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Critical role for free radicals on sprint exercise-induced CaMKII and AMPKα phosphorylation in human skeletal muscle

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Morales-Alamo D, Ponce-González JG, Guadalupe-Grau A, Rodríguez-García L, Santana A, Cusso R, Guerrero M, Dorado C, Guerra B, Calbet JA. Critical role for free radicals on sprint exercise-induced CaMKII and AMPKα phosphorylation in human skeletal muscle. *J Appl Physiol* 114: 566–577, 2013. First published January 3, 2013; doi:10.1152/japplphysiol.01246.2012.—The extremely high energy demand elicited by sprint exercise is satisfied by an increase in O_2 consumption combined with a high glycolytic rate, leading to a marked lactate accumulation, increased AMP-to-ATP ratio, and reduced NAD^+/NADH.H^+ and muscle pH, which are accompanied by marked Thr^{172} AMP-activated protein kinase (AMPK)-α phosphorylation during the recovery period by a mechanism not fully understood. To determine the role played by reactive nitrogen and oxygen species (RNOS) on Thr^{172}-AMPKα phosphorylation in response to cycling sprint exercise, nine voluntary participants performed a single 30-s sprint (Wingate test) on two occasions: one 2 h after the ingestion of placebo and another after the intake of antioxidants (α-lipoic acid, vitamin C, and vitamin E) in a double-blind design. Vastus lateralis muscle biopsies were obtained before, immediately postsprint, and 30 and 120 min postsprint. Performance and muscle metabolism were similar during both sprints. The NAD^+-to-NADH.H^+ ratio was similarly reduced (84%) and the AMP-to-ATP ratio was similarly increased (×21-fold) immediately after the sprints. Thr^{286} Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and Thr^{172}-AMPKα phosphorylations were increased after the control sprint (with placebo) but not when the sprints were preceded by the ingestion of antioxidants. Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, a known inhibitory mechanism of Thr^{172}-AMPKα phosphorylation, was increased only with antioxidant ingestion. In conclusion, RNOS play a crucial role in AMPK-mediated signaling after sprint exercise in human skeletal muscle. Antioxidant ingestion 2 h before sprint exercise abrogates the Thr^{172}-AMPKα phosphorylation response observed after the ingestion of placebo by reducing CaMKII and increasing Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation. Sprint performance, muscle metabolism, and AMP-to-ATP and NAD^+-to-NADH.H^+ ratios are not affected by the acute ingestion of antioxidants.

AMP-actived protein kinase; antioxidants; exercise; fatigue; sprint

IN SKELETAL MUSCLE, AMP-activated protein kinase (AMPK) intervenes in the regulation of fat oxidation (67), glucose transport (40), mitochondrial biogenesis (46, 76), and Na^+-K^+-ATPase activity (32), among other functions (69). Thr^{172}-AMPKα phosphorylation is required for activation of AMPK (25). This can be elicited by several AMPK kinases, among which liver kinase B1 (LKB1) plays an important role in skeletal muscle, since it responds to increases of the AMP-to-ATP ratio (24) and can be also activated through deacetylation by sirtuin 1 (SIRT1) in response to an increase in the NAD^+-to-NADH.H^+ ratio (29). Whether reactive nitrogen and oxygen species (RNOS) may also play a role in contraction-mediated Thr^{172}-AMPKα phosphorylation in skeletal muscle remains controversial (41, 62). Moreover, the influence that RNOS may have on the regulation of Thr^{172}-AMPKα phosphorylation in response to sprint exercise has not been studied in humans, despite that sprint exercise due to the combination of high O_2 consumption with full activation of the anaerobic pathways leads to a fast production of RNOS (8, 43).

Free radicals may modulate Thr^{172}-AMPKα phosphorylation by several mechanisms that may involve activation/inhibition of AMPK kinases and phosphatases. The main two AMPK kinases producing Thr^{172}-AMPKα phosphorylation in response to sprint exercise that could be regulated by RNOS are Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (11, 13, 71) and CaMK kinase-β (44). However, the impact RNOS produced during sprint exercise on the activity of these two kinases remains unknown.

Recent studies have shown that AMPK phosphorylation in Ser^{485} of the α_1-subunit and Ser^{491} of the α_2-subunit mitigates or completely blunts Thr^{172}-AMPKα phosphorylation. For example, insulin antagonizes anoxia or ischemia-induced AMPKα phosphorylation through Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation (28), which may be produced by Akt (also known as PKB) (34, 65). Phenylephrine induces Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation in cardiomyocytes, preventing Thr^{172}-AMPKα phosphorylation in response to adenosine agonists (48). In brown adipose tissue, reducing both α- and β-adrenergic signaling in vivo activates Akt and PKA, which, in turn, increase Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation to reduce AMPK activity (51).

Although a single 30-s sprint (Wingate test) elicits Thr^{172}-AMPKα phosphorylation 30 min after the sprint (during the passive recovery period), Thr^{172}-AMPKα phosphorylation is prevented when the exercise is preceded by the ingestion of 75 g glucose (19) or when the sprint is performed in severe acute hypoxia (fraction of inspired O_2: 0.105) (43). In both circumstances, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation was increased with a time pattern adequate to inhibit Thr^{172}-AMPKα phosphorylation. Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2

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phosphorylation may be regulated via changes in RNOS production (43, 51). Immediately after and during the first 30 min after a single bout of sprint exercise, acetyl-CoA carboxylase (ACC) is phosphorylated on Ser^{221} (14, 19), enabling fat oxidation during recovery. Ser^{221}-ACCβ phosphorylation may be elicited by AMPK (7) and unknown AMPK-independent mechanisms (10, 53). In cell cultures, treatment with H_2O_2 elicits Ser^{221}-ACCβ phosphorylation in an AMPK-dependent manner (12). However, whether Ser^{221}-ACCβ phosphorylation in response to sprint exercise is modulated by RNOS remains unknown.

Therefore, the main aim of this study was to determine the role that RNOS may have on Thr^{172}-AMPKα phosphorylation in response to sprint exercise. For this purpose, antioxidants were administered before a single sprint, and muscle biopsies and blood samples were obtained to examine potential endocrine, metabolic, and signaling mechanisms that could regulate Thr^{172}-AMPKα phosphorylation, with special emphasis on LKB1, CaMKII, and Ser^{485}-AMPKα$_1$/Ser^{491}-AMPKα$_2$ phosphorylation-dependent mechanisms. Since LKB1 activity depends on both the AMP-to-ATP ratio and deacetylation by SIRT1, we also measured muscle metabolite changes to determine sarcoplasmic AMP-to-ATP and NAD^+-to-$NADH.H^+$ ratios. Ser^{473}/Thr^{308}-Akt phosphorylation was determined as an upstream signal of Ser^{485}-AMPKα$_1$/Ser^{491}-AMPKα$_2$ phosphorylation and Ser^{221}-ACCβ phosphorylation as a downstream signal of Thr^{172}-AMPKα phosphorylation.

