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Increased oxidative stress and anaerobic energy release, but blunted Thr^{172}-AMPKα phosphorylation, in response to sprint exercise in severe acute hypoxia in humans

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Morales-Alamo D, Ponce-González JG, Guadalupe-Grau A, Rodríguez-García L, Santana A, Cusso MR, Guerrero M, Guerra B, Dorado C, Calbet JA. Increased oxidative stress and anaerobic energy release, but blunted Thr^{172}-AMPKα phosphorylation, in response to sprint exercise in severe acute hypoxia in humans. *J Appl Physiol* 113: 917–928, 2012. First published August 2, 2012; doi:10.1152/japplphysiol.00415.2012.—AMP-activated protein kinase (AMPK) is a major mediator of the exercise response and a molecular target to improve insulin sensitivity. To determine if the anaerobic component of the exercise response, which is exaggerated when sprint is performed in severe acute hypoxia, influences sprint exercise-elicited Thr^{172}-AMPKα phosphorylation, 10 volunteers performed a single 30-s sprint (Wingate test) in normoxia and in severe acute hypoxia (inspired P_{O_2}: 75 mmHg). Vastus lateralis muscle biopsies were obtained before and immediately after 30 and 120 min postsprint. Mean power output and O_2 consumption were 6% and 37%, respectively, lower in hypoxia than in normoxia. O_2 deficit and muscle lactate accumulation were greater in hypoxia than in normoxia. Carbonylated skeletal muscle and plasma proteins were increased after the sprint in hypoxia. Thr^{172}-AMPKα phosphorylation was increased by 3.1-fold 30 min after the sprint in normoxia. This effect was prevented by hypoxia. The NAD^+-to-$NADH.H^+$ ratio was reduced (by 24-fold) after the sprints, with a greater reduction in hypoxia than in normoxia ($P < 0.05$), concomitant with 53% lower sirtuin 1 (SIRT1) protein levels after the sprint in hypoxia ($P < 0.05$). This could have led to lower liver kinase B1 (LKB1) activation by SIRT1 and, hence, blunted Thr^{172}-AMPKα phosphorylation. Ser^{485}-AMPKα$_1$/Ser^{491}-AMPKα$_2$ phosphorylation, a known negative regulating mechanism of Thr^{172}-AMPKα phosphorylation, was increased by 60% immediately after the sprint in hypoxia, coincident with increased Thr^{308}-Akt phosphorylation. Collectively, our results indicate that the signaling response to sprint exercise in human skeletal muscle is altered in severe acute hypoxia, which abrogated Thr^{172}-AMPKα phosphorylation, likely due to lower LKB1 activation by SIRT1.

sprint; AMP-activated protein kinase; signaling; muscle; metabolism

AMP-ACTIVATED PROTEIN KINASE (AMPK) is a metabolic energy sensor activated by Thr^{172} phosphorylation of the α-subunit, mainly in response to an increase of the AMP-to-ATP ratio (25). AMPK is involved in the regulation of feeding and body weight (42), lipid metabolism (26), glucose homeostasis (62), and mitochondrial biogenesis (69) and is a key player in the adaptation to exercise training (48). AMPKα phosphorylation of Thr^{172} increases markedly in response to sprint exercise (22), most likely due to the elevation of the AMP-to-ATP ratio (11). Whether free radicals may also play a role in contraction-mediated Thr^{172}-AMPKα phosphorylation in skeletal muscle remains controversial (41, 52). In cell cultures, hypoxia and anoxia increase Thr^{172}-AMPKα phosphorylation more through the release of free radicals than through an increase in the AMP-to-ATP ratio (15). In contrast, chronic hypoxia (5 and 12 days of exposure to 5,500 m above sea level) did not increase skeletal muscle Thr^{172}-AMPKα phosphorylation in rats (10).

The influence of the inspired O_2 fraction ($F_{I_{O_2}}$) on exercise-induced Thr^{172}-AMPKα phosphorylation has been scarcely studied in humans (63). After 30 min of exercise at 73% of peak O_2 consumption ($\dot{V}_{O_2\,peak}$), Thr^{172}-AMPKα phosphorylation was greater in normoxia than in hypoxia. This apparent unexpected finding was explained by the higher absolute intensity during the exercise in normoxia, which was associated with a higher calculated free ADP muscular concentration, without significant effects on the AMP-to-ATP ratio attributable to hypoxia (63). Thus, exercise intensity is an important factor determining the Thr^{172}-AMPKα phosphorylation response to exercise. The intensity factor may be controlled by asking the subjects to perform an all-out exercise, such as the Wingate test. During Wingate tests in severe acute hypoxia, peak and mean power output are barely affected; however, anaerobic energy release is increased (8, 37).

In this background, we hypothesized that the combination of high-intensity exercise (sprint exercise) and hypoxia would result in additive or synergistic effects on Thr^{172}-AMPKα phosphorylation, due to the convergence of a high energy turnover with a greater glycolytic rate (38) and, potentially, greater release of free radicals (9) in hypoxia than in normoxia.

Thr^{172}-AMPKα phosphorylation by a rise of the AMP-to-ATP ratio is liver kinase B1 (LKB1) dependent (25). LKB1 is also activated by the deacetylase sirtuin 1 (SIRT1) an NAD^+-dependent deacetylase that is activated by the increase of $NAD^+/NADH.H^+$ (58). Although most human studies have reported reduction of $NAD^+/NADH.H^+$, little is known about the effect of sprint exercise on $NAD^+/NADH.H^+$ (64). Since $NAD^+/NADH.H^+$ is expected to be reduced at high glycolytic rates, and since greater glycolytic rates are attained during sprint exercise in hypoxia than in normoxia (38), we hypothesized that cytosolic (and nuclear) $NAD^+/NADH.H^+$ would be lower after sprint exercise in hypoxia. If this mechanism

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Table 1. *Physical characteristics and ergoespirometric variables during sprint exercise in normoxia and severe acute hypoxia*

	Normoxia	Hypoxia
Age, yr	25.1 ± 4.5	
Height, cm	176.7 ± 5.3	
Weight, kg	80.2 ± 9.9	
Body fat, %	18.2 ± 6.3	
Two-legs lean mass, kg	19.73 ± 2.37	
Maximal heart rate, beats/min	188.1 ± 5.6	171.8 ± 9.2*
Peak $\dot{V}O_2$, l/min	4.018 ± 0.249	2.634 ± 0.248*
W_{max}, W	332.4 ± 110.2	288.5 ± 32.0*
W_{peak}, W	997.4 ± 121.7	950.5 ± 125.3
W_{peak}/LLM, W/kg	50.9 ± 6.3	48.2 ± 3.6
W_{mean}, W	573.8 ± 56.2	544.8 ± 68.0*
W_{mean}/LLM, W/kg	29.4 ± 3.0	27.5 ± 2.0*
O_2 demand, l/min	8.437 ± 0.767	7.908 ± 0.763*
Accumulated $\dot{V}O_2$, liters	1.345 ± 0.549	0.853 ± 0.118*
O_2 deficit, liters	2.874 ± 0.599	3.101 ± 0.391
O_2 deficit/W_{mean}	5.02 ± 1.06	5.74 ± 0.44*
Wingate hemoglobin saturation	96.8 ± 3.7	81.0 ± 6.5*
Wingate end-tidal P_{O_2}	114.0 ± 6.5	48.7 ± 3.1*

Values are means ± SD; $n = 10$ subjects. $\dot{V}O_2$, O_2 consumption; W_{max}, maximal intensity during the incremental exercise test to exhaustion; W_{peak}, peak power output during the Wingate test; LLM, lean mass of the lower extremities; W_{mean}, mean power output during the Wingate test; accumulated $\dot{V}O_2$, $\dot{V}O_2$ during the 30-s Wingate test. *$P < 0.05$ compared with normoxia.

prevails, this could result in lower or no phosphorylation of AMPKα after sprint exercise in hypoxia, due to lower LKB1 activity.

