Eur J Appl Physiol (2010) 109:731–743
DOI 10.1007/s00421-010-1413-y

ORIGINAL ARTICLE

SIRT1, AMP-activated protein kinase phosphorylation and downstream kinases in response to a single bout of sprint exercise: influence of glucose ingestion

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Accepted: 17 February 2010 / Published online: 9 March 2010
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Abstract This study was designed to examine potential in vivo mechanisms of AMP-activated protein kinase (AMPK) phosphorylation inhibition and its downstream signaling consequences during the recovery period after a single bout of sprint exercise. Sprint exercise induces Thr^{172}-AMPK phosphorylation and increased PGC-1α mRNA, by an unknown mechanism. Muscle biopsies were obtained in 15 young healthy men in response to a 30-s sprint exercise (Wingate test) randomly distributed into two groups: the fasting ($n = 7$, C) and the glucose group ($n = 8$, G), who ingested 75 g of glucose 1 h before exercising to inhibit AMPKα phosphorylation. Exercise elicited different patterns of Ser^{221}-ACCβ, Ser^{473}-Akt and Thr^{642}-AS160 phosphorylation, during the recovery period after glucose ingestion. Thirty minutes after the control sprint, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation was reduced by 33% coinciding with increased Thr^{172}-AMPKα phosphorylation (both, $P < 0.05$). Glucose abolished the 30-min Thr^{172}-AMPKα phosphorylation. Ser^{221}-ACCβ phosphorylation was elevated immediately following and 30 min after exercise in C and G, implying a dissociation between Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation. Two hours after the sprint, PGC-1α protein expression remained unchanged while SIRT1 (its upstream deacetylase) was increased. Glucose ingestion abolished the SIRT1 response without any significant effect on PGC-1α protein expression. In conclusion, glucose ingestion prior to a sprint exercise profoundly affects Thr^{172}-AMPKα phosphorylation and its downstream signaling during the recovery period.

Keywords Signaling · Wingate · Human · Skeletal muscle · Fatigue · Performance

Communicated by Susan Ward.

Electronic supplementary material The online version of this article (doi:10.1007/s00421-010-1413-y) contains supplementary material, which is available to authorized users.

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Introduction

Recent studies indicate that exercise-induced muscle signaling and training adaptations are affected by the pre-exercise diet (De Bock et al. 2008; Deldicque et al. 2009; Wilkinson et al. 2008; Yeo et al. 2008). However, it remains unknown if pre-exercise diet influences muscle signaling in response to sprint exercise, in which the AMP-activated protein kinase (AMPK) plays a crucial role (Carling 2004; Davies et al. 1994; Hardie et al. 2006; Long and Zierath 2008).

AMPK is activated by cellular stress associated with ATP depletion, together with a concomitant rise in AMP/ATP ratio (Carling 2004; Hardie et al. 2003), as observed

during repeated sprint exercise in human skeletal muscle (Gibala et al. 2009). In skeletal muscle, the activity of AMPK is dependent on the Thr^{172} phosphorylation in the activation loop of the α-subunit by the upstream kinase LKB1 (Sakamoto et al. 2005). AMP acts allosterically via the γ-subunit to enhance phosphorylation of Thr^{172}-AMPKα by LKB1 kinase (Hawley et al. 2003; Woods et al. 2003a) and to suppress dephosphorylation by protein phosphatases PP2A and PP2C in vitro (Davies et al. 1995). The LKB1/AMPK signaling axis is activated by SIRT1, an NAD-dependent deacetylase that acts as a master metabolic sensor of NAD^+ (Hou et al. 2008). SIRT1 plays an important role in mitochondrial biogenesis, fatty acid oxidation and glucose homeostasis through deacetylation of PGC-1α (Gerhart-Hines et al. 2007). It has been shown that endurance exercise increases SIRT1 protein expression in rat skeletal muscle (Suwa et al. 2008) and this up-regulation of SIRT1 protein expression correlates with its activity (Gerhart-Hines et al. 2007). In contrast, Gurd et al. (2009) and Chabi et al. (2009) have recently reported no association between muscle oxidative capacity and SIRT1 protein expression or deacetylase activity. It remains unknown if sprint exercise may affect SIRT1 protein expression in human skeletal muscle and how this may influence PGC-1α protein levels.

