr^{642} (H NE; n: −1 and P NE; n: −1) (*E*), Tre-2/BUB2/cdc 1 domain family 1 (TBC1D1) phosphorylation at Ser^{237} (*F*), whole cell Glut4 protein expression in H NE, P NE, H HE, and P HE pre-exercise and postexercise (*G*), and representative Western blots (*H*). Values are presented as means ± SE. $*P < 0.05$; $***P < 0.001$ vs. pre-exercise; $\#P < 0.05$ vs. healthy. Pre: pre-exercise; Post: postexercise. "Fold basal" means fold changes compared to the pre-exercise value from the healthy group in normoxia.

conditions, hypoxia did not regulate this pathway. Finally, we tested the p38 MAPK pathway, a possible way by which exercise (11) and hypoxia (22, 24) can stimulate glucose transport. However, p38 phosphorylation increased after exercise in all groups, except in the healthy subjects exercising in normoxia. Finally, we looked at the HIF1α pathway, which is known to regulate glucose metabolism during hypoxia (25). However, neither the expression of HIF1α protein nor the expression of its target genes was modified by our hypoxic conditions during exercise in skeletal muscle. This is not totally surprising due to the moderate hypoxic dose (F_{IO_2}: 14% for 60 min) and previous results in similar hypoxic conditions (32). It is also possible that we missed HIF1α stabilization due to extremely rapid degradation even though the second muscle biopsy was taken within 3 min after exercise completion and exposure to hypoxia. At least, if the pathway was activated during hypoxic exercise, we should have measured changes in the mRNA levels of the downstream targets of HIF1α pathway. Anyway, an activation of the HIF1α pathway by hypoxia would have resulted in an increased use of