We hypothesized that antioxidant administration would increase Ser^{485}-AMPKα$_1$/Ser^{491}-AMPKα$_2$ phosphorylation, blunting the normal skeletal muscle Thr^{172}-AMPKα phosphorylation response to a single sprint (14–15, 19, 43) and the phosphorylation of its target, ACCβ. We also hypothesized that antioxidant administration would reduced exercise-induced Thr^{286}-CaMKII phosphorylation, blunting Thr^{172}-AMPKα phosphorylation.

MATERIALS AND METHODS

Materials. Complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor were obtained from Roche Diagnostics (Mannheim, Germany). All primary antibodies used were from Cell Signaling Technology (Denvers, MA) except the anti-CaMKII antibody (no. sc-13082), which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal mouse anti-α-tubulin antibody (no. T-5168-ML), which was obtained from Biosigma (Sigma, St. Louis, MO). The corresponding catalogue numbers of the antibodies from Cell Signaling were as follows: anti-phospho-AMPKα (Thr^{172}), no. 2531; anti-AMPKα, no. 2532; anti-phospho-AMPKα$_1$ (Ser^{485})/AMPKα$_2$ (Ser^{491}), no. 4185; anti-phospho-AMPKα$_1$ (Ser^{485}), no. 4184; anti-AMPKα$_1$, no. 2795; anti-phospho-ACC (Ser^{221}), no. 3661; anti-ACC, no. 3662; anti-SIRT1, no. 2310; anti-phospho-Akt (Ser^{473}), no. 9271; anti-phospho-Akt (Thr^{308}), no. 9275; anti-Akt, no. 9272; anti-phospho-CaMKII (Thr^{286}), no. 3361; and AS160, no. 2447. 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). Immun-Blot polyvinylidene difluoride (PVDF) membranes and the Immun-Star WesternC kit were from Bio-Rad Laboratories. The ChemiDoc XRS system and the image-analysis software Quantity One were obtained from Bio-Rad Laboratories.

Subjects. Nine healthy male physical education students (age: 25 ± 5 yr, height: 176.0 ± 5.1 cm, body mass: 79.4 ± 10.1 kg, and body fat: 18.3 ± 6.7%) agreed to participate in this investigation (Table 1). All subjects were involved in regular sports and physical activity at a rate of 2–4 h/wk, but none of them was consistently training. Subjects were instructed not to participate in any strenuous exercise sessions other than those prescribed by the study protocol. Before volunteering, subjects received full oral and written information 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). Baseline values corresponded to 9 of 10 subjects previously studied in normoxia and hypoxia, as reported elsewhere (43).

Table 1. *Physical characteristics and ergoespirometric variables during sprint under control conditions (placebo) and after the ingestion of antioxidants*

	Placebo	Antioxidants
Age, yr	25.2 ± 4.7	
Height, cm	176.0 ± 5.1	
Weight, kg	79.4 ± 10.1	
Body fat, %	18.3 ± 6.7	
Two-legs lean mass, kg	19.5 ± 2.4	
Maximal heart rate, beats/min	188 ± 6	
Maximal O_2 consumption, l/min	3.99 ± 0.25	
W_{max}, W	359 ± 34	
W_{peak}, W	999 ± 129	979 ± 114
W_{peak}/kg LLM, W/kg	51.6 ± 6.2	50.5 ± 4.3
W_{mean}, W	575 ± 61	572 ± 61
W_{mean}/kg LLM, W/kg	29.7 ± 3.0	29.5 ± 2.3
O_2 demand, l/min	8.390 ± 0.798	8.257 ± 0.838
Accumulated O_2 consumption, liters	1.192 ± 0.406	1.207 ± 0.391
O_2 deficit, liters	3.003 ± 0.498	2.921 ± 0.513
O_2 deficit/W_{mean}, ml/W	5.24 ± 0.83	5.11 ± 0.72
Wingate end-tidal P_{O_2}, mmHg	114 ± 7	115 ± 5

Values represent means ± SD; n = 9 subjects for all variables and conditions. 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 O_2 consumption, amount of O_2 consumed during the 30-s Wingate test.

General procedures. The subjects' body composition was determined by dual-energy X-ray absorptiometry (Hologic QDR-1500, Hologic, software version 7.10, Waltham, MA), as described elsewhere (1, 50). Subjects reported to the laboratory to complete different test on separate days. First, their maximal O_2 uptake ($\dot{V}O_{2\,max}$), maximal heart rate, and maximal power output (the power reached at exhaustion) were assessed with ramp incremental exercise tests to exhaustion (50W/min) on a Lode Excalibur Sport 925900 (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, after a 12-h overnight fast, nine subjects performed one 30-s isokinetic Wingate test at 100 RPM after the ingestion of either placebo or antioxidants in a double-blind design. Antioxidants were administered split into two doses, with the first dose ingested 2 h before the sprint (at 07:00 AM) followed by the second dose 30 min later, i.e., 90 min before the sprint. The first dose consisted of 300 mg α-lipoic acid, 500 mg vitamin C, and 200 IU vitamin E, whereas the second dose included 300 mg α-lipoic acid, 500 mg vitamin C, and 400 IU vitamin E (water dispersible). This cocktail was chosen because there is solid evidence, obtained directly in vivo in humans [using electron paramagnetic resonance (EPR) spectroscopy], showing that this antioxidant is effective in decreasing free radical levels at rest and in response to exercise (54). Placebo microcrystalline cellulose capsules were of similar taste, color, and appearance and were likewise consumed in two similarly timed doses (54). For each trial, subjects reported to the laboratory at 08:00 AM after an overnight fast, and an antecubital vein was catheterized. After 10-min rest in the supine position, a 20-ml blood sample was withdrawn from the subjects and used to measure serum glucose, insulin,

and plasma carbonylated proteins. Immediately after this, a muscle biopsy was obtained from the middle portion of the vastus lateralis muscle using Bergstrom's technique with suction.

During both sprints, subjects attempted to pedal as fast and hard as possible (i.e., all out and loaded since the start of pedaling) from the start to the end of the exercise while receiving strong verbal encouragement. Since the cycle ergometer was set on 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.