Incubation of cells at high glucose concentration reduces SIRT1 protein amount and AMPK phosphorylation (Suchankova et al. 2009). Conversely, incubation in a pyruvate-enriched medium results in increases in SIRT1 protein expression and AMPK phosphorylation (Suchankova et al. 2009), likely linked to an elevation of the ratio NAD^+/$NADH.H^+$. It remains unknown if glucose ingestion may affect the SIRT1 response to sprint exercise.

Insulin inhibits AMPK activity and fatty acid oxidation in Fao hepatoma cells (Witters and Kemp 1992) and in rat heart (Gamble and Lopaschuk 1997). Moreover, insulin antagonizes anoxia or ischemia-induced Thr^{172}-AMPKα phosphorylation (Horman et al. 2006; Kovacic et al. 2003) without a detectable change in the AMP/ATP ratio (Beauloye et al. 2001). The effect of insulin is blocked by wortmannin, an inhibitor of phosphatidylinositol-3-kinase; and only occurs when insulin is bathing the cells during some time before anoxia, suggesting a hierarchical control. Insulin inhibits AMPKα Thr^{172} phosphorylation through Ser^{485}-AMPKα1 and Ser^{491}-AMPKα2 phosphorylation (Horman et al. 2006) by increasing Akt activity (Kovacic et al. 2003; Soltys et al. 2006). In transgenic mice overexpressing constitutively active Akt and in neonatal cardiomyocytes infected with adenovirus expressing constitutively active Akt isoforms, Thr^{172}-AMPKα phosphorylation is decreased (Kovacic et al. 2003).

It remains unknown if glucose ingestion may also attenuate Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation after one bout of high-intensity exercise. The inhibitory effect of glucose ingestion on Thr^{172}-AMPKα phosphorylation could be mediated by the concomitant phosphorylation of $Ser^{485/491}$ in the catalytic α1/α2-subunits, which may blunt Thr^{172} phosphorylation by LKB1 (Horman et al. 2006; Woods et al. 2003b). Glucose ingestion before sprint exercise increases insulin concentration which increases pyruvate dehydrogenase (PDH) activity, facilitating pyruvate conversion to acetyl CoA (Abbot et al. 2005).

Thus, we hypothesized that by increasing plasma insulin concentrations prior to an all-out sprint test, via the ingestion of 75 g of glucose 1 h before the sprint, Akt would be activated inducing $Ser^{485/491}$ phosphorylation in the catalytic AMPKα1/α2-subunits limiting Thr^{172}-AMPKα phosphorylation immediately after the exercise or during the recovery period. In addition, we also hypothesized that sprint exercise will result in an increase in SIRT1 protein expression in human skeletal muscle and that this effect will be attenuated by glucose ingestion prior to an all-out sprint test, by reducing lactate production and/or blunting AMPKα Thr^{172} phosphorylation. This hypothesis is based on the fact that insulin may blunt AMPKα Thr^{172} phosphorylation and may increase PDH activity (Abbot et al. 2005), and with a higher PDH activity at the start of the sprint we expect a lower lactate production and lower increase of the ratio NAD^+/$NADH.H^+$ with exercise.

Therefore, the aims of this study were (1) to determine the effect of a single bout of 30-s all-out exercise on the cycle ergometer (Wingate test) on skeletal muscle Thr^{172}-AMPKα phosphorylation immediately after the exercise and during the recovery period; (2) to find out if glucose ingestion prevents Thr^{172}-AMPKα and ACCβ phosphorylation in response to high-intensity exercise, immediately after and during the recovery period; (3) to determine if exposure of human skeletal muscle to physiologically elevated insulin levels for 1 h prior to sprint exercise elicits phosphorylation of Akt and its downstream kinase Akt substrate of 160 kDa (AS160), leading to phosphorylation of Ser^{485}-AMPKα1 and/or Ser^{491}-AMPKα2 and, hence, prevents Thr^{172}-AMPKα phosphorylation immediately at the end of exercise and during the recovery period; and (4) to determine if SIRT1 and PGC-1α protein expression is increased by sprint exercise and the effect that glucose ingestion prior to the sprint exercise may have on this response.