Among others, alternative kinases upstream to AMPK are Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and transforming growth factor-β-activated kinase 1 (TAK-1) (14, 68). Upon activation by phosphorylation, CaMKII can phosphorylate AMPK during exercise (14). Likewise, TAK-1, which is activated by phosphorylation, has been shown to phosphorylate AMPKα in vitro (43). In cardiac myocytes, TAK-1 is likely acts upstream of LKB1 (68).

Insulin antagonizes anoxia or ischemia-induced AMPKα phosphorylation through Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation (31), which may be produced by Akt (34, 55). In agreement with these findings in cardiac myocytes (31, 34, 55), we observed that sprint exercise-elicited AMPKα phosphorylation in skeletal muscle was prevented by the ingestion of 75 g of glucose 1 h before the start of the sprint (22). Although it is well established that circulating levels of glucose and insulin increase immediately after sprint exercise (18, 22), it remains unknown if this response is modified when the sprint is performed in severe acute hypoxia. Greater or prolonged exercise-induced elevation of both plasma glucose and insulin could blunt the Thr^{172}-AMPKα phosphorylation normally observed 30 min after sprint exercise (23) through Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, while a reduced insulin response could be associated with even greater Thr^{172}-AMPKα phosphorylation.

Therefore, the main aim of this study was to determine if hypoxia increases skeletal muscle Thr^{172}-AMPKα phosphorylation in response to sprint exercise, by examining potential endocrine, metabolic, and signaling mechanisms. More specifically, by determining muscle lactate (Lac) responses and the accumulated O_2 deficit incurred during sprints (8), combined with the assessment of muscle and plasma carbonylated proteins (49), we expected to determine whether potential changes in sprint-exercise elicited Thr^{172}-AMPKα phosphorylation are associated with oxidative stress and the anaerobic component of the exercise response, which should be exaggerated when the sprint is performed in severe acute hypoxia (8). By assessing the protein levels of SIRT1, we determined if sprint exercise changes in SIRT1 protein levels could, by modifying LKB1 activity, regulate Thr^{172}-AMPKα phosphorylation. Moreover, by determining glucose and plasma insulin responses, combined with the assessment of Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 and Ser^{473}/Thr^{308}-Akt phosphorylation, we expected to determine if the degree of Thr^{172}-AMPKα phosphorylation is modulated by hypoxia through a Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation-dependent mechanism.

MATERIALS AND METHODS

Materials. The complete protease inhibitor cocktail and the PhosSTOP phosphatase inhibitor were obtained from Roche Diagnostics (Mannheim, Germany). All primary antibodies used were from Cell Signaling Technology (Denvers, MA) except for polyclonal anti-phospho-AS160 [Thr^{642}, no. AT-7079; molecular mass: 160 kDa], which was obtained from MBL (Woburn, MA), anti-CaMKII antibody (no. sc-13082, molecular mass: 50 kDa), which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal mouse anti-α-tubulin antibody (no. T-5168-ML, molecular mass: 50 kDa), which was obtained from Biosigma (Sigma, St. Louis, MO). The corresponding catalog numbers of the antibodies from Cell Signaling were as follows: anti-phospho-AMPKα (Thr^{172}), no. 2531 (molecular mass: 62 kDa); anti-AMPKα, no. 2532 (molecular mass: 62 kDa); anti-phospho-AMPKα_1 (Ser^{485})/AMPKα_2 (Ser^{491}), no. 4185 (molecular mass:62 kDa); anti-phospho-AMPKα_1 (Ser^{485}), no. 4184 (molecular mass: 62 kDa); anti-AMPKα_1, no. 2795 (molecular mass: 62 kDa); anti-phospho-acetyl-CoA carboxylase (ACC; Ser^{221}), no. 3661 (molecular mass: 280 kDa); anti-ACC, no. 3662 (molecular mass: 280 kDa); anti-SIRT1, no. 2310 (molecular mass: 120 kDa); anti-phospho-Akt (Ser^{473}), no. 9271 (molecular mass: 60 kDa); anti-phospho-Akt

Table 2. *Glucose and insulin concentrations before and during the recovery period after the sprint exercise in normoxia and hypoxia*

		Time After Sprint Exercise			
	Resting Value	0 min	30 min	120 min	$F_{I_{O_2}}$ × Time Interaction
Glucose, mg/dl					
Normoxia	89.8 ± 6.3	97.8 ± 6.7*	96.9 ± 8.2*	87.01 ± 6.5	$P = 0.07$
Hypoxia	88.5 ± 4.3	100.6 ± 11.3*	101.8 ± 12.7*	87.02 ± 7.4	
Insulin, μIU/ml					
Normoxia	4.7 ± 2.1	7.6 ± 3.0*	10.8 ± 3.7*	4.4 ± 2.5	$P = 0.57$
Hypoxia	5.2 ± 3.2	5.9 ± 3.0	11.3 ± 5.8*	6.0 ± 4.0	

Values are means ± SD. 0 min corresponds to immediately after the Wingate test. $F_{I_{O_2}}$, fraction of inspired O_2. *$P < 0.05$ vs. resting values.

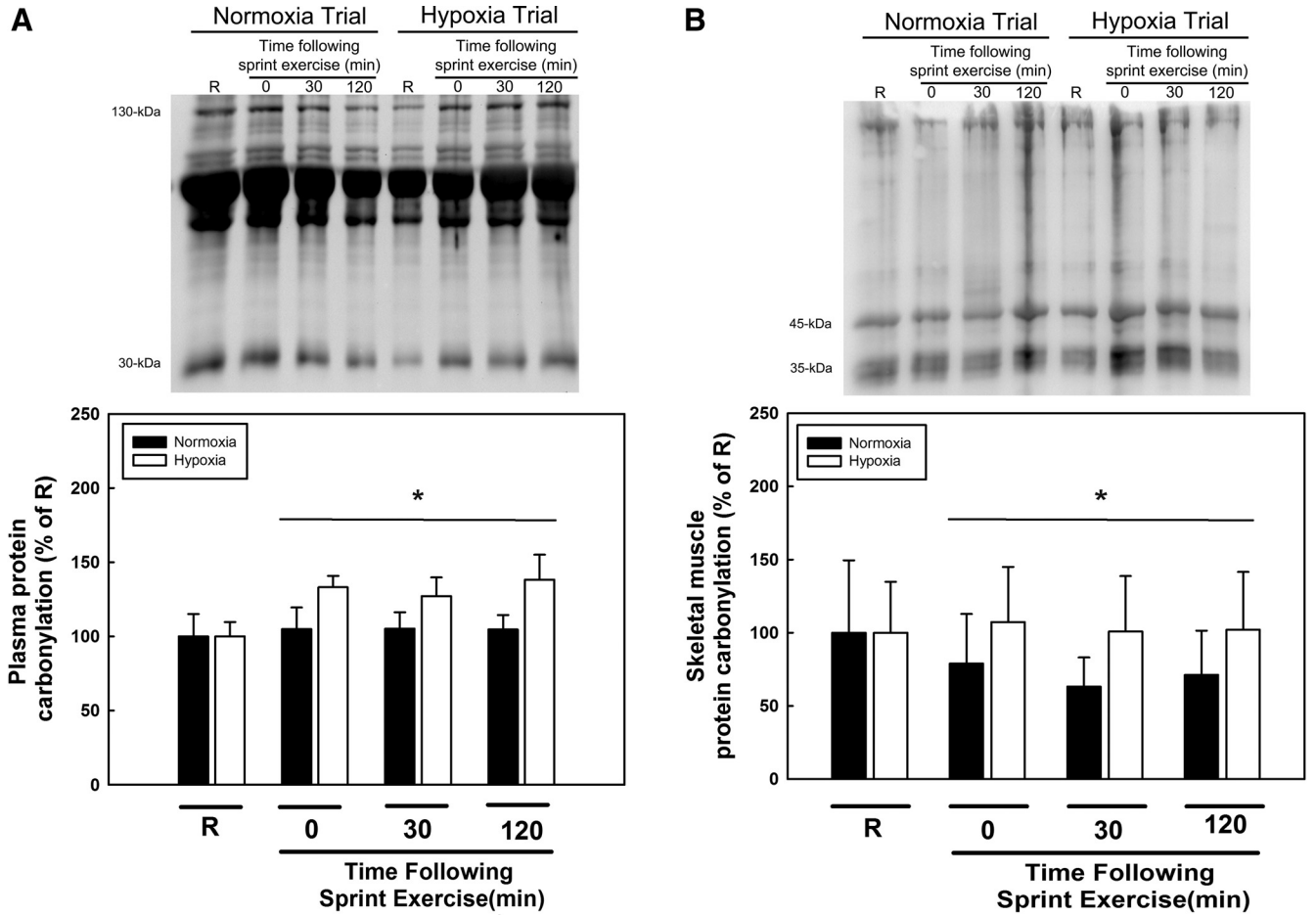


Fig. 1. Levels of carbonylated proteins in plasma (*A*) and skeletal muscle (*B*) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to those observed in the biopsies obtained immediately before the sprint exercise [resting (R) values], which were assigned a value of 100%. *A*: Western blot and densitometry analysis (130 and 30 kDa) showing carbonylated proteins in plasma extracts. *$P < 0.05$, normoxia vs. hypoxia. *B*: Western blot and densitometry analysis (45 and 35 kDa) showing carbonylated proteins in skeletal muscle extracts. Statistical analysis was performed with logarithmically transformed data. *$P < 0.05$, normoxia vs. hypoxia. $N = 10$ subjects in both experimental conditions.

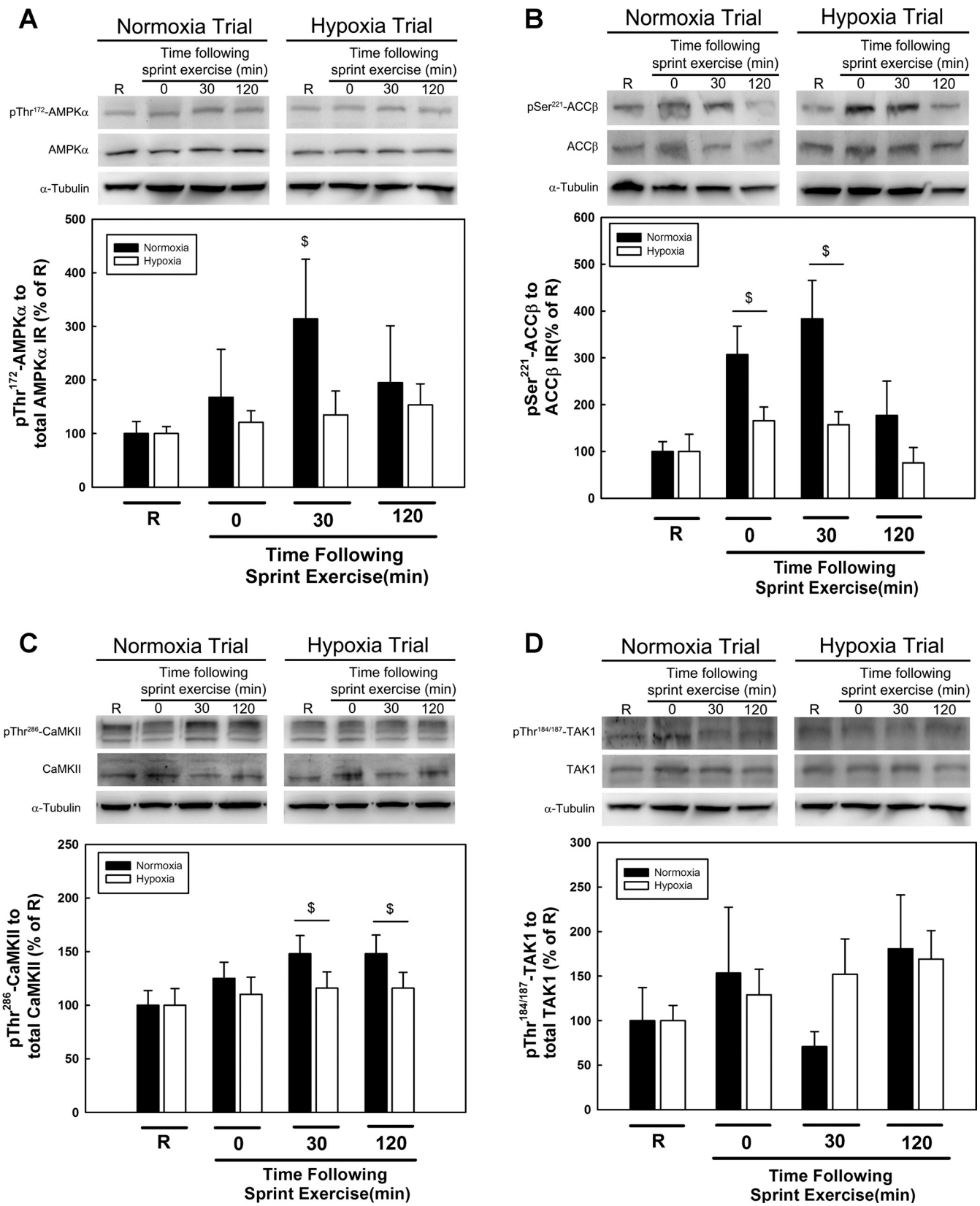
(Thr^{308}), no. 9275 (molecular mass: 60 kDa); anti-Akt, no. 9272 (molecular mass: 60 kDa); anti-phospho-TAK-1 (Thr$^{184/187}$), no. 4531 (molecular mass: 82 kDa); anti-TAK-1, no. 4505 (molecular mass: 82 kDa); anti-phospho-CaMKII (Thr^{286}), no. 3361 (molecular mass: 50 kDa); and AS160, no. 2447 (molecular mass: 160 kDa). The secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit (no. 111-035-144) and HRP-conjugated donkey anti-mouse (no. 715-035-150) antibodies were from Jackson ImmunoResearch (West Grove, PA). The Immun-Blot polyvinylidene difluoride membranes, Inmmun-Star WesternC, ChemiDoc XRS System, and image-analysis software (Quantity One) were obtained from Bio-Rad Laboratories (Hertfordshire, UK).

Subjects. Ten healthy male physical education students (age: 25 ± 4 yr, height: 176.7 ± 5.3 cm, body weight: 80.2 ± 9.9 kg, body fat: 18.2 ± 6.3%) agreed to participate in this investigation (Table 1). Before volunteering, subjects received full oral and written informa-

Table 3. *Muscle metabolites before and immediately after a 30-s sprint in normoxia and severe acute hypoxia*

	Normoxia		Hypoxia	
	Resting	Postsprint	Resting	Postsprint
ATP, mmol/kg	4.99 ± 1.80	2.46 ± 0.77*	4.78 ± 0.96	3.10 ± 1.56*
AMP/ATP, mmol/mol	7.5 ± 7.1	155.0 ± 302.0*	5.4 ± 2.2	174.3 ± 242.9*
Phosphocreatine, mmol/kg	16.10 ± 2.66	5.34 ± 2.43*	16.56 ± 1.45	5.83 ± 3.18*
Creatine, mmol/kg	12.31 ± 2.66	23.08 ± 2.43*	11.85 ± 1.45	22.59 ± 3.18*
Pyruvate, mmol/kg‡	0.09 ± 0.05	0.25 ± 0.12*	0.14 ± 0.05	0.23 ± 0.07*
Lactate, mmol/kg‡	2.5 ± 1.9	36.2 ± 13.2*	2.2 ± 1.2	53.2 ± 20.3*†
Lactate/pyruvate‡	48.3 ± 69.9	179.0 ± 129.5*	17.1 ± 13.5	247.2 ± 118.9*†
$NAD^+/NADH.H^+$ concentration, $\times 10^7$	470 ± 313	64 ± 24*	860 ± 640	45 ± 21*†

Values are mean ± SD. The AMP-to-ATP ratio was calculated from the creatine kinase and adenilate kinase apparent equilibrium constants for free AMP and ADP. Statistical analyses for lactate/pyruvate and $NAD^+/NADH.H^+$ were performed with normalized values, taking the resting values as 100%. *$P < 0.05$, postsprint vs. resting values (same condition); †$P < 0.05$, postsprint in normoxia vs. hypoxia; ‡$P < 0.05$ for FI_{O_2} × time interaction.



tion about the experiments and possible risks associated with participation. Written consent was obtained from each subject. This study was performed in accordance with the Helsinki Declaration and was approved by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2010-01).

General procedures. The subjects' body composition was determined by dual X-ray absorptiometry (Hologic QDR-1500, Hologic, software version 7.10, Waltham, MA) as previously described (2, 47). Subjects reported to the laboratory to complete different tests on separate days. First, their $\dot{V}O_{2\ peak}$, maximal heart rate, and maximal power output in normoxia (FI_{O_2}: 0.21; barometric pressure: 735–745 mmHg) and hypoxia (FI_{O_2}: 0.104; barometric pressure: 735–745 mmHg) were assessed with ramp incremental exercise tests to exhaustion (50 W/min) on an Excalibur Sport 925900 (Lode, Groningen, The Netherlands). One week before the exercise, subjects were familiarized with the experimental protocol (a single 30-s isokinetic Wingate test at 100 rpm). On separate days and in random order, they performed one 30-s isokinetic Wingate test at 100 rpm in normoxia and another test in hypoxia. On each trial day, subjects reported to the laboratory at 8.00 AM, after an overnight fast, and an antecubital vein was catheterized. After a 10-min resting supine period, a 20-ml blood sample was withdrawn and used to measure serum glucose and insulin. Right after, a muscle biopsy was obtained from the middle portion of the vastus lateralis muscle using Bergstrom's technique with suction, as previously described (46). After the preexercise muscle biopsy, subjects sat on the cycle ergometer for 4 min. During this period, they breathed either room air (normoxia) or a hypoxic gas mixture from a Douglas bag containing 10.4% O_2 in N_2 (hypoxia). The Douglas bag was replenished with gas from a cylinder specially prepared for the experiment (Carburos metálicos, gas mixture 206030, Las Palmas de Gran Canaria).

During both sprints, subjects attempted to pedal as fast and hard as possible (i.e., all out) from the start to the end of the exercise. Since the cycle ergometer (Excalibur Sport, Lode) was set to isokinetic mode, the braking force was a servo controlled by the Ergometer applying the braking force needed to maintain a fixed pedaling rate of 100 rpm. The latter was possible because as subjects fatigued, the ergometer automatically decreased the braking force.

Peak power output was calculated as the highest work output performed during 1-s interval, and mean power output was calculated from the average work performed during the 30 s. Warm up was not allowed before the start of the Wingate test, and stop-start Wingate tests were performed by both groups, meaning that the Wingate test was not preceded by a phase of unloaded pedaling (7, 8, 23).

Within 10 s from the end of the sprint, a second muscle biopsy was taken, and another blood sample was then obtained. During the following 2 h, subjects fasted but had free access to water and sat quietly in the laboratory. During the recovery period, two additional muscle biopsies and blood samples were obtained at 30 and 120 min. For the last two biopsies, a new incision was performed in the contralateral leg. To avoid injury-triggered activation of signaling cascades, the muscle biopsies were obtained at least 3 cm apart, using the procedures described by Guerra et al. (21). Muscle specimens were cleaned to remove any visible blood, fat, or connective tissue. Muscle tissue was the immediately frozen in liquid nitrogen and stored at −80°C for later analysis. The time needed to obtain and freeze the muscle biopsies was below 30 s.

Cycling economy tests. Cycling economy was determined on two different days using 8–11 submaximal workloads at intensities between 50% and 90% of $\dot{V}O_{2\ peak}$ at 100 rpm. Exercise intensities and pedaling rates were administered in random order, separated by rest periods of 6 min. To reduce thermal stress and minimize water losses due to sweating, subjects were fan cooled and ingested fresh water during the resting periods ad libitum. The duration of each submaximal bout was set at 10 min. The mean $\dot{V}O_2$ registered during the last 2 min was taken as representative of each submaximal exercise intensity. To relate $\dot{V}O_2$ to power, linear regression equations were calculated by least-square linear fit.

O_2 uptake and hemoglobin O_2 saturation. O_2 uptake was measured with a metabolic cart (Vmax N29, Sensormedics) calibrated immediately before each test according to the manufacturer's instructions. Respiratory variables were analyzed breath by breath and averaged every 5 s during the Wingate test and every 20 s during the incremental and cycling economy tests. The highest 20-s averaged $\dot{V}O_2$ recorded in normoxia was taken as $\dot{V}O_{2\ peak}$. The same criterion was applied to determine $\dot{V}O_{2\ peak}$ in severe acute hypoxia. The hemoglobin O_2 saturation was determined with a finger pulse oxymeter (Excalibur Sport 925900, Lode).

Muscle metabolites. From each muscle biopsy, 30 mg of wet tissue were treated with 0.5 M $HClO_4$ and centrifuged at 15,000 *g* at 4°C for 15 min. The supernatant was neutralized with $KHCO_3$ (2.1 M), and ATP, phosphocreatine (PCr), creatine (Cr), pyruvate (Pyr), and Lac were enzymatically determined in neutralized extracts by fluorometric analysis (20, 36). Muscle metabolite concentrations were adjusted to the individual mean total Cr (PCr + Cr) because this mean should remain constant during exercise (27). The adjustment to total Cr content accounts for the variability in solid nonmuscle constituents, which may be present in the biopsies (45). The glycolytic rate was calculated as follows: glycolytic rate = $0.5 \times (\Delta Lac + \Delta Pyr)$ (57). The free AMP-to-ATP molar ratio was estimated after we calculated the ADP concentration using the creatine kinase equilibrium apparent constant for resting conditions and exhaustion after a Wingate test (45), as described by Sahlin et al. (51). Subsequently, the AMP concentration was calculated using the adenilate kinase apparent equilibrium constant for the same conditions (45). The $NAD^+/NADH.H^+$ concentration was calculated using the equilibrium constant for Lac dehydrogenase (6, 66).

Total protein extraction, electrophoresis, and Western blot analysis. Muscle protein extracts were prepared as previously described (24), and total protein content was quantified using the bicinchoninic acid assay (54). Briefly, proteins were solubilized in sample buffer containing 0.0625 M Tris·HCl (pH 6.8), 2.3% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. Equal amounts (50 μg) of each sample were electrophoresed with 7.5–10% SDS-PAGE using the system of Laemmli (35) and transferred to Hybond-P membranes. To determine Thr^{172}-AMPKα, Ser^{485}-AMPKα$_1$/Ser^{491}-AMPKα$_2$, Ser^{485}-AMPKα$_1$, Ser^{221}-ACCβ, Ser^{473}-Akt, Thr^{308}-Akt, $Thr^{184/187}$-TAK-1, Thr^{286}-CaMKII, and Thr^{642}-AS160 phosphorylation levels, antibodies directed against the phosphorylated and total forms of these

Fig. 2. Levels of Thr^{172}-AMP-activated protein kinase (AMPKα) (*A*), Ser^{221}-acetyl-CoA carboxylase (ACCβ) (*B*), Thr^{286}-Ca^{2+}/calmodulin-dependent kinase II (CaMKII; *C*), and $Thr^{184/187}$-transforming growth factor-β-activated kinase-1 (TAK-1) (*D*) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to R values, which were assigned a value of 100%. *A*, *top*: representative Western blot with antibodies against AMPKα, phosphorylated (p-)AMPKα, and α-tubulin. *Bottom*, AMPKα phosphorylation densitometric values relative to total AMPKα. $\$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. *B*, *top*: representative Western blot with antibodies against ACCβ, p-ACCβ, and α-tubulin. *Bottom*, ACCβ phosphorylation values relative to total ACCβ. $\$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. *C*, *top*: representative Western blot with antibodies against CaMKII, p-CaMKII, and α-tubulin. *Bottom*, CaMKII phosphorylation values relative to total CaMKII. $\$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. *D*, *top*: representative Western blot with antibodies against TAK-1, p-TAK-1, and α-tubulin. *Bottom*, TAK-1 phosphorylation values relative to total TAK-1. $\$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. $N = 10$ subjects in both experimental conditions.

kinases were diluted in 5% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T; BSA-blocking buffer). SIRT1 was assessed in membranes incubated with a SIRT1 antibody (diluted in BSA-blocking buffer). To control for differences in loading and transfer efficiency across membranes, membranes were incubated with a monoclonal mouse anti-α-tubulin antibody diluted in TBS-T with 5% blotting grade blocker nonfat dry milk (blotto blocking buffer). No significant changes were observed in α-tubulin protein levels during the experiments (data not shown). Antibody-specific labeling was revealed by an incubation with HRP-conjugated goat anti-rabbit antibody (1:20,000) or HRP-conjugated donkey anti-mouse antibody (1:10,000), with both diluted in 5% blotto blocking buffer, and visualized with the Immun-Star WesternC kit (Bio-Rad Laboratories). Densitometry analyses were carried out immediately before saturation of the immunosignals. Specific bands were visualized with the Immun-Star WesternC kit using the ChemiDoc XRS system (Bio-Rad Laboratories) and analyzed with Quantity One (Bio-Rad Laboratories). Muscle signaling data are represented as percentages of the immunostaining values obtained for the phosphorylated form of each kinase relative the respective total form.

Samples from each subject were run on the same gel (hypoxic and normoxic conditions). In addition, in all gels, a human muscle sample obtained from a healthy young man was used as an internal control, to reduce intergel variability.

Insulin measurements. Serum insulin was measured by an ECL immunoassay intended for use on Modular Analytics analyzer E170 using insulin kit reagents (Roche/Hitachi, Indianapolis, IN). Insulin sensitivity was 0.20 μIU/ml.

Serum glucose. Serum glucose was measured by the hexokinase method using Gluco-quant reagents (11876899216, Roche/Hitachi) with a sensitivity of 2 mg/dl.

Protein carbonylation. Protein carbonylation in skeletal muscle and plasma was assessed by immunoblot detection of protein carbonyl groups using the "OxyBlot" protein oxidation kit (Intergen, Purchase, NY) as previously described (49). Protein carbonylation data are represented as percentages of immunostaining values.

Statistics. Variables were checked for normal distribution using the Kolmogorov-Smirnov test with the Lilliefors correction. When necessary, the analysis was carried out on logarithmically transformed data. For between-trial comparisons, individual responses were normalized to the level of phosphorylation observed just before the start of the Wingate test. Repeated-measures ANOVA over time and oxygenation condition with two levels (normoxia vs. hypoxia) was used to compare the responses with the values before the start of the Wingate test. When there was a significant condition effect or condition × time interaction ($F_{I_{O_2}}$ × time), pairwise comparisons at specific time points were adjusted for multiple comparisons with the Holm-Bonferroni method. The relationship between variables was determined using linear regression analysis. Areas under the curve (AUCs) were determined using the trapezoidal rule and compared between conditions with paired Student *t*-tests. Values are reported as means ± SE (unless otherwise stated). *P* values of ≤0.05 were considered significant. Statistical analysis was performed using SPSS (version 15.0) for Windows (SPSS, Chicago, IL).

RESULTS

Performance and ergospirometric variables. Ergoespirometric and performance-related variables are shown in Table 1. Compared with normoxia, mean power output and accumulated $\dot{V}O_2$ were reduced in hypoxia by 6% and 37%, respectively ($P < 0.05$).

Serum glucose, insulin, and carbonylated plasma proteins. Compared with resting values, the serum glucose concentration was increased by 11% immediately after the sprints and remained at this level 30 min later ($P < 0.05$). Compared with resting values, the serum insulin concentration was elevated by 62% immediately after the sprint in normoxia ($P < 0.05$) and was further increased to 2.2-fold (the preexercise concentration) 30 min postsprint, with a similar response in both conditions ($P < 0.05$). Serum glucose and insulin concentrations decreased to values similar to those observed before the sprint 120 min after the exercise (Table 2).

Compared with normoxia, carbonylated plasma protein AUCs were 26% greater after the sprints in hypoxia ($P < 0.05$; Fig. 1*A*).

Muscle metabolites. The changes observed in muscle metabolites are shown in Table 3. The AMP-to-ATP molar ratio was similarly increased (×24-fold) immediately after the sprints, regardless of $F_{I_{O_2}}$. The glycolytic rate was 50% greater in hypoxia than in normoxia ($P < 0.05$). Compared with resting values, immediately after the sprint, the muscle pyruvate concentration was increased by 2.7- and 1.7-fold in normoxia and hypoxia, respectively ($F_{I_{O_2}}$ × time interaction, $P < 0.05$). The corresponding muscle Lac concentration changes were 14- and 24-fold ($F_{I_{O_2}}$ × time interaction, $P < 0.05$). Consequently, the muscle Lac concentration after the sprint was 48% higher in hypoxia than in normoxia ($P < 0.05$). After the sprints, the Lac-to-Pyr ratio was increased by 4- and 14-fold in normoxia and hypoxia ($P < 0.05$, $F_{I_{O_2}}$ × time interaction, $P < 0.05$ by ANOVA). Consequently, immediately after the sprints, the NAD^+-to-$NADH.H^+$ ratio was reduced by 87% and 95% in normoxia and hypoxia, respectively (both $P < 0.05$). This reduction was more marked in hypoxia than in normoxia ($P < 0.05$).

Skeletal muscle carbonylated proteins. The AUC for carbonylated proteins in skeletal muscle was 50% greater after the sprint in hypoxia compared with in normoxia ($P < 0.05$; Fig. 1*B*).

Muscle signaling. Thr^{172}-AMPKα phosphorylation was increased by 3.1-fold 30 min after the Wingate test in normoxia ($P < 0.05$; Fig. 2*A*). Hypoxia prevented the exercise-induced AMPKα phosphorylation at 30 min ($P < 0.05$). However, ACCβ phosphorylation was increased by 2.4- and 2.7-fold immediately after and 30 min into the recovery period, respectively (both $P < 0.05$), with a similar response in both conditions (Fig. 2*B*).

Compared with resting values, Thr^{286}-CaMKII phosphorylation was increased by 32% at 30 and 120 min after the sprints (both $P < 0.05$; Fig. 2*C*), without significant differences between conditions. No statistically significant changes were observed in $Thr^{184/187}$-TAK-1 phosphorylation after both sprints (Fig. 2*D*).

Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation levels were increased by 60% immediately after the sprint performed in hypoxia ($P < 0.05$), whereas they remained unchanged after the normoxic sprint ($F_{I_{O_2}}$ × time interaction, $P < 0.05$ by ANOVA; Fig. 3*A*). No significant changes in Ser^{485}-AMPKα_1 phosphorylation were observed after both sprints (Fig. 3*B*). Compared with normoxia, the AUC for SIRT1 protein expression was 53% lower after the sprint in hypoxia ($P < 0.05$; Fig. 3*C*).

No significant between-condition differences were observed in Ser^{473}-Akt phosphorylation. Ser^{473}-Akt phosphorylation was increased by 2.2- and 1.14-fold at 30 and 120 min after the sprint, respectively (both $P < 0.05$; Fig. 4*A*). Thr^{308}-Akt phosphorylation was increased by 2.4-fold just after the exercise ($P < 0.05$). This response was more accentuated in

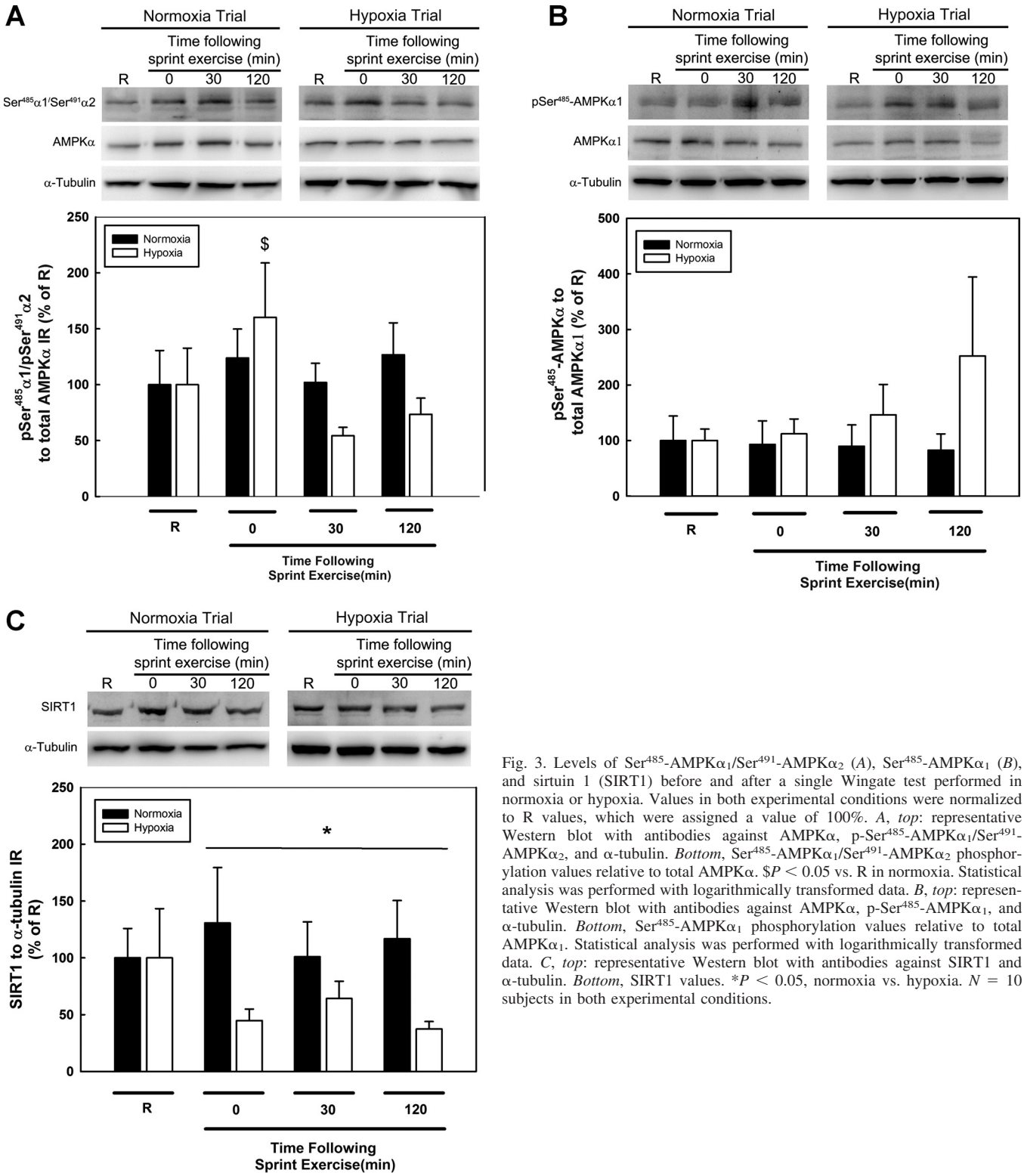


Fig. 3. Levels of Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 (*A*), Ser^{485}-AMPKα_1 (*B*), and sirtuin 1 (SIRT1) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to R values, which were assigned a value of 100%. *A*, *top*: representative Western blot with antibodies against AMPKα, p-Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2, and α-tubulin. *Bottom*, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation values relative to total AMPKα. $\$P < 0.05$ vs. R in normoxia. Statistical analysis was performed with logarithmically transformed data. *B*, *top*: representative Western blot with antibodies against AMPKα, p-Ser^{485}-AMPKα_1, and α-tubulin. *Bottom*, Ser^{485}-AMPKα_1 phosphorylation values relative to total AMPKα_1. Statistical analysis was performed with logarithmically transformed data. *C*, *top*: representative Western blot with antibodies against SIRT1 and α-tubulin. *Bottom*, SIRT1 values. $^*P < 0.05$, normoxia vs. hypoxia. $N = 10$ subjects in both experimental conditions.

hypoxia, as reflected by the Thr^{308}-Akt phosphorylation AUC, which was 87% higher after the sprint in hypoxia ($P < 0.05$; Fig. 4*B*).

Thr^{642}-AS160 phosphorylation was increased by 47% and 35% at 30 and 120 min after the sprints (both $P < 0.05$ compared with the value immediately postexercise), with comparable responses in both sprints (Fig. 4*C*).

DISCUSSION

This study examined the influence of $F_{I_{O_2}}$ on the regulation of skeletal muscle AMPKα phosphorylation in response to a 30-s sprint exercise in humans. In contrast to our hypothesis, hypoxia blunted the expected AMPKα phosphorylation 30 min after exercise, and this effect was preceded by Ser^{485}-

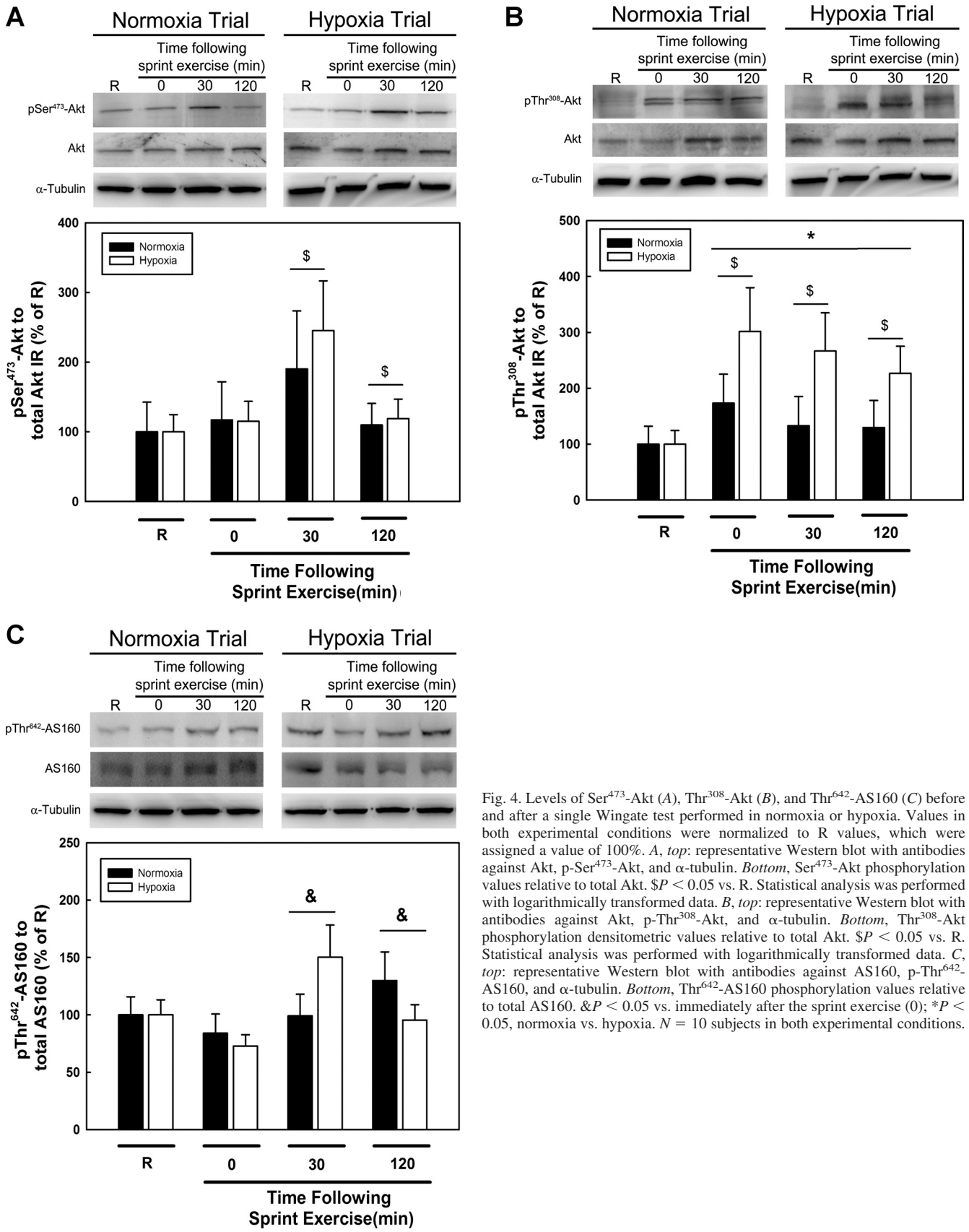


Fig. 4. Levels of Ser^{473}-Akt (*A*), Thr^{308}-Akt (*B*), and Thr^{642}-AS160 (*C*) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to R values, which were assigned a value of 100%. *A*, *top*: representative Western blot with antibodies against Akt, p-Ser^{473}-Akt, and α-tubulin. *Bottom*, Ser^{473}-Akt phosphorylation values relative to total Akt. $\$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. *B*, *top*: representative Western blot with antibodies against Akt, p-Thr^{308}-Akt, and α-tubulin. *Bottom*, Thr^{308}-Akt phosphorylation densitometric values relative to total Akt. $\$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. *C*, *top*: representative Western blot with antibodies against AS160, p-Thr^{642}-AS160, and α-tubulin. *Bottom*, Thr^{642}-AS160 phosphorylation values relative to total AS160. $\&P < 0.05$ vs. immediately after the sprint exercise (0); $*P < 0.05$, normoxia vs. hypoxia. $N = 10$ subjects in both experimental conditions.

AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, a known inhibitory mechanism of Thr^{172}-AMPKα phosphorylation (31). The sprint performed in severe acute hypoxia elicited greater reductions of the NAD^+-to-$NADH.H^+$ ratio combined with a higher glycolytic rate and oxidative stress, as reflected by the increased accumulation of intramuscular Lac and the elevated levels of carbonylated proteins in muscle and plasma during the recovery period. We have also shown that sprint exercise in hypoxia reduced SIRT1 protein levels, which combined with a lower NAD^+-to-$NADH.H^+$ ratio at the end of the sprint, could have led to lower LKB1 activation by SIRT1 and, hence, reduced Thr^{172}-AMPKα phosphorylation.

Lac-to-Pyr and NAD^+-to-$NADH.H^+$ ratios and muscle signaling. The observed intramuscular accumulation of Lac is due to the enormous energy demand generated during the sprint, which was accounted for by an extremely high glycolytic rate surpassing the mitochondrial capacity to oxidize pyruvate. In the present investigation, hypoxia reduced the mean power by only 6%, despite a 37% lower $\dot{V}O_2$, reflecting a lower mitochondrial capacity to oxidize Pyr in hypoxia due to reduced O_2 delivery. To compensate for the reduction in oxidative energy yield, the glycolytic rate was increased by 50% during the sprint in hypoxia compared with the sprint in normoxia. These findings agree with the observed 14% greater O_2 deficit (ml O_2/wt) during the sprint in hypoxia (8, 39).

The increased glycolytic rate in hypoxia elicited higher muscle Lac accumulation and a greater Lac-to-Pyr ratio. Consequently, the calculated cytoplasmatic NAD^+-to-$NADH.H^+$ ratio (50, 66) was largely reduced after the sprint in hypoxia than in normoxia.

During exercise, the main mechanism leading to Thr^{172}-AMPKα phosphorylation is an increase of free AMP (relative to ATP) (67), which acts allosterically via the γ-subunit to enhance the phosphorylation of Thr^{172}-AMPKα by LKB1 kinase (28) and to suppress dephosphorylation by protein phosphatases 2A and 2C in vitro (12). Despite the greater anaerobic energy yield during the sprint in hypoxia, the calculated free AMP-to-ATP ratio was increased to the same extent as in normoxia. Thus, our findings cannot be explained in terms of blunted AMP-dependent activation. In addition to AMP, SIRT1, an NAD-dependent deacetylase that acts as a master metabolic sensor of NAD^+, may deacetylate (and activate) LKB1 (32). Incubation of HepG2 cells in a Pyr-enriched medium results in increased SIRT1 protein expression and Thr^{172}-AMPKα phosphorylation (58), likely linked to an elevation of the ratio of NAD^+ to $NADH.H^+$. SIRT1 activity may be reduced by lower NAD^+ and loss of SIRT1 protein (19), which were both observed in the present investigation after the sprint exercise in hypoxia. Thus, the combined effect of lower NAD^+ and loss of SIRT1 protein could have blunted the activation of LKB1 in hypoxia and, hence, the phosphorylation of AMPKα at Thr^{172}.

In agreement with our results, insulin or glucose induces JNK1 phosphorylation in cell cultures, which, in turn, causes Ser^{47}-SIRT1 phosphorylation (19). SIRT1 phosphorylation causes its translocation to the nucleus and an increase of its deacetylase activity while committing SIRT1 to degradation at the proteasome (19). The latter reduces SIRT1 protein levels within 30 min (19). Although in the present investigation insulin and plasma glucose responses to the sprints could not explain the changes observed in SIRT1 protein levels, it is known that in cell cultures H_2O_2 induces JNK1 phosphorylation, which then phosphorylates SIRT1 (44). Thus, the greater oxidative stress triggered by the sprint exercise in hypoxia may have led to a loss of SIRT1 protein through JNK1 and SIRT1 phosphorylation, followed by proteasome degradation of SIRT1.

The observed reduction of SIRT1 protein levels after the sprint in hypoxia was accompanied by increased Thr^{308}-Akt phosphorylation. Interestingly, resveratrol (a SIRT1 activator) decreases phosphoinositide 3-kinase activation in cultured muscle cell lines (17). Since phosphoinositide 3-kinase is an upstream kinase for Thr^{308}-Akt phosphorylation (30), the reduced SIRT1 protein levels after the sprint in hypoxia could account, at least in part, for the increased Thr^{308}-Akt phosphorylation.

Free radicals. Free radicals have been found to be inducers of Thr^{172}-AMPKα phosphorylation by several mechanisms (56), and hypoxia has been shown to increase Thr^{172}-AMPKα phosphorylation by a mechanism involving mitochondrial ROS independently of the AMP-to-ATP ratio (15). In the present investigation, during the 120 min after the sprint in hypoxia, carbonylated muscle and plasma proteins were increased, indicating greater oxidative stress after the sprint in hypoxia than in normoxia. The fact that carbonylated proteins increased not only in muscle but also in plasma indicates that some free radicals reached the circulation (3). Protein carbonylation is a good marker of increased oxidative stress (4, 53); however, free radicals could have reacted with other molecules (3), implying that the actual difference in oxidative stress between normoxia and hypoxia could have been even greater.

Increased levels of ROS may activate CaMKII through modification of the Met-$^{281/282}$ pair within the regulatory domain, blocking reassociation with the catalytic domain and preserving kinase activity via a similar but parallel mechanism to Thr^{286} autophosphorylation (16). Moreover, the ability of CaMKII to respond to Ca^{2+} elevation is enhanced under prooxidant conditions (16). However, despite increased oxidative stress after the sprint performed in hypoxia, no significant differences were observed between conditions in the exercise-induced CaMKII phosphorylation. Thus, the lack of Thr^{172}-AMPKα phosphorylation in response to the sprint in hypoxia cannot be attributed to lower CaMKII phosphorylation.

Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 is increased after the sprint in hypoxia: a potential inhibitory mechanism of Thr^{172}-AMPKα phosphorylation. This finding confirms previous work from our laboratory showing an abrogation of Thr^{172}-AMPKα phosphorylation by enhanced Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation induced by the ingestion of 75 g of glucose before sprint exercise (22). Like in Guerra et al. (22), sprint exercise elicited a twofold elevation of serum insulin levels 30 min after the sprints. This increase in circulating insulin could account for the Akt and AS160 phosphorylation observed at the same time point (30 min after the sprint). However, immediately after the sprints, Akt phosphorylation (Thr^{308}) was observed in hypoxia only, and this occurred with minor changes in circulating insulin. Thus, the effect of FI_{O_2} on Thr^{172}-AMPKα and Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation does not seem mediated by the insulin response. The latter, together with the fast Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 response (already present 10 s after the sprint), indicates that this phosphorylation is likely triggered by an intracellular mechanism. In fact, PKB (Akt) (31) and PKA (33) can

phosphorylate AMPKα_1/α_2 at Ser$^{485/491}$, which inhibits AMPK phosphorylation at Thr^{172}. Thirty minutes after the sprint in hypoxia, the expected Thr^{172}-AMPKα phosphorylation (18, 22) was absent, and this was preceded by increased Thr^{308}-Akt phosphorylation immediately after the sprint in hypoxia. As expected with greater Akt phosphorylation, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation levels were increased immediately after the sprint exercise in hypoxia. This finding agrees with results from our previous study (22), in which glucose ingestion 1 h before a similar sprint prevented the expected Thr^{172}-AMPKα phosphorylation through a Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation-dependent mechanism. The ultimate mechanism leading to Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation remains to be elucidated, since the present results do not support insulin as the main mechanism causing the observed Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation immediately after the exercise in hypoxia. The fact that Thr^{308}-Akt phosphorylation was elevated 30 min after the sprint in hypoxia indicates that additional mechanisms regulate Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation.

Increased levels of cellular cAMP may blunt Thr^{172}-AMPKα phosphorylation by inhibition of Ca^{2+}/calmodulin-dependent kinase kinase-β [an AMPK kinase (29)] but not LKB1 (33). Moreover, Hurley et al. (33) have shown that phosphorylation of the Ser$^{485/491}$ site appears to be required but not sufficient to inhibit AMPK under conditions of elevated cAMP. It remains to be determined if muscle cAMP levels are differently altered by sprint exercise depending on F_{IO_2}.

TAK-1 acts like an AMPK kinase (68), which may be stimulated by hypoxia (5, 40). However, in the present work, Thr$^{184/187}$-TAK-1 phosphorylation did not change after the sprint, regardless of F_{IO_2}, i.e., our results cannot be attributed to differences in TAK-1 phosphorylation.

Thr^{642}-AS160 phosphorylation response to sprint exercise. Muscle contraction, insulin, hypoxia, and other stimuli raising intracellular Ca^{2+} promote muscle glucose uptake by increasing plasma membrane glucose transporter 4 (GLUT4) content (59, 65). Most of these signals evoke AS160 phosphorylation, which increases GLUT4 translocation to the membrane (59). Akt, AMPKα_2, PKC, and CaMKII may phosphorylate AS160 (59). There is little information on the effects of exercise on AS160 phosphorylation in human skeletal muscle (13, 59–61). These results confirm those of our previous study (22), i.e., small AS160 phosphorylation in response to a single bout of sprint exercise at least at Thr^{642} with the PAS antibody. As a new finding, we have shown that despite the higher anaerobic component and greater oxidative stress after the sprint in hypoxia, these two factors did not influence the Thr^{642}-AS160 phosphorylation response to sprint exercise. The small increase in AS160 phosphorylation from 30 to 120 min after the sprint could have enhanced membrane GLUT4 abundance for at least 2 h after the sprint. Another interesting finding is that Thr^{642}-AS160 phosphorylation does not require increased Thr^{172}-AMPKα phosphorylation, since hypoxia blunted the Thr^{172}-AMPKα phosphorylation without any significant effect on Thr^{642}-AS160 phosphorylation. Moreover, Thr^{308}-Akt phosphorylation was increased after the sprint in hypoxia without apparent effects on Thr^{642}-AS160 phosphorylation. Although Ser^{473}-Akt was increased 30 and 120 min after the sprints, for full activation of Akt, both Thr^{308} and Ser^{473} phosphorylations are required (1). Thus, other mechanisms should also play a role in exercise-induced Thr^{642}-AS160 phosphorylation. Moreover, it remains unknown what the effects of sprint exercise are on other AS160 phosphorylation sites (61).

Conclusions. In summary, we have shown that sprint exercise in severe acute hypoxia elicits a greater glycolytic rate with a higher accumulation of muscle Lac, increased Lac-to-Pyr ratios, and, hence, reduced NAD^+-to-NADH.H^+ ratios. Consequently, the human skeletal muscle signaling response to sprint exercise is modified in hypoxia. Acute hypoxia blunted the expected increase in Thr^{172}-AMPKα phosphorylation, and this article provides results with two potential mechanisms to explain this response. First, the lower NAD^+-to-NADH.H^+ ratio after the sprint in hypoxia combined with the reduced SIRT1protein levels may have blunted the SIRT1/LKB1-mediated phosphorylation of AMPKα. Second, Thr^{172}-AMPKα phosphorylation may have been blunted due to increased Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, a known mechanism of Thr^{172}-AMPKα phosphorylation inhibition mediated by Akt. The latter concords with the greater increase of Akt phosphorylation observed immediately after the sprint in hypoxia. Finally, we have shown that AS160 phosphorylation was elevated 30–120 min after the sprints, regardless of F_{IO_2} and despite the differences in AMPK and Akt phosphorylation responses. These findings may have important implications in terms of better understanding the potential differences in acute and adaptive responses to exercise in humans with chronic hypoxia or increased oxidative stress, such as in patients with chronic lung diseases or in chemotherapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.M.-A., J.G.P.-G., A.G.-G., L.R.-G., A.S., B.G., and J.A.L.C. performed experiments; D.M.-A., J.G.P.-G., A.G.-G., L.R.-G., A.S., M.R.C., M.G., B.G., and J.A.L.C. analyzed data; D.M.-A., J.G.P.-G., A.G.-G., A.S., M.R.C., M.G., B.G., and J.A.L.C. interpreted results of experiments; D.M.-A. and B.G. prepared figures; D.M.-A. and J.A.L.C. drafted manuscript; D.M.-A., J.G.P.-G., A.G.-G., M.R.C., B.G., C.D., and J.A.L.C. edited and revised manuscript; D.M.-A., J.G.P.-G., A.G.-G., L.R.-G., A.S., M.R.C., M.G., B.G., C.D., and J.A.L.C. approved final version of manuscript; A.S., B.G., C.D., and J.A.L.C. conception and design of research.

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