Maximal power output was calculated as the highest work output performed during the 1-s interval, and mean power output was calculated from the average work performed during 30 s. A 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 (5, 6, 20).

Within 10 s from the end of the sprint, a second muscle biopsy was taken, and another blood sample was then obtained. During the next 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, muscle biopsies were obtained at least 3 cm apart, using procedures previously described by Guerra et al. (18). Muscle specimens were cleaned to remove any visible blood, fat, or connective tissue. Muscle tissue was then immediately frozen in liquid nitrogen and stored at −80°C for later analysis. The time needed to obtain and freeze the muscle biopsies was under 30 s.

Cycling economy tests and O_2 deficit. Cycling economy was determined on two different days using 8–11 submaximal workloads at intensities between 50 to 90% of peak $\dot{V}O_2$ at 100 RPM (43). 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 a least-squares linear fit. From this linear relationship, the individual O_2 demand corresponding to each Wingate test was calculated, and the accumulated O_2 deficit was determined as the difference between the O_2 demand and the Wingate $\dot{V}O_2$ (5, 6).

O_2 uptake and respiratory variables. O_2 uptake was measured with a metabolic cart (Vmax N29, Sensormedics), which was 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 $\dot{V}O_{2\,max}$.

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). ATP, phosphocreatine, creatine, pyruvate (Pyr), and lactate (Lac) were enzymatically determined in neutralized extracts by fluorometric analysis (17, 36). Muscle metabolite concentrations were adjusted to the individual mean total creatine (phosphocreatine + creatine) because this mean should remain constant during the exercise (26). The adjustment to total creatine content accounts for the variability in solid nonmuscle constituents, which may be present in the biopsies (49). 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 the ADP concentration was calculated using the creatine kinase equilibrium apparent constant for resting conditions and exhaustion after a Wingate test (49), as previously described by Sahlin et al. (61). Subsequently, the AMP concentration was calculated using the adenylate kinase apparent equilibrium constant for the same conditions (49). The NAD^+-to-$NADH.H^+$ ratio was calculated using the equilibrium constant for lactate dehydrogenase (3, 73).

Total protein extraction, electrophoresis, and Western blot analysis. Muscle protein extracts were prepared as previously described (21), and total protein content was quantified using the bicinchoninic acid assay (64). Briefly, proteins were solubilized in sample buffer containing 0.0625 M Tris·HCl (pH 6.8), 2.3% (wt/vol) SDS-polyacrylamide, 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 using 7.5–10% SDS-PAGE using the system of Laemmli (35) and transferred to Immun-Blot PVDF 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, and Thr^{286}-CaMKII 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 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 incubation with HRP-conjugated goat anti-rabbit antibody (1:20,000) or HRP-conjugated donkey anti-mouse antibody (1:10,000), which were both diluted in 5% Blotto-blocking buffer and visualized with the Immun-Star WesternC kit (Bio-Rad Laboratories). Densitometric 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 the image-analysis program Quantity One (Bio-Rad laboratories). Muscle signaling data were represented as a percentage of 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 (under antioxidant and placebo 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 electrochemiluminescence 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 an OxyBlot protein oxidation kit (Intergen, Purchase, NY), as previously described (56). Protein carbonylation data were represented as a percentage of immunostaining values.

Statistics. Variables were checked for normal distribution using the Shapiro-Wilks test. When necessary, the analysis was carried out on logarithmically transformed data. First, preexercise values were compared between the two conditions using a Student's *t*-test. Since nonsignificant differences between conditions were observed before the start of the sprint, individual responses were normalized to the band densities or level of phosphorylation observed just before the start of the Wingate test. Repeated-measures ANOVA over time and antioxidant conditions with two levels (placebo vs. antioxidants) was used to compare the responses with the value before the start of the Wingate test. When there was a significant condition effect or condition × time interaction, 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

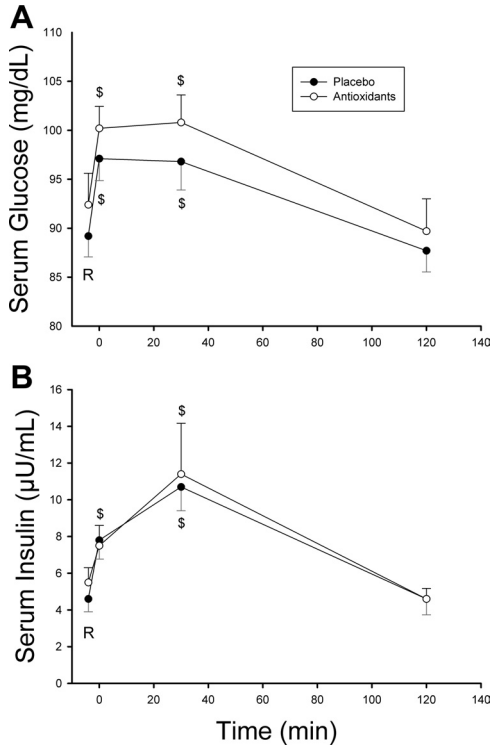

Fig. 1. Serum glucose (*A*) and insulin concentrations (*B*) before and after sprint exercise under control conditions (placebo) and after the ingestion of antioxidants. 0 min corresponds to immediately after the Wingate test. $n = 9$ for all variables and conditions. $\$P < 0.05$ vs. resting (R) values.

analysis. Areas under the curve 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 respiratory variables. Exercise performance, O_2 deficit, and respiratory variables were not significantly affected by the ingestion of antioxidants before the Wingate test (Table 1).

Serum glucose and insulin. Basal serum glucose and insulin concentrations were not altered by the ingestion of antioxidants. Compared with rest, the serum glucose concentration was increased by 9% just after the exercise and remained elevated until 30 min into the recovery period (time effect, $P < 0.05$; Fig. 1*A*). Serum insulin was increased by 50% and 2.2-fold just after the test and 30 min into the recovery period, respectively (time effect, $P < 0.05$; Fig. 1*B*). Both variables decreased to values similar to those observed before the sprint 120 min after the exercise.

Muscle metabolites. The changes observed in muscle metabolites are shown in Table 2. Basal muscle metabolites were not altered by the ingestion of antioxidants. Antioxidants did not change the metabolic response to sprint exercise. The AMP-to-ATP molar ratio was similarly increased (×21-fold) immediately after the sprints (time effect, $P < 0.05$). Compared with rest, immediately after the sprint, muscle Pyr and Lac concentrations were increased by 3- and 14-fold, respectively (time effect, $P < 0.05$). After both sprints, the Lac-to-Pyr ratio was similarly increased, by 4.1-fold (time effect, $P < 0.05$), and, consequently, the NAD^+-to-$NADH.H^+$ ratio was similarly reduced, by 84% (time effect, $P < 0.05$).

Plasma and skeletal muscle carbonylated proteins. Antioxidants did not alter the basal levels of plasma or muscle carbonylated proteins, which remained unchanged after the sprints. No statistically significant changes were observed in plasma and skeletal muscle carbonylated proteins in response to both sprints (Fig. 2).

Muscle signaling. Ingestion of antioxidants did not alter the basal levels of Thr^{172}-AMPKα phosphorylation, ACCβ phosphorylation, Thr^{286}-CaMKII phosphorylation, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, Ser^{485}-AMPKα_1 phosphorylation, Ser^{473}-Akt phosphorylation, and Thr^{308}-Akt phosphorylation, or total SIRT1 protein content. As shown in Fig. 3, Thr^{172}-AMPKα phosphorylation was increased by 3.2-fold 30 min after the control Wingate test. This effect was not observed when the sprint was performed after the ingestion of antioxidants (condition × time interaction, $P < 0.05$; Fig. 3*A*). However, Ser^{221}-ACCβ phosphorylation was similarly enhanced (3.9- and 3.4-fold; Fig. 3*B*) immediately and 30 min after the sprints, respectively (time effect, $P < 0.05$).

Thr^{286}-CaMKII phosphorylation was increased by 53% and 58% at 30 and 120 min after the control sprints, whereas it remained unchanged after the ingestion of antioxidants (condition × time interaction, $P < 0.05$; Fig. 3*C*).

Compared with rest, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation levels were increased by 57% but only immediately

Table 2. *Muscle metabolites before and immediately after a 30-s sprint under control conditions (placebo) and after the ingestion of antioxidants*

	Placebo		Antioxidants	
	Resting	Postsprint	Resting	Postsprint
ATP, mmol/kg	5.08 ± 1.88	2.43 ± 0.81*	4.96 ± 1.59	3.51 ± 1.40*
AMP/ATP, mmol/mol	7.9 ± 7.5	170.7 ± 316.0*	4.2 ± 2.4	67.5 ± 55.7*
Phosphocreatine, mmol/kg	15.97 ± 2.78	4.95 ± 2.23*	17.77 ± 2.35	6.04 ± 2.95*
Creatine, mmol/kg	12.44 ± 2.78	23.46 ± 2.23*	10.64 ± 2.35	22.37 ± 2.95*
Pyr, mmol/kg	0.09 ± 0.04	0.28 ± 0.11*	0.10 ± 0.04	0.28 ± 0.09*
Lac, mmol/kg	2.6 ± 2.2	38.5 ± 13.1*	2.5 ± 2.4	35.5 ± 15.3*
Lac/Pyr	30.1 ± 27.8	145.3 ± 36.9*	37.5 ± 36.4	129.8 ± 53.6*
$[NAD^+]/([NADH.H^+])$, $\times 10^7$	450.4 ± 223.9	65.5 ± 16.5*	496.6 ± 387.9	87.9 ± 54.4*

Values represent means ± SD; $n = 9$ subjects for all variables and conditions except for pyruvate (Pyr), lactate (Lac), Lac/Pyr, and $NAD^+/NADH.H^+$ data, where $n = 8$ subjects. AMP/ATP was calculated from the creatine kinase and adenylate kinase apparent equilibrium constants for free AMP and ADP. *Significant for postsprint vs. resting (same condition).

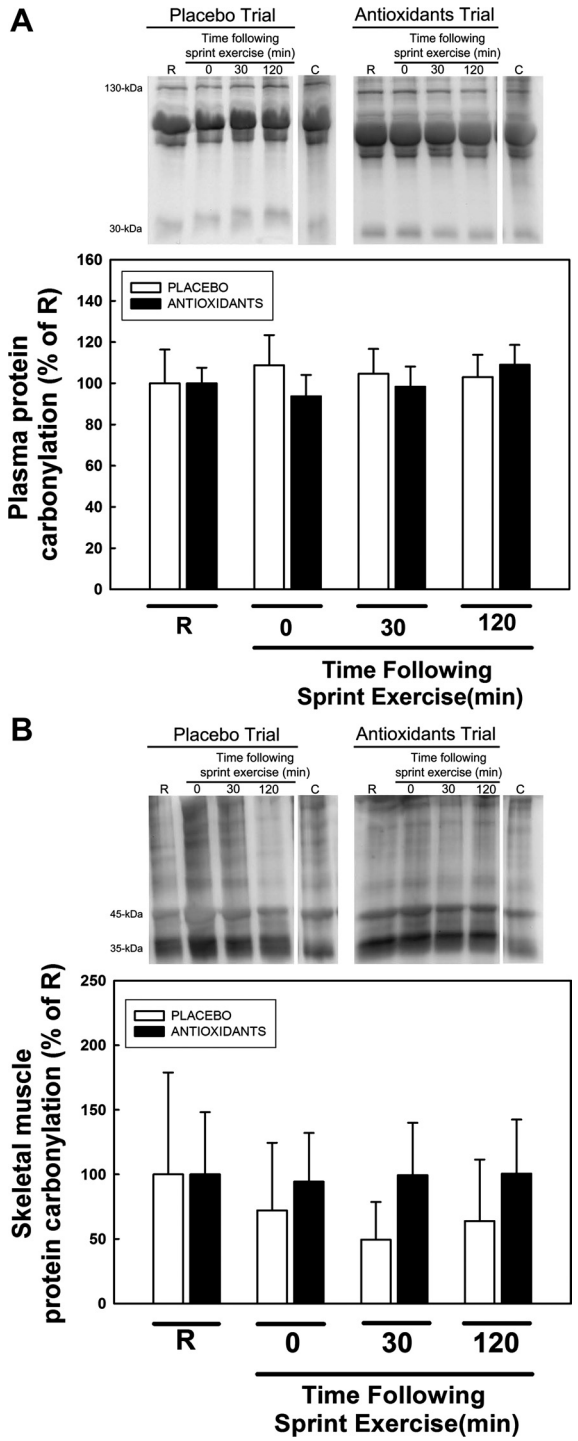

Fig. 2. Levels of carbonylated proteins in plasma (*A*) and skeletal muscle (*B*) before and after a single Wingate test performed after ingestion of placebo (open bars) or antioxidants (solid bars). Values under both experimental conditions were normalized to those observed in the biopsies obtained immediately before the sprint exercise (R), which were assigned a value of 100%. *A*: Western blot (*top*) and densitometry analysis (*bottom*; 130 and 30 kDa) showing carbonylated proteins in plasma extracts. *B*: Western blot (*top*) and densitometry analysis (*bottom*; 45 and 35 kDa) showing carbonylated proteins in skeletal muscle extracts. Statistical analysis was performed with logarithmically transformed data. C, control sample. Error bars represent SEs. $n = 9$ in both experimental conditions.

after the sprint with antioxidants, returning to preexercise levels 30 min later (Fig. 4*A*). Subsequently, 120 min after the sprints with antioxidants, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation levels were increased by 76% (time effect, $P < 0.05$). Ser^{485}-AMPKα_1 phosphorylation and SIRT1 protein levels were unchanged after the sprints (Fig. 4, *B* and *C*).

Ser^{473}-Akt phosphorylation was increased by 2.1-fold and 33% 30 and 120 min after the sprints, respectively (time effect, $P < 0.05$; Fig. 5*A*). Thr^{308}-Akt phosphorylation was increased by 75% and 16% just after the exercise and 30 min into the recovery period, respectively (time effect, $P < 0.05$), without significant differences between conditions (Fig. 5*B*).

DISCUSSION

This study examined the role of RNOS on the regulation of skeletal muscle AMPKα phosphorylation in response to a single 30-s cycling sprint exercise in humans. Ingestion of antioxidants before the sprint prevented Thr^{172}-AMPKα phosphorylation by two potential mechanisms. First, antioxidant ingestion blunted Thr^{286}-CaMKII phosphorylation, an upstream kinase for AMPK, in skeletal muscle. Second, antioxidants increased Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation immediately after the sprint. These findings indicate that RNOS may play a critical role in the Thr^{172}-AMPKα phosphorylation response to cycling sprint exercise in humans. Despite the fact that the antioxidant cocktail administered in this study has been shown to effectively reduce oxidative stress (54), in the present investigation, antioxidants did not alter exercise performance, muscle aerobic and anaerobic metabolism, AMP-to-ATP and NAD^+-to-$NADH.H^+$ ratios, or the degree of protein carbonylation in plasma and skeletal muscle. Thus, since the metabolic signals leading to Thr^{172}-AMPKα phosphorylation were similar in both conditions, it is likely that the main mechanism explaining the blunted that Thr^{172}-AMPKα phosphorylation after the ingestion of antioxidants is reduced RNOS-mediated signaling. This is further supported by the blunted Thr^{286}-CaMKII phosphorylation after the ingestion of antioxidants.

AMPK regulation by Thr^{286}-CaMKII phosphorylation: role of RNOS. RNOS have been found to be inducers of Thr^{172}-AMPKα phosphorylation by several mechanisms (66), and hypoxia has been shown to increase Thr^{172}-AMPKα phosphorylation by a mechanism involving mitochondrial RNOS independent of the AMP-to-ATP ratio (12). Because of this, we hypothesized that antioxidant ingestion before sprint exercise could blunt or mitigate the expected Thr^{172}-AMPKα phosphorylation normally observed in the vastus lateralis muscle after a single sprint (15, 19). Gomez-Cabrera et al. (16) have shown in rats that vitamin C supplementation decreases training-induced mitochondrial biogenesis and the increase of antioxidant capacity in skeletal muscle. Another study (55) has shown that vitamin C and E administration decreases insulin sensitivity and peroxisome proliferator-activated receptor PPAR-γ coactivator PGC-1α adaptations in humans (55). These two studies have indicated that RNOS are necessary signals for some exercise-induced adaptations and, in particular, to stimulate mitochondrial biogenesis (16, 55), which is in part mediated by AMPK (45). However, more recent studies using vitamin C either alone (70) or in combination with E vitamin (27) have

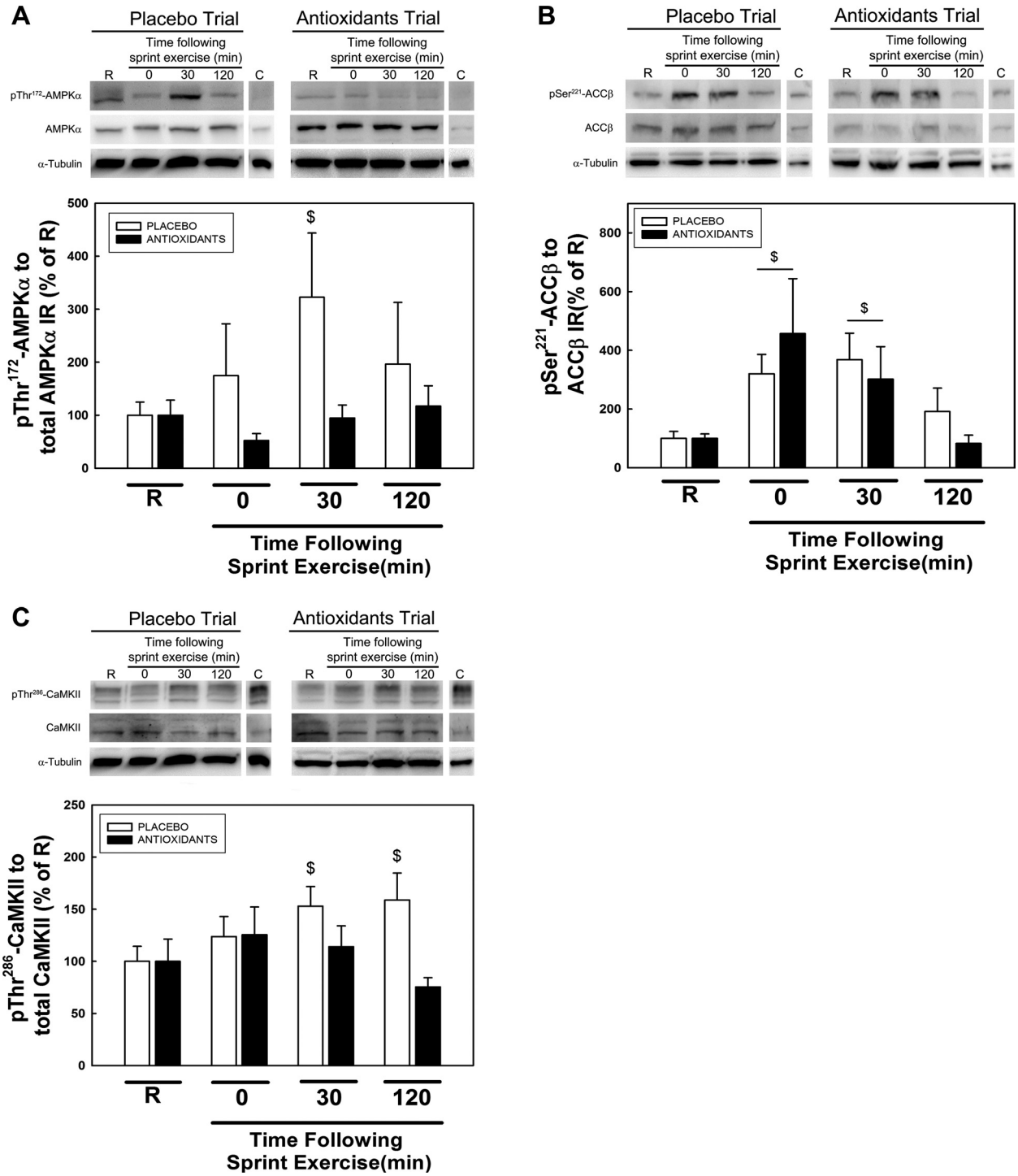

Fig. 3. Levels of Thr^{172}-AMP-activated protein kinase (AMPK)α (*A*), Ser^{221}-acetyl-Co carboxylase (ACC)-β (*B*), and Thr^{286}-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (*C*) before and after a single Wingate test performed after ingestion of placebo (open bars) or antioxidants (solid bars). Values under both experimental conditions were normalized to those observed in the biopsies obtained immediately before the sprint exercise (R), 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. *B*, *top*: representative Western blot with antibodies against ACCβ, pACCβ, 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. Error bars represent SEs. $n = 9$ in both experimental conditions.

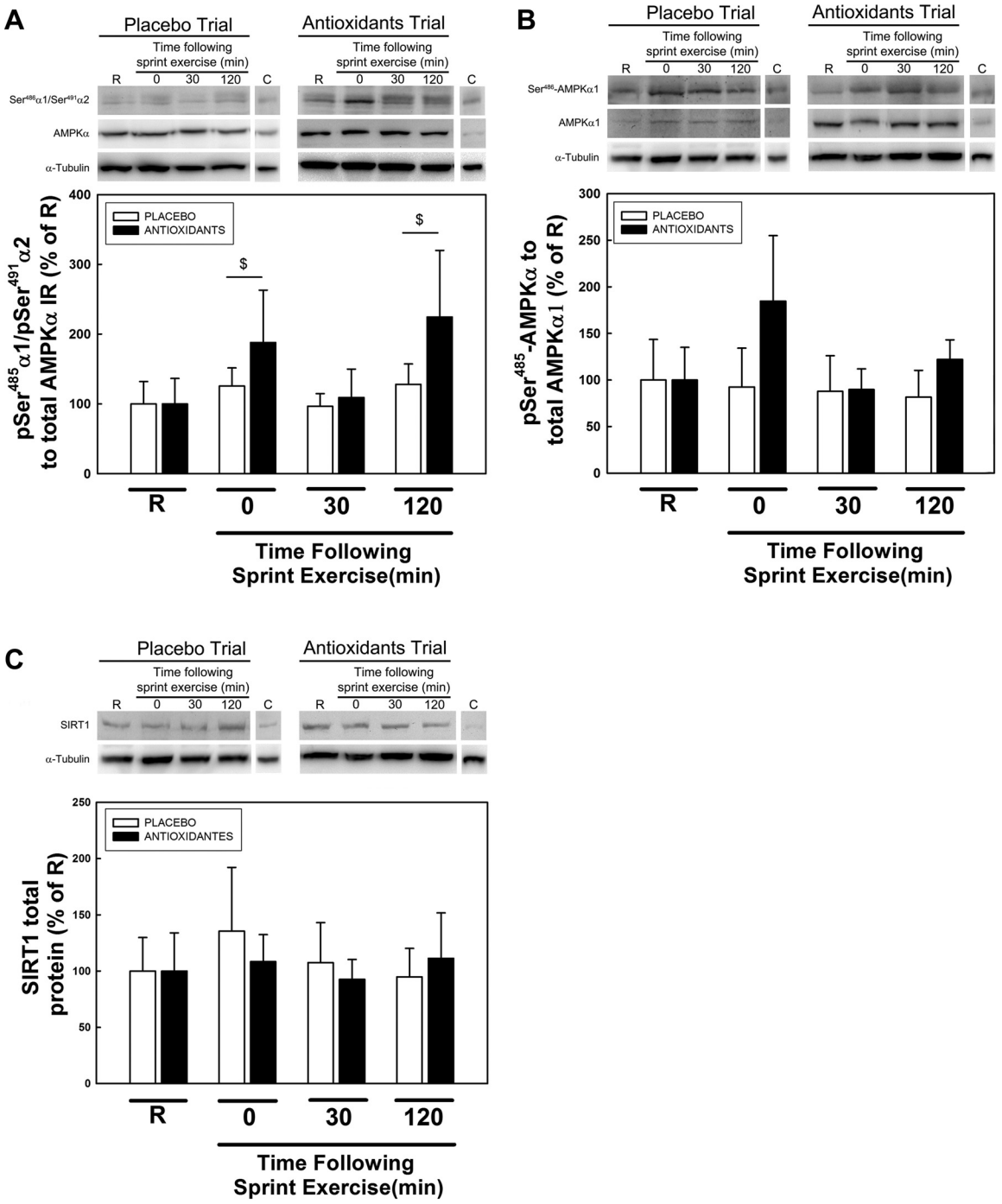

Fig. 4. Levels of Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 (*A*), Ser^{485}-AMPKα_1 (*B*), and sirtuin 1 (SIRT1) (*C*) before and after a single Wingate test performed after ingestion of placebo (open bars) or antioxidants (solid bars). Values under both experimental conditions were normalized to those observed in the biopsies obtained immediately before the sprint exercise (R), which were assigned a value of 100%. *A*, *top*: representative Western blot with antibodies against AMPKα, pSer^{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, pSer^{485}-AMPKα_1, and α-tubulin. *Bottom*, Ser^{485}-AMPKα_1 phosphorylation values relative to total AMPKα_1. *C*, *top*: representative Western blot with antibodies against SIRT1 and α-tubulin. *Bottom*, SIRT1 values. \$$P < 0.05$ vs. R. Statistical analysis was performed with logarithmically transformed data. Error bars represent SEs. $n = 9$ in both experimental conditions.