Materials and methods

Materials

The complete protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany). All the primary antibodies used were from Cell Signaling Technology

(Danvers, MA, USA) except for the polyclonal rabbit anti-phospho-acetyl CoA carboxylase (Ser[79]) antibody that was obtained from Upstate Biotechnology (Lake Placid, NY, USA), the polyclonal anti-phospho-AS160 (Thr[642]) that was obtained from MBL International Corporation (Woburn, MA, USA), the polyclonal rabbit anti-PGC-1α that was obtained from Abcam (Cambridge, UK) and the monoclonal mouse anti-alpha-tubulin antibody that was obtained from Biosigma (Sigma, St. Louis, MO, USA). The specificity of PGC-1α was tested with a competitive assay (supplementary Figure 1). The secondary HRP-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch (West Grove, PA, USA). The Hybond-P transfer membranes and the ECL plus Western Blotting Detection System were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). The ChemiDoc XRS System and the image analysis software Quantity One© were obtained from Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, UK).

Subjects

Fifteen healthy male physical education students (age 23.4 ± 0.6 years, height 178 ± 1.8 cm, body mass 77.5 ± 2.1 kg, body fat 14.7 ± 1.9%) agreed to participate in this investigation (Table 1). Before volunteering, subjects were given full oral and written information about the course of the study and possible risks associated with participation. Written consent was obtained from each subject. The study was performed in accordance with the Helsinki Declaration and approved by the Ethical Committee of the University of Las Palmas de Gran Canaria.

General procedures

The body composition of each subjects was determined by DXA (Hologic QDR-1500, Hologic Corp., software version 7.10, Waltham, MA) as described elsewhere (Ara et al. 2004; Perez-Gomez et al. 2008b). On a different day, subjects reported to the laboratory at 8.00 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 and used to measure serum glucose and insulin. Then a muscle biopsy was obtained from the middle portion of the vastus lateralis muscle using the Bergstrom's technique with suction, as described elsewhere (Guerra et al. 2007; Perez-Gomez et al. 2008a). Subjects were randomly divided into a control group (seven subjects) and the glucose group (eight subjects), matched for physical characteristics and performance (Table 1). Three minutes after the resting muscle biopsy and blood sample, the control group performed a 30-s Wingate test with a braking force equivalent to 10% of their body mass as described elsewhere (Calbet et al. 1997, 2003). No warm up was allowed prior to the start of the Wingate test. Immediately after the Wingate test, the subjects were moved rapidly to a bed placed nearby the ergometer and another muscle biopsy and blood sample were obtained. The time needed to obtain and freeze the muscle biopsies immediately after the Wingate test was always below 30 s in all cases. During the following 4 h, the subjects were fasting and sat quietly in the laboratory or in the library of our faculty. During the recovery period, additional muscle biopsies and blood samples were obtained at 30, 120 and 240 min. The last two or three muscle biopsies were obtained from the contralateral leg. Only one incision was practiced in each thigh. Although it has been reported that using the same incision may elicit injury-induced activation of some enzymes like the extracellular-regulated kinases (ERK1 and ERK2) or p38 mitogen-activated protein kinase (p38MAPK), this only happens in the muscle biopsies obtained close to a muscle injury (Aronson et al. 1998). In eight subjects, we obtained muscle biopsies from the same incision 60 min apart, but changing the direction of the needle immediately after crossing the fascia as carried out by Drummond et al. (2009). With this procedure, no changes were observed in ERK1–2 phosphorylation (data not shown). In addition, we also determined the phosphorylation of p38MAPK (pp38MAPK) in the glucose group in the last three consecutive biopsies which were obtained from the same incision at 30, 120 and 240 min after the Wingate test. Non-significant differences in pp38MAPK were observed (supplementary Figure 2). The glucose group ingested 75 g of glucose (Glucomedics-75 orange, Biomedics, Madrid, Spain) immediately following the resting muscle biopsy and blood sample and then rested on the bed or seated on chair for 60 min (not reported). At the end of this period, another muscle biopsy and another blood sample were obtained. Three minutes after the muscle biopsy, subjects performed a Wingate test, also without warming up, and muscle biopsies and blood samples were