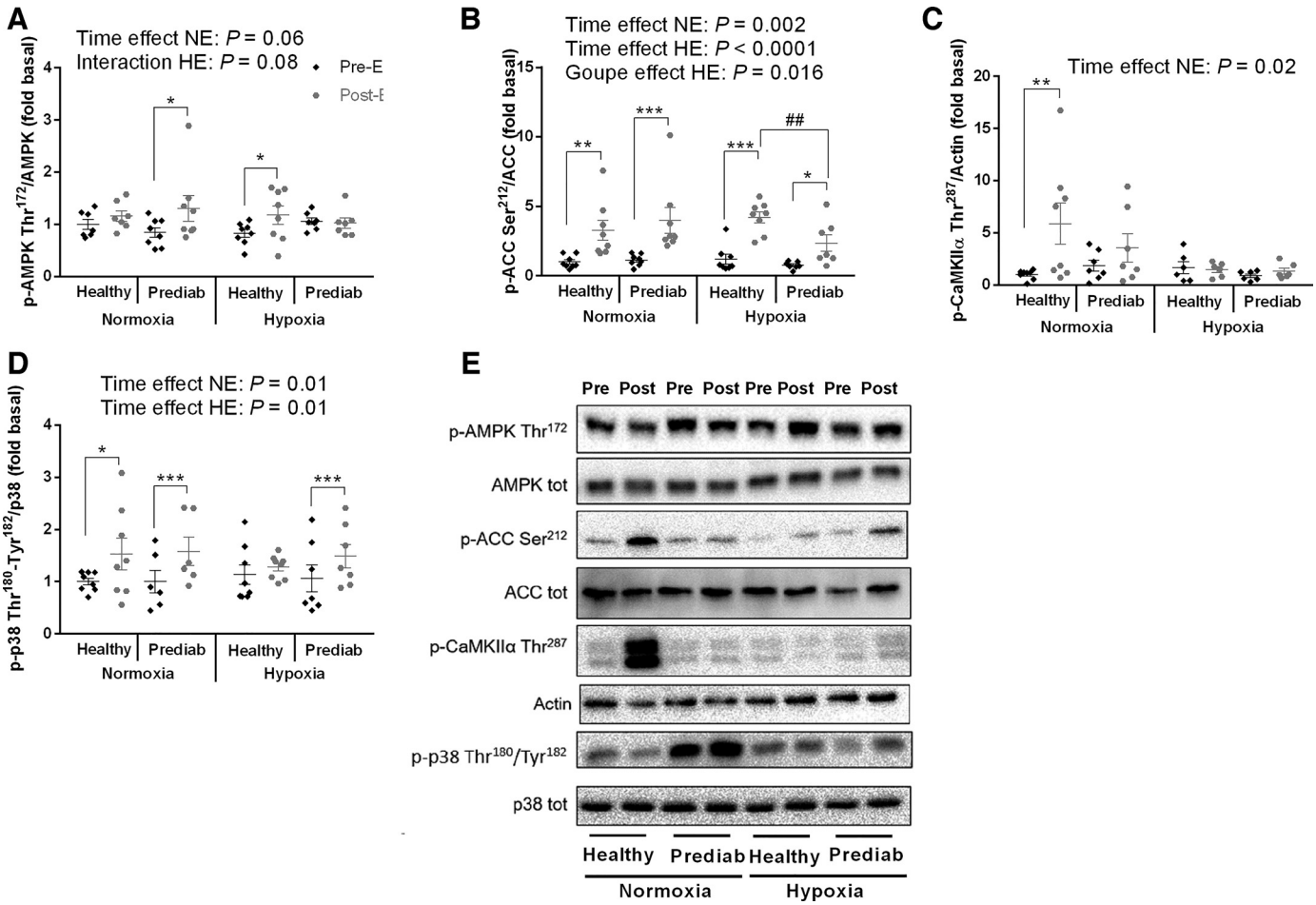

Figure 5. Effect of environmental hypoxia and exercise on key regulators of exercise-stimulated glucose transport. Healthy normoxia (H NE, $n = 8$), prediabetic normoxia (P NE, $n = 8$), healthy hypoxia (H HE, $n = 8$), and prediabetic hypoxia (P HE, $n = 7$) except when specified. AMPK phosphorylation at Thr^{172} (H NE; *n:* −1) (*A*), ACC phosphorylation at Ser^{212} (*B*), CamKIIα phosphorylation at Thr^{287} (H HE; *n*: −2; P NE; *n*: −1; and P HE; *n*: −1) (*C*), and p38 phosphorylation at Thr^{180}-Tyr^{182} (P NE; *n*: −2) (*D*), in H NE, P NE, H HE, and P HE pre-exercise and postexercise. *E:* representative Western blots. Values are presented as means ± SE. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. pre-exercise. $\#\#P < 0.01$ vs. healthy. Pre: pre-exercise; Post: postexercise. "Fold basal" means fold changes compared to the pre-exercise value from the healthy group in normoxia.

glucose in skeletal muscle during exercise and a likely decrease in plasma glucose levels during exercise, which was not the case. Skeletal muscle is a potent tissue to face a lack of oxygen. It is likely that a F_{IO_2} of 14.0% was not severe enough to induce hypoxia at a cellular level despite a lower SpO_2 in the hypoxic group. The lack of effect on tissue oxygenation does not preclude hypoxemia to initiate intramuscular molecular mechanisms via, for example, indirect regulation of cytokines levels (33). Additional mechanisms probably remain to be discovered to link environmental hypoxia, hypoxemia, and intramuscular molecular targets. Altogether, the molecular mechanisms tested here in skeletal muscle cannot explain the increase in glucose AUC during OGTT after exercise in hypoxia.

Since the severity of hypoxia (F_{IO_2}), the timing of blood sampling as well as the duration of exercise used in our experiment was the same as those used in the studies of Mackenzie et al. (7, 8), we wondered whether the intensity of the exercise or the type of population might be the cause of the divergent results. Prediabetic subjects are an intermediate category between healthy subjects and subjects with T2D. In subjects with T2D, Mackenzie et al. (7, 8) showed that an acute hypoxic exercise was more effective than normoxic exercise at improving glucose tolerance. Similar beneficial effects were found after a whole exercise training of 4 wk (3 sessions/wk) in healthy subjects, where hypoxic, but not normoxic, aerobic exercise training decreased plasma insulin levels (34) as well as in obese adolescents trained for 6 wk, three times a week (35). In the latter study, plasma insulin and blood glucose levels were decreased in the hypoxic group but remained unchanged in the normoxic group. On the other hand, no additional benefit of hypoxia versus normoxia on glucose tolerance was observed in elderly people after 8 wk of training (36) and in men with metabolic syndrome after 6 wk of training (37). Of note, none of the aforementioned studies included a proper control group, i.e., a healthy young group exercising in normoxia, which we did.

Beyond the population, it is also important to consider the intensity of the exercise used here. We chose to work with an equal relative intensity between normoxia and hypoxia as exercise performed at the same absolute intensity is perceived to be more difficult in hypoxia than in normoxia (38,

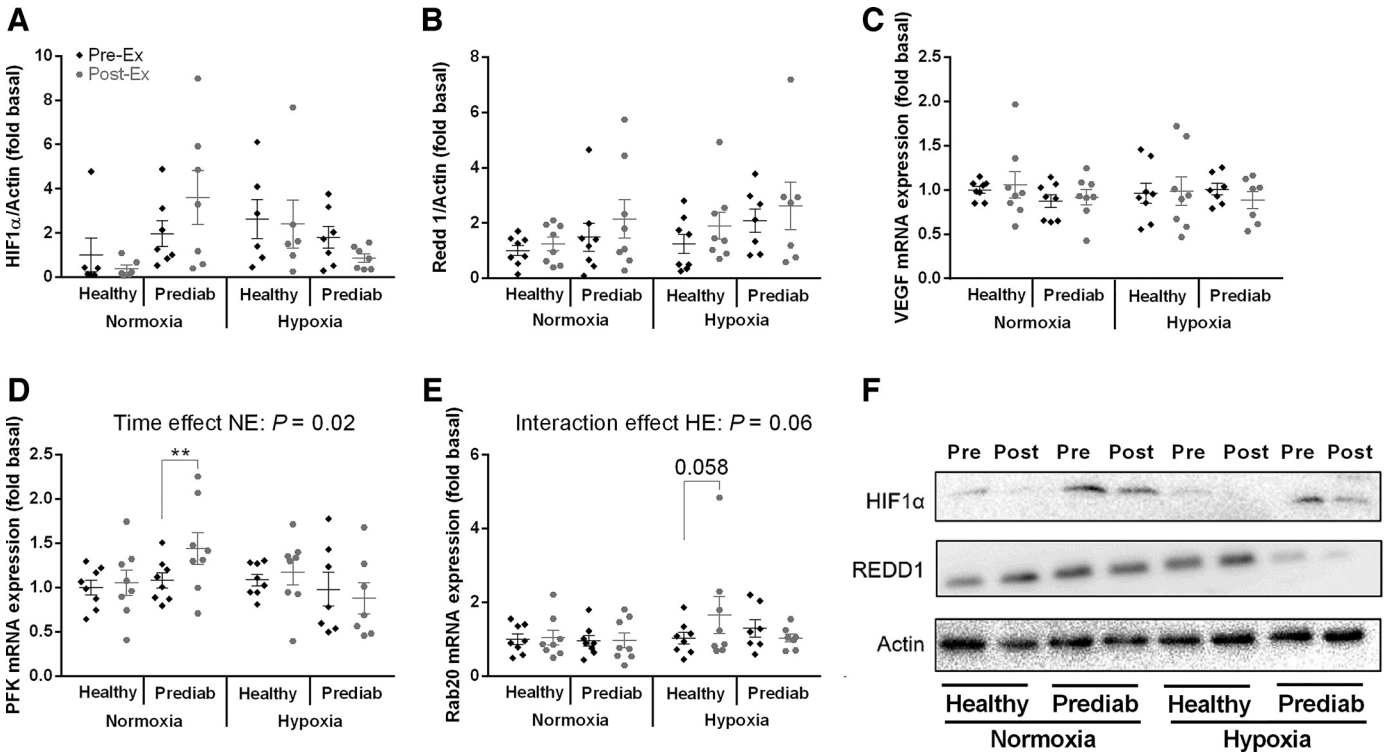

Figure 6. Regulation of the hypoxia-inducible factor 1 alpha (HIF1α) pathway by hypoxia and exercise. Healthy normoxia (H NE, $n = 8$), prediabetic normoxia (P NE, $n = 8$), healthy hypoxia (H HE, $n = 8$), and prediabetic hypoxia (P HE, $n = 7$) except when specified. HIF1α protein expression (H NE; n: −2; P NE; n: −1 and H HE n: −2) (*A*), Redd1 protein expression (*B*), vascular endothelial growth factor (VEGF) mRNA expression (*C*), PFK mRNA expression (*D*), and Rab20 mRNA expression (*E*) in H NE, P NE, H HE, and P HE pre-exercise and postexercise. *F*: representative Western blots. Values are presented as means ± SE. $^{**}P < 0.01$ vs. pre-exercise. Pre: pre-exercise; Post: postexercise. "Fold basal" means fold changes compared to the pre-exercise value from the healthy group in normoxia.

39). Knowing that obese and sedentary people have a higher risk to develop cardiovascular issues and orthopedic injuries while exercising (40, 41), getting similar health benefits with a lower exercise intensity is certainly an added value. The intensity was set to a HR corresponding to 55% of $\dot{V}O_{2max}$ measured in normoxia. Thus, the absolute intensity was constantly adjusted throughout the exercise to maintain the target HR corresponding to the same relative intensity. By measuring the rate of perceived exertion using the Borg scale, we confirmed that there was no difference in the perception of exercise intensity between hypoxia and normoxia. We chose to work at 55% of $\dot{V}O_{2max}$ based on a previous study reporting an improvement in glucose tolerance as well as insulin sensitivity following an acute exercise in hypoxia in T2D (7). In the latter study, the absolute workload was the same in both normoxia and hypoxia. Consequently, the subjects in hypoxia were exercised at a higher relative intensity than those in normoxia. The authors acknowledged this limitation to their protocol at the end of the discussion as the improvement in insulin sensitivity observed in that study might have been due to a higher relative intensity of exercise. Nevertheless, other studies using equal relative exercise intensity between hypoxia and normoxia showed opposite results regarding the effect of hypoxia on glucose tolerance (34, 37). In the context of hypoxic exercise and glucose tolerance, both plasma catecholamines and muscle lactate levels, which are closely associated with relative exercise intensity (16), can be informative. Based on the aforementioned study, similar lactate levels were expected in our four conditions as they exercised at the same relative intensity. Although the pattern of regulation was equal between normoxic and hypoxic exercise in the prediabetic group, higher lactate levels were measured in the hypoxic compared with the normoxic healthy group. The latter indicates that energy provision during exercise relies on a greater proportion of anaerobic metabolism in hypoxia than in normoxia in healthy, which has been shown before (42), but not in prediabetic subjects. Of note, based on our results, intramuscular glycogen did not seem to be the major substrate to fuel the enhanced anaerobic metabolism in the healthy group in hypoxia as the levels were not decreased after exercise. Anyway, beyond this discussion on absolute versus relative intensity, we cannot exclude that the exercise intensity chosen here was simply not sufficient to induce beneficial effects on glucose tolerance in healthy and prediabetic subjects, which could explain the divergent results observed in this study compared with the data reported by Makenzie et al. (7, 8).

In addition, it is important to consider the effects of hypoxia on other insulin-sensitive peripheral tissues such as the adipose tissue or the liver as they may play a role in exercise-induced regulation of glucose metabolism as well. Exercise-induced lipolysis was indirectly assessed by measuring plasma triglycerides levels before and after exercise. Only in the healthy group exercising in normoxia, an increase in plasma triglycerides levels was observed, suggesting that

exercising in hypoxia had no effect on lipolysis. Moreover, the release of hyperglycemic hormones such as catecholamines and cortisol has been shown to occur after physical exercise (43) and following exposure to hypoxia resulting in a transient decrease in insulin sensitivity (44, 45). An increase in the levels of those hormones could have therefore contributed to the transient increase in glycemia observed in our two hypoxic groups. Although catecholamines were not determined here, the cortisol levels during exercise increased only in the prediabetic hypoxic group and tended to be higher than in the healthy hypoxic group. The higher cortisol levels in the prediabetic hypoxic group could have contributed to the reduced glucose tolerance observed postexercise in that group but not in the healthy hypoxic group. Finally, we based our experimental protocol on two previous studies reporting a positive effect of a single exercise on whole body glucose tolerance in diabetic patients to understand the underlying molecular mechanisms in skeletal muscle. Nevertheless, other studies found oral glucose tolerance to be negatively regulated immediately postexercise (46), probably due to a quicker entry of glucose into the systemic circulation from the gut or an accelerated release from the liver. It is possible that hypoxia exacerbated this phenomenon in the present study, though we did not test it as we concentrated on skeletal muscle.

One limitation of our study is that our two groups of subjects (healthy vs. prediabetic) are not age matched. However, it represents the global reality that prediabetes affects older more than young people and still allows reliable pre-post exercise comparisons. Despite some previous studies reporting a positive effect of a single exercise on whole body glucose tolerance in diabetic patients (7, 27), it is possible that in prediabetic volunteers, repeated sessions are needed to make the same observation. However, we could have expected to, at least, detect changes in the intramuscular mechanisms known to regulate glucose homeostasis under normoxic and hypoxic conditions as previously reported after exercise in rodents (47).

In conclusion, our results show that an acute bout of exercise in hypoxia reduces glucose tolerance in healthy and prediabetic subjects. At a molecular level, some adaptations regulating glucose transport in muscle were found in all groups without associations with glucose tolerance after exercise. The results suggest that hypoxia negatively affects glucose tolerance postexercise through unidentified mechanisms.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

E.D.G., F.A.B., J-P.T., and L.D. conceived and designed the study. E.D.G., F.A.B., E.B., G.W., and H.N. conducted the experiment. E.D.G. and L.S. performed the laboratory analysis and analyzed and interpreted the data. E.D.G., L.S., and L.D. wrote the manuscript. E.D.G., F.A.B., E.B., G.W., J-P.T., H.N., L.S., and L.D. commented on and approved the final version of the manuscript.

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