reported no interference of antioxidants with exercise-induced mitochondrial biogenesis in response to training in rats. Since vitamin C, particularly when given alone, may have prooxidant effects inducing increased the expression of antioxidants in skeletal muscle (70), the stimulus added by training in rats receiving vitamin C may be insufficient to further increase the expression of antioxidant proteins in skeletal muscle. It is also possible that the effects of vitamin C supplementation on skeletal muscle mitochondrial biogenesis and antioxidant enzymes depend on the duration of the supplementation (days or

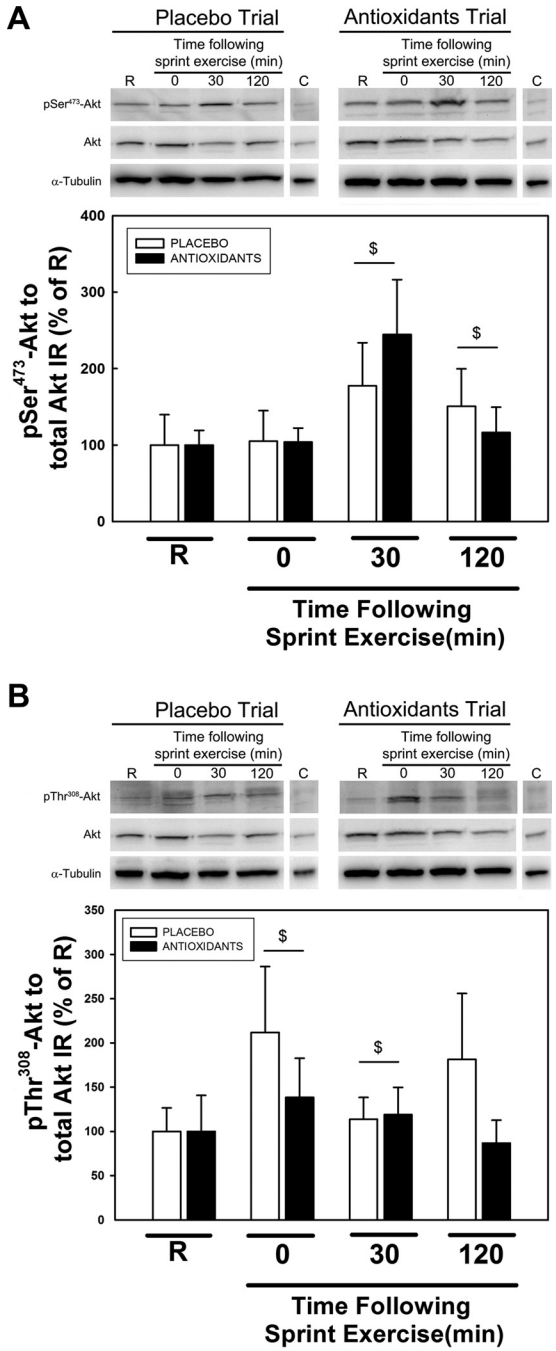

Fig. 5. Levels of Ser^{473}-Akt (*A*) and Thr^{308}-Akt (*B*) before and after a single Wingate test performed after ingestion of placebo (open bars) or antioxidants (solid bars). Values under both experimental conditions were normalized to those observed in the biopsies obtained immediately before the sprint exercise (R), which were assigned a value of 100%. *A*, *top*: representative Western blot with antibodies against Akt, pSer^{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, pThr^{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. Error bars represent SEs. $n = 9$ in both experimental conditions.

weeks) (68, 70). The fact that rodents produce endogenous vitamin C, which could be downregulated in response to supplementation in rats, further complicates the interpretation of these experiments.

In the present investigation, we administered an antioxidant cocktail containing α-lipoic acid, vitamin C, and vitamin E, which has been shown to decrease circulating free radicals in humans at rest (~98%) and during exercise (~85%) (54). These effects were assessed by EPR, a high sensitive technique (54). Vitamin C is a water-soluble antioxidant capable of quenching mitochondrial RNOS (38). Vitamin E is a potent lipid-soluble antioxidant preventing lipid peroxidation (4). α-Lipoic acid is an 8-carbon fatty acid containing a disulfide bond that, upon cellular entry, is reduced, generating dithiol dihydrolipoic acid. Dithiol dihydrolipoic acid is a water-soluble antioxidant that quenches hypochlorous acid, hydroxyl radicals, peroxyl radicals, and singlet oxygen and can also recycle ascorbate, glutathione, and vitamin E from their oxidized forms (23, 42, 47, 59). In combination, vitamin E and α-lipoic acid are considered as a potent antioxidant mixture (39).

The lack of change in protein carbonylation in response to sprint exercise does not imply lack of RNOS production; it may simply indicate that RNOS have been captured by other molecules and antioxidant systems. We (43) have previously observed an increase in protein carbonylation after sprint exercise in severe hypoxia. However, the level of protein carbonylation induced by a single sprint in normoxia may be too low to be detected by Western blot analysis in plasma and muscle (63).

Thr^{286}-CaMKII could phosphorylate AMPK on Thr^{172} (by a direct or indirect mechanism), and this may be physiologically relevant during high-intensity exercise (11). Thr^{286}-CaMKII phosphorylation is regulated in Ca^{2+}-dependent manner (52), and the increase of sarcoplasmic Ca^{2+} during sprint exercise depends to great extend on the opening of the ryanodine receptor, which is activated by RNOS and changes of the antioxidant status (75). However, the fact that peak power output was not affected by the ingestion of antioxidants is against a major change in sarcoplasmic Ca^{2+} transients.

RNOS may also 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 (13). Thus, reducing RNOS by antioxidant administration could result in lower CaMKII activity by a mechanism unrelated to sarcoplasmic Ca^{2+}.

Wright et al. (74) have shown that tyrosine and Ser/Thr phosphatases may be inhibited by exposure to RNOS in skeletal muscle. Antioxidant administration before exercise may attenuate the inhibitory RNOS influence on protein phosphatases, leading to increased Thr^{286}-CaMKII and Thr^{172}-AMPKα phosphorylation. Against such a mechanism is the observed lack of change of basal levels of Thr^{286}-CaMKII/Thr^{172}-AMPKα phosphorylation after the ingestion of antioxidants. Moreover, after sprint exercise, Thr^{286}-CaMKII/Thr^{172}-AMPKα phosphorylations were abrogated, indicating that any inhibitory effect of antioxidants on protein phosphatases was minor and outweighed by the stimulating effect of RNOS on Thr^{286}-CaMKII/Thr^{172}-AMPKα phosphorylations.

Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation may mediate the blunting effect of antioxidant ingestion on sprint-induced Thr^{172}-AMPKα phosphorylation in skeletal muscle. Here, we showed that Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation is increased immediately after a sprint exercise when preceded by the ingestion of antioxidants. A similar response was observed after a 30-s cycling sprint performed with severe acute hypoxia (43). A fast intracellular mechanism should account for Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, since this phosphorylation was present 10 s after the sprint. Sprint exercise in severe acute hypoxia elicits a higher glycolytic rate, with lower muscle pH and a greater degree of protein carbonylation, than a similar sprint in normoxia, suggesting increased RNOS production in hypoxia (43). The results of the present study indicate that block of RNOS-mediated signaling with antioxidants increased Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation. Thus, it seems that an excessively high or low level of RNOS during sprint exercise may end causing the same outcome, i.e., Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation and subsequent blunting of the expected Thr^{172}-AMPKα phosphorylation. This dual effect of RNOS is not new; in fact, it has been reported that high RNOS (30, 58), in addition to preventing RNOS signaling (55), are associated with insulin resistance. Thus, it seems that an optimal level of RNOS-mediated signaling during sprint exercise is required to elicit Thr^{172}-AMPKα phosphorylation.

Akt (PKB) (28) and PKA (31) can phosphorylate AMPKα_1/α_2 at $Ser^{485/491}$, which inhibits AMPK phosphorylation at Thr^{172} (28, 31, 48, 65). In the present investigation, Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation occurred immediately after the sprint, whereas Ser^{473}-Akt phosphorylation was increased 30 min after the end of the sprint, suggesting that alternative mechanisms should account for the immediate postexercise Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation. In our previous study (43), the temporal pattern of Thr^{308}-Akt phosphorylation after a sprint performed in hypoxia coincided with AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation. In the present study, Thr^{308}-Akt phosphorylation also occurred immediately after the sprint exercise but without significant differences attributable to the ingestion of antioxidants. An alternative mechanism for exercise-induced Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation could involve PKA, but this is uncertain (69).

Lac-to-Pyr and NAD^+-to-$NADH.H^+$ ratios and SIRT1/LKB1/AMPK signaling. Previous studies (2, 49) have also reported similar glycolytic rates and metabolite changes during Wingate tests (2, 49). As a novelty, the results of this study show that antioxidant administration before the sprints does not seem to modify the energy metabolism during a short sprint and has no impact on muscle fatigue. This may not be the case during submaximal contractions, which are more sensitive to RNOS-mediated fatigue mechanisms (72). In both trials, the contribution of the anaerobic metabolism was comparable, as reflected by the similar O_2 deficits and intra-muscular accumulation of Lac.

We have recently shown that during sprint exercise in severe hypoxia, the glycolytic rate is markedly increased to compensate for the reduction in oxidative energy yield, leading to greater Lac accumulation and Lac-to-Pyr ratios (43) and, hence, a large reduction of the NAD^+-to-$NADH.H^+$ ratio (60, 73). Consequently, the sprint exercise in hypoxia was associated with reduced SIRT1-mediated signaling and blunted Thr^{172}-AMPKα phosphorylation during the recovery period compared with a similar sprint in normoxia. One of the most important upstream kinases for Thr^{172}-AMPKα phosphorylation is LKB1, whose activity parallels the changes of the NAD^+-to-$NADH.H^+$ ratio (29). A larger reduction of SIRT1 activity is expected when the NAD^+-to-$NADH.H^+$ ratio is lowered. Since SIRT1 deacetylates (and activates) LKB1 (29), lower LKB1 activity is expectable when the NAD^+-to-$NADH.H^+$ ratio is reduced, and this mechanism could explain the blunted Thr^{172}-AMPKα phosphorylation after the sprint exercise in hypoxia (43). However, in the present investigation, Lac-to-Pyr and cytoplasmatic NAD^+-to-$NADH.H^+$ ratios as well as SIRT1 protein content were similar after both sprints. Consequently, the stimulus provided by SIRT1 to activate LKB1 should have been similar, which means that the blunting Thr^{172}-AMPKα phosphorylation by antioxidants is unlikely to be caused by lower LKB1 activity after the ingestion of antioxidants. Nevertheless, a definitive conclusion regarding the role played by SIRT1 in the blunted Thr^{172}-AMPKα phosphorylation by antioxidants requires a direct assessment of SIRT1 activities (22).

The ingestion of a combination of vitamin C (1,000 mg/day) and vitamin E (400 IU/day) prevents the positive effects of exercise (twenty 85-min sessions × 5 days/wk × 4 wk) on fasting plasma insulin concentrations, insulin sensitivity, and plasma adiponectin levels and attenuates the induction of PGC-1α, PGC-1β, PPAR-γ, and antioxidant enzyme gene expression in skeletal muscle (55). What the present investigation adds is that we have shown that the ingestion of antioxidants before a single sprint, which we have shown is able to induce PGC-1α (20), prevents Thr^{172}-AMPKα phosphorylation, a critical step for the induction of PGC-1α gene expression (9, 33). Therefore, ingestion of antioxidants before sprint exercise may also prevent some of the expected positive muscle adaptations on insulin sensitivity and antioxidant capacity or blunt the improvement in performance (37).

Ser^{221}-ACCβ phosphorylation is dissociated from Thr^{172}-AMPKα phosphorylation. Ser^{221}-ACCβ phosphorylation has been often used as an indicator of AMPK activity. However, evidence is accumulating showing that, at least during sprint exercise, Thr^{172}-AMPKα phosphorylation is not required for Ser^{221}-ACCβ phosphorylation (14, 19, 43). In agreement with this view, the present investigation clearly demonstrates that abrogation of Thr^{172}-AMPKα phosphorylation through the administration of antioxidants has no effect on the Ser^{221}-ACCβ phosphorylation response to sprint exercise. This result also agrees with the reported lack of effects of *N*-acetylcysteine infusion on fat oxidation during prolonged exercise (41).

In conclusion, we have shown that RNOS play a critical role in AMPK-mediated signaling after sprint exercise in human skeletal muscle. Antioxidant ingestion 2 h before sprint exercise abrogates the Thr^{172}-AMPKα phosphorylation response observed after the ingestion of placebo by reducing CaMKII and increasing Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation. This could explain the blunted response to sprint training with antioxidant ingestion reported by others (37). Finally, we have shown that the ingestion of antioxidants immediately before a single sprint has no influence on exercise performance, muscle aerobic and anaerobic metabolism, and AMP-

to-ATP and NAD^+-to-$NADH.H^+$ ratios. These findings could help to explain why antioxidants may blunt some adaptations to sprint training.

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

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