Table 1 Physical characteristics and performance

	Mean ± SD	
	Control group (n = 7)	Glucose group (n = 8)
Age (years)	23 ± 2	23 ± 2
Height (cm)	176.2 ± 9.1	180.1 ± 3.9
Body mass (kg)	78.1 ± 10.4	76.9 ± 6.3
% Body fat	17.7 ± 6.6	12.0 ± 7.2
Pmax (W)	1,018.6 ± 138.7	1,032.2 ± 135.2
Pmax (W/kg body mass)	13.2 ± 2.1	13.3 ± 1.4
Pmean (W)	676.9 ± 91.1	706.9 ± 37
Pmean (W/kg body mass)	8.7 ± 1.1	9.2 ± 0.7

Pmax, *Pmean* peak and mean power output in the Wingate test

Values are means ± SD. All $P > 0.05$

obtained with the same time frame as for the control group. The muscle specimens were cleaned to remove any visible blood, fat, or connective tissue. Then, the muscle tissue was immediately frozen in liquid nitrogen and stored at −80°C for later analysis.

Western blot analysis

Muscle protein extracts were prepared as described previously (Guerra et al. 2007) and total protein content was quantified using the bicinchoninic acid assay (Smith et al. 1985). Equal amounts (50 μg) of each sample were subjected to immunoblotting protocol as described previously (Guerra et al. 2007). To determine Thr^{172}-AMPKα, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, Ser^{485}-AMPKα1, Ser^{221}-ACCβ, Ser^{473}-Akt and Thr^{642}-AS160 phosphorylation levels, antibodies directed against the phosphorylated and total form of these kinases were used all diluted in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T) (BSA-blocking buffer). AMPKα2 was immunoprecipitated to test the specificity of Ser^{485}-AMPKα1 and Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 antibodies (data not shown). To detect PGC-1α and SIRT1 protein expression, membranes were incubated with a polyclonal-specific anti-human PGC-1α and SIRT1 antibodies (diluted in BSA-blocking buffer). To control for differences in loading and transfer efficiency across membranes, membranes were incubated with a monoclonal mouse anti-alpha-tubulin antibody diluted in TBS-T with 5% blotting grade blocker non-fat dry milk (blotto-blocking buffer). Antibody-specific labeling was revealed by incubation with a HRP-conjugated goat anti-rabbit antibody (1:20,000) or a HRP-conjugated donkey anti-mouse (1:10,000) antibody both diluted in blotto-blocking buffer and visualized with the ECL chemiluminescence kit (Amersham Biosciences). Specific bands were visualized with the ECL chemiluminescence kit, using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with the image analysis program Quantity One© (Bio-Rad laboratories, Hercules, CA, USA). For immunosignal quantification, band densities were normalized to the values obtained from the biopsies taken immediately before the start of the sprint. Data were represented as a percentage of immunostaining values obtained for the phosphorylated form of each kinase relative to those obtained for, respectively, total form or as a percentage of immunostaining values obtained for PGC-1α or SIRT1 relative to those obtained for alpha-tubulin. Alpha-tubulin content in the muscle biopsies of the two experimental groups was similar (data not shown, $P > 0.05$). Western blot analysis of all proteins studied was performed in triplicate for each muscle biopsy with a variation coefficient less than 10%. Samples from each subject were running in the same gel.

Insulin measurements

Serum insulin was measured by an electrochemiluminescence immunoassay (ECLIA) intended for use on Modular Analytics analyzer E170 using Insulin kit reagents (Roche/Hitachi, Indianapolis, USA). Insulin sensitivity was 0.20 μU/ml.

Glucose and lactate measurements

Serum glucose was measured by the hexokinase method using Gluco-quant reagents (Roche/Hitachi, 11876899216, Indianapolis, USA) with a sensitivity of 2 mg/dl. Blood lactate concentration was determined in capillary blood obtained from the ear lobe hyperemized with Finalgon, prior to the start of the sprint and at 3, 5, 7 and 10 min into the recovery period, using a Lactate Pro analyzer (Arkay, JA).

Statistics

Variables were checked for normal distribution using a Kolmogorov–Smirnov test with the Lilliefors correction, and for equality of variances with the Levene's test. When necessary, the analysis was done on logarithmically transformed data. For between-group comparisons, the individual responses were normalized to the level of phosphorylation observed just before the start of the Wingate test. A mixed-model ANOVA with repeated measures over time and one factor (treatment, not repeated) with two levels (control vs. glucose) was used to compare the responses with the value just before the start of the Wingate test, using values normalized to the level of phosphorylation observed just before the start of the Wingate test. When there was a significant treatment × time interaction, intra-group effects were tested using one-way ANOVA separately in each group, and pairwise comparisons were carried out using the Holm–Bonferroni method. Unpaired *t* tests were used for planned comparisons to test between-group differences at specific time points, the corresponding *P* values were adjusted for multiple comparisons with the Holm–Bonferroni method. Since we missed three muscle biopsies corresponding to the 240-min time point (two in the glucose group and one in the control group), these analyses were limited to the first 120 min. The 240-min point was compared to the pre-exercise condition using a paired *t* test. The relationship between variables was determined using linear regression analysis with calculation of the Pearson's correlation coefficient. Values are reported as the mean ± standard error of the mean (unless otherwise stated). $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL).

Results

Serum insulin and glucose, and blood lactate responses

Compared to the control group, insulin concentration was elevated by 4.5-fold in the glucose group prior to the start of the Wingate test (Fig. 1), whereas plasma glucose concentrations were similar in both groups (Fig. 1b). The serum insulin responses to the Wingate test were significantly different (time × group interaction $P < 0.05$). Compared to pre-exercise values, 30 min after exercise, insulin concentration was increased (by 123%, $P < 0.05$) and decreased (by 53%, $P < 0.05$) in the control and glucose groups, respectively (Fig. 1a).

During the recovery period, there was a significant group × time interaction ($P < 0.01$) for glucose concentration, which increased in the control group to peak at 30 min while it decreased in the glucose group to reach a nadir at 30 min (both, $P < 0.05$, compared to pre-exercise values). Thus, at 30-min post-exercise, the plasma glucose concentration was 34% higher in the control compared to the glucose group (105.9 ± 3.1 and 79.0 ± 6.7 mg/dl, respectively, $P < 0.05$; Fig. 1b).

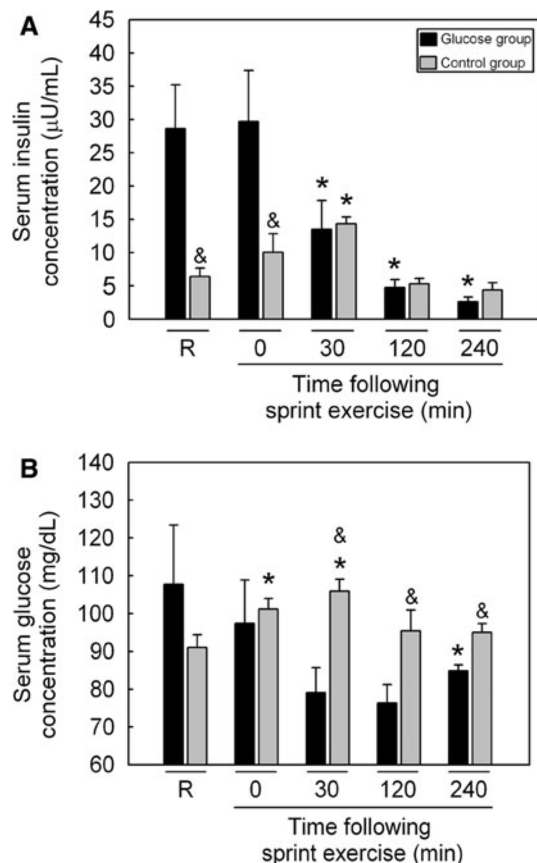


Fig. 1 Serum insulin (**a**) and glucose (**b**) responses, before and after a Wingate test. The glucose group (*black bars*) ingested 75 g of glucose 60 min prior to the start of the Wingate test. The control group (*gray bars*) performed the test under fasting conditions. R represents the value just prior to the start of the Wingate test. *$P < 0.05$ versus rest (R); $^{\&}P < 0.05$ versus glucose group ($n = 7$ for the control group and $n = 8$ for the glucose group, except for the 240-min sample for which $n = 6$ in both conditions)

The mean blood lactate concentration between the third and tenth minutes of the recovery was 12% lower in the glucose compared to the control group (14.4 ± 0.5 and 12.6 ± 0.4 mmol l^{-1}, respectively, $P < 0.05$).

Influence of glucose ingestion on Thr^{172}-AMPKα and Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation after high-intensity exercise

Thr^{172}-AMPKα phosