Increased oxidative stress and anaerobic energy release, but blunted Thr<sup>172</sup>-AMPKα phosphorylation, in response to sprint exercise in severe acute hypoxia in humans

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Abstract

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<sup>172</sup>-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<sup>172</sup>-AMPKα phosphorylation, 10 volunteers performed a single 30-s sprint (Wingate test) in normoxia and in severe acute hypoxia (inspired Po2: 75 mmHg). Vastus lateralis muscle biopsies were obtained before and immediately after 30 and 120 min post sprint. Mean power output and O2 consumption were 6% and 37%, respectively, lower in hypoxia than in normoxia. O2 deficit and muscle lactate accumulation were greater in hypoxia than in normoxia. Carbohydrate skeletal muscle and plasma proteins were increased after the sprint in hypoxia. Thr<sup>172</sup>-AMPKα phosphorylation was increased by 3.1-fold 30 min after the sprint in normoxia. This effect was prevented by hypoxia. The NAD<sup>+</sup>/NADH.H<sup>+</sup> 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<sup>172</sup>-AMPKα phosphorylation. Ser<sup>485</sup>-AMPKα/Ser<sup>491</sup>-AMPKα phosphorylation, a known negative regulating mechanism of Thr<sup>172</sup>-AMPKα phosphorylation, was increased by 60% immediately after the sprint in hypoxia, coincident with increased Thr<sup>108</sup>-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<sup>172</sup>-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<sup>172</sup> 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<sup>172</sup> 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<sup>172</sup>-AMPKα phosphorylation in skeletal muscle remains controversial (41, 52). In cell cultures, hypoxia and anoxia increase Thr<sup>172</sup>-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<sup>172</sup>-AMPKα phosphorylation in rats (10).

The influence of the inspired O2 fraction (FiO2) on exercise-induced Thr<sup>172</sup>-AMPKα phosphorylation has been scarcely studied in humans (63). After 30 min of exercise at 73% of peak O2 consumption (Vo<sub>2</sub>peak), Thr<sup>172</sup>-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<sup>172</sup>-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<sup>172</sup>-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<sup>172</sup>-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<sup>+</sup>-dependent deacetylase that is activated by the increase of NAD<sup>+</sup>/NADH.H<sup>+</sup> (58). Although most human studies have reported reduction of NAD<sup>+</sup>/NADH.H<sup>+</sup>, little is known about the effect of sprint exercise on NAD<sup>+</sup>/NADH.H<sup>+</sup> (64). Since NAD<sup>+</sup>/NADH.H<sup>+</sup> 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<sup>+</sup>/NADH.H<sup>+</sup> would be lower after sprint exercise in hypoxia. If this mechanism
TAK-1 is likely acts upstream of LKB1 (68). \(\text{H9251}\) which is activated by phosphorylation, has been shown to phosphorylate AMPK during exercise (14). Likewise, TAK-1, -activated kinase 1 (TAK-1) (14, \(\text{H9252}\) transforming growth factor- \(\text{H9253}\) performed in severe acute hypoxia. Greater or prolonged exercise-induced elevation of both plasma glucose and insulin could blunt the Thr\(^{172}\)-AMPK\(\alpha\) phosphorylation normally observed 30 min after sprint exercise (23) through Ser\(^{485}\) AMPK\(\alpha_1\)/Ser\(^{491}\), AMPK\(\alpha_2\) phosphorylation, while a reduced insulin response could be associated with even greater Thr\(^{172}\)-AMPK\(\alpha\) phosphorylation.

Therefore, the main aim of this study was to determine if hypoxia increases skeletal muscle Thr\(^{172}\)-AMPK\(\alpha\) 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 \(\text{O}_2\) 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\(^{172}\)-AMPK\(\alpha\) 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\(^{172}\)-AMPK\(\alpha\) phosphorylation. Moreover, by determining glucose and plasma insulin responses, combined with the assessment of Ser\(^{485}\)-AMPK\(\alpha_1\)/Ser\(^{491}\)-AMPK\(\alpha_2\) and Ser\(^{173}\)/Thr\(^{308}\)-Akt phosphorylation, we expected to determine if the degree of Thr\(^{172}\)-AMPK\(\alpha\) phosphorylation is modulated by hypoxia through a Ser\(^{485}\)-AMPK\(\alpha_1\)/Ser\(^{491}\), AMPK\(\alpha_2\) 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 (Denver, MA) except for polyclonal anti-phospho-AS160 [Thr642, 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-\(\alpha\)-tubulin antibody (no. T-5168-ML, molecular mass: 50 kDa), which was obtained from Biosigma (St. Louis, MO). The corresponding catalog numbers of the antibodies from Cell Signaling were as follows: anti-phospho-AMPK\(\alpha\) (Thr\(^{172}\)), no. 2531 (molecular mass: 62 kDa); anti-AMPK\(\alpha_1\), no. 2532 (molecular mass: 62 kDa); anti-phospho-AMPK\(\alpha_1\) (Ser\(^{485}\))/AMPK\(\alpha_2\) (Ser\(^{491}\)), no. 4185 (molecular mass:62 kDa); anti-phospho-AMPK\(\alpha_1\) (Ser\(^{485}\)), no. 4184 (molecular mass: 62 kDa); anti-AMPK\(\alpha_1\), no. 2795 (molecular mass: 62 kDa); anti-phospho-acetyl-CoA carboxylase (ACC; Ser\(^{214}\)), 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 1. Physical characteristics and ergospirometric variables during sprint exercise in normoxia and severe acute hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>25.1 ± 4.5</td>
<td>25.6 ± 4.7</td>
</tr>
<tr>
<td>Height, cm</td>
<td>176.7 ± 5.3</td>
<td>175.1 ± 4.8</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>80.2 ± 9.9</td>
<td>81.3 ± 9.1</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>18.2 ± 6.3</td>
<td>19.0 ± 6.8</td>
</tr>
<tr>
<td>Two-legs lean mass, kg</td>
<td>19.7 ± 2.37</td>
<td>20.1 ± 2.42</td>
</tr>
<tr>
<td>Maximal heart rate, beats/min</td>
<td>188.1 ± 5.6</td>
<td>171.8 ± 9.2*</td>
</tr>
<tr>
<td>Peak (V\dot{O}_2), l/min</td>
<td>4.018 ± 0.249</td>
<td>2.634 ± 0.248*</td>
</tr>
<tr>
<td>(W_{\text{max}}), W</td>
<td>332.4 ± 110.2</td>
<td>288.5 ± 32.0*</td>
</tr>
<tr>
<td>(W_{\text{peak}}), W</td>
<td>997.4 ± 121.7</td>
<td>950.5 ± 125.3</td>
</tr>
<tr>
<td>(W_{\text{mean}}/\text{LLM}, W/kg</td>
<td>50.9 ± 6.3</td>
<td>48.2 ± 3.6</td>
</tr>
<tr>
<td>(W_{\text{mean}}, W</td>
<td>573.8 ± 56.2</td>
<td>544.8 ± 68.0*</td>
</tr>
<tr>
<td>(W_{\text{mean}}/\text{LLM}, W/kg</td>
<td>29.4 ± 3.0</td>
<td>27.5 ± 2.0*</td>
</tr>
<tr>
<td>(O_2) demand, l/min</td>
<td>8.437 ± 0.767</td>
<td>7.908 ± 0.763*</td>
</tr>
<tr>
<td>Accumulated (V\dot{O}_2), liters</td>
<td>1.345 ± 0.549</td>
<td>0.853 ± 0.118*</td>
</tr>
<tr>
<td>(O_2) deficit, liters</td>
<td>2.874 ± 0.599</td>
<td>3.0 ± 0.391</td>
</tr>
<tr>
<td>(O_2) deficit/(W_{\text{mean}})</td>
<td>5.02 ± 1.06</td>
<td>5.74 ± 0.44*</td>
</tr>
<tr>
<td>Wingate hemoglobin saturation</td>
<td>96.8 ± 3.7</td>
<td>81.0 ± 6.5*</td>
</tr>
<tr>
<td>Wingate end-tidal (P_F)</td>
<td>114.0 ± 6.5</td>
<td>48.7 ± 3.1*</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = 10\) subjects. \(V\dot{O}_2\), \(O_2\) consumption; \(W_{\text{max}}\), maximal intensity during the incremental exercise test to exhaustion; \(W_{\text{peak}}\), peak power output during the Wingate test; LLM, lean mass of the lower extremities; \(W_{\text{mean}}\), mean power output during the Wingate test; accumulated \(V\dot{O}_2\), \(O_2\) consumed during the 30-s Wingate test. *\(P < 0.05\) compared with normoxia.

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

<table>
<thead>
<tr>
<th></th>
<th>Resting Value</th>
<th>0 min</th>
<th>30 min</th>
<th>120 min</th>
<th>(F\dot{O}_2) × Time Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>8.98 ± 6.3</td>
<td>97.8 ± 6.7*</td>
<td>96.9 ± 8.2*</td>
<td>87.01 ± 6.5*</td>
<td>0.07</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>88.5 ± 4.3</td>
<td>100.6 ± 11.3*</td>
<td>101.8 ± 12.7*</td>
<td>87.02 ± 7.4*</td>
<td></td>
</tr>
<tr>
<td>Insulin, IU/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>4.7 ± 1.1</td>
<td>7.6 ± 3.0*</td>
<td>10.8 ± 3.7*</td>
<td>4.4 ± 2.5*</td>
<td>0.57</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>5.2 ± 3.2</td>
<td>5.9 ± 3.0</td>
<td>11.3 ± 5.8*</td>
<td>6.0 ± 4.0</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. 0 min corresponds to immediately after the Wingate test. \(F\dot{O}_2\), fraction of inspired \(O_2\). *\(P < 0.05\) vs. resting values.

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Thr308, no. 9275 (molecular mass: 60 kDa); anti-Akt, no. 9272 (molecular mass: 60 kDa); anti-phospho-TAK-1 (Thr184/187), no. 4531 (molecular mass: 82 kDa); anti-TAK-1, no. 4505 (molecular mass: 82 kDa); anti-phospho-CaMKII (Thr286), 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, Immun-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 informed consent. The Ethics Committee of the University of the Basque Country approved the study protocol (10004/106).

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

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resting</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP, mmol/kg</td>
<td>4.99 ± 1.80</td>
<td>2.46 ± 0.77*</td>
</tr>
<tr>
<td>AMP/ATP, mmol/mol</td>
<td>7.5 ± 7.1</td>
<td>155.0 ± 302.0*</td>
</tr>
<tr>
<td>Phosphocreatine, mmol/kg</td>
<td>16.10 ± 2.66</td>
<td>5.34 ± 2.43*</td>
</tr>
<tr>
<td>Creatine, mmol/kg</td>
<td>12.31 ± 2.66</td>
<td>23.08 ± 2.43*</td>
</tr>
<tr>
<td>Pyruvate, mmol/kg‡</td>
<td>0.09 ± 0.05</td>
<td>0.25 ± 0.12*</td>
</tr>
<tr>
<td>Lactate, mmol/kg‡</td>
<td>2.5 ± 1.9</td>
<td>36.2 ± 13.2*</td>
</tr>
<tr>
<td>Lactate/pyruvate‡</td>
<td>48.3 ± 69.9</td>
<td>179.0 ± 129.5*</td>
</tr>
<tr>
<td>NAD+/NADH.H⁺ concentration, ×10⁷</td>
<td>470 ± 313</td>
<td>64 ± 24*</td>
</tr>
</tbody>
</table>

| **Postprint**    |          |         |
| ATP, mmol/kg     | 4.78 ± 0.96 | 3.10 ± 1.56* |
| AMP/ATP, mmol/mol| 5.4 ± 2.2 | 174.3 ± 242.9* |
| Phosphocreatine, mmol/kg | 16.56 ± 1.45 | 5.83 ± 3.18* |
| Creatine, mmol/kg | 11.85 ± 1.45 | 22.59 ± 3.18* |
| Pyruvate, mmol/kg‡ | 0.14 ± 0.05 | 0.23 ± 0.07* |
| Lactate, mmol/kg‡ | 2.2 ± 1.2 | 53.2 ± 20.3*† |
| Lactate/pyruvate‡ | 17.1 ± 13.5 | 247.2 ± 118.9*† |
| NAD+/NADH.H⁺ concentration, ×10⁷ | 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, postprint vs. resting values (same condition); †P < 0.05, postprint in normoxia vs. hypoxia; ‡P < 0.05 for FIO2 × 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 VO2peak, maximal heart rate, and maximal power output in normoxia (FiO2; 0.21; barometric pressure: 735–745 mmHg) and hypoxia (FiO2; 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% O2 in N2 (hypoxia). The Douglas bag was replenished with gas from a cylinder specially prepared for the experiment (Carburcos 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 VO2peak 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 VO2 registered during the last 2 min was taken as representative of each submaximal exercise intensity. To relate VO2 to power, linear regression equations were calculated by least-square linear fit.

O2 uptake and hemoglobin O2 saturation. O2 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 VO2 recorded in normoxia was taken as VO2peak. The same criterion was applied to determine VO2peak in severe acute hypoxia. The hemoglobin O2 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 HClO4 and centrifuged at 15,000 g at 4°C for 15 min. The supernatant was neutralized with KHC03 (2.1 M), and ATP, phosphocreatine (PCr), creatine (Cr), pyruvate (Pyru), 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 × (∆LaC + ∆Pyru) (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 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 Thr172-AMPK, Ser485-AMPKα, Ser491-AMPKα, Ser845-AMPKα, Ser212-ACCβ, Ser373-Akt, Thr183/T184/T185 phosphorylation, Thr183/T184/T185, TAK-1, Thr386, CaMKII, and Thr422/AS160 phosphorylation levels, antibodies directed against the phosphorylated and total forms of these
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 to the respective total form.

Samples from each subject were run on the same gel (hypoxic and normoxic conditions). 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 immunonassay 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 (Roche/Hitachi, Indianapolis, IN) 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 performed. No statistically significant changes were observed after both sprints (Fig. 2A).

** complared 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 post sprint, 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. 1A).

**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 FIO\(_2\). 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 (FIO\(_2\) × time interaction, P \( < 0.05 \)). The corresponding muscle Lac concentration changes were 14- and 24-fold (FIO\(_2\) × 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 \( < 0.05 \); FIO\(_2\) × time interaction, P \( < 0.05 \) by ANOVA). Consequently, immediately after the sprints, the NAD\(^+\)/NADH 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. 1B).

**Muscle signaling.** Thr\(^{172}\)-AMPK\(\alpha\) phosphorylation was increased by 3.1-fold 30 min after the Wingate test in normoxia (P \( < 0.05 \); Fig. 2A). Hypoxia prevented the exercise-induced AMPK\(\alpha\) phosphorylation at 30 min (P \( < 0.05 \)). However, ACC\(\beta\) 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. 2B).

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. 2C), without significant differences between conditions. No statistically significant changes were observed in Thr\(^{184/187}\)-TAK-1 phosphorylation after both sprints (Fig. 2D).

Ser\(^{85}\)-AMPK\(\alpha\)/Ser\(^{491}\)-AMPK\(\alpha\) phosphorylation levels were increased by 60% immediately after the sprint performed in hypoxia (P \( < 0.05 \)), whereas they remained unchanged after the normoxic sprint (FIO\(_2\) × time interaction, P \( < 0.05 \) by ANOVA; Fig. 3A). No significant changes in Ser\(^{85}\)-AMPK\(\alpha\) phosphorylation were observed after both sprints (Fig. 3B). Compared with normoxia, the AUC for SIRT1 protein expression was 53% lower after the sprint in hypoxia (P \( < 0.05 \); Fig. 3C).

No significant between-condition differences were observed in Ser\(^{77}\)-Akt phosphorylation. Ser\(^{173}\)-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. 4A). Thr\(^{308}\)-Akt phosphorylation was increased by 2.4-fold just after the exercise (P \( < 0.05 \)). This response was more accentuated in
hypoxia, as reflected by the Thr^{308}-Akt phosphorylation AUC, which was 87% higher after the sprint in hypoxia (P < 0.05; Fig. 4B).

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. 4C).

DISCUSSION

This study examined the influence of FIO2 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 Ser473-Akt (A), Thr308-Akt (B), and Thr642-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-Ser473-Akt, and α-tubulin. Bottom, Ser473-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-Thr308-Akt, and α-tubulin. Bottom, Thr308-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-Thr642-AS160, and α-tubulin. Bottom, Thr642-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/Ser491-AMPKα2 phosphorylation, a known inhibitory mechanism of Thr172-AMPK phosphorylation (31). The sprint performed in severe acute hypoxia elicited greater reductions of the NAD+/to-NADH 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 ratio at the end of the sprint, could have led to lower LKB1 activation by SIRT1 and, hence, reduced Thr172-AMPK phosphorylation.

Lac-to-Pyr and NAD+/to-NADH 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 V̇O2, reflecting a lower mitochondrial capacity to oxidize Pyr in hypoxia due to reduced O2 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 O2 deficit (ml O2/sWt) 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 ratio (50, 66) was largely reduced after the sprint in hypoxia than in normoxia. During exercise, the main mechanism leading to Thr172-AMPKα phosphorylation is an increase of free AMP (relative to ATP) (67), which acts allosterically via the γ-subunit to enhance the phosphorylation of Thr172-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 Thr172-AMPKα phosphorylation (58), likely linked to an elevation of the ratio of NAD+ to NADH+. 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 Thr172.

In agreement with our results, insulin or glucose induces JNK1 phosphorylation in cell cultures, which, in turn, causes Ser47-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 H2O2 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 Thr308-Akt phosphorylation. Interestingly, resveratrol (a SIRT1 activator) decreases phosphorynosidate 3-kinase activation in cultured muscle cell lines (17). Since phosphorynosidate 3-kinase is an upstream kinase for Thr308-Akt phosphorylation (30), the reduced SIRT1 protein levels after the sprint in hypoxia could account, at least in part, for the increased Thr308-Akt phosphorylation.

Free radicals. Free radicals have been found to be inducers of Thr172-AMPK phosphorylation by several mechanisms (56), and hypoxia has been shown to increase Thr172-AMPK 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 Thr286 autophosphorylation (16). Moreover, the ability of CaMKII to respond to Ca2+ 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 Thr172-AMPKα phosphorylation in response to the sprint in hypoxia cannot be attributed to lower CaMKII phosphorylation.

Ser491-AMPKα/ Ser491-AMPKα2 is increased after the sprint in hypoxia: a potential inhibitory mechanism of Thr172-AMPKα phosphorylation. This finding confirms previous work from our laboratory showing an abrogation of Thr172-AMPKα phosphorylation by enhanced Ser485-AMPKα/ Ser485-AMPKα2 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 (Thr308) was observed in hypoxia only, and this occurred with minor changes in circulating insulin. Thus, the effect of Fio2 on Thr172-AMPKα and Ser485-AMPKα/ Ser491-AMPKα2 phosphorylation does not seem mediated by the insulin response. The latter, together with the fast Ser485-AMPKα/ Ser491-AMPKα2 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...
phosphorylation was increased after the sprint in hypoxia without over, Hurley et al. (33) have shown that phosphorylation of the nase kinase-Thr172. Thirty minutes after the sprint in hypoxia, Akt phosphorylation was increased at Thr642 with the PAS antibody. As a result from our previous study (22), in which glucose ingestion 1 h before a similar sprint prevented the expected Thr172-AMPKα phosphorylation through a Ser485-AMPKα1/Ser491-AMPKα2 phosphorylation-dependent mechanism. The ultimate mechanism leading to Ser485-AMPKα1/Ser491-AMPKα2 phosphorylation remains to be elucidated, since the present results do not support insulin as the main mechanism causing the observed Ser485-AMPKα1/Ser491-AMPKα2 phosphorylation immediately after the exercise in hypoxia. The fact that Thr308-Akt phosphorylation was elevated 30 min after the sprint in hypoxia indicates that additional mechanisms regulate Ser485-AMPKα1/Ser491-AMPKα2 phosphorylation.

Increased levels of cellular cAMP may blunt Thr172-AMPKα phosphorylation by inhibition of Ca2+/calmodulin-dependent kinase-β [an AMPK kinase (29)] but not LKB1 (33). Moreover, Hurley et al. (33) have shown that phosphorylation of the Ser485/491 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 FIO2.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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REFERENCES


Hemming BA.
Hypoxia Blunts AMPK Phosphorylation • Morales-Alamo D et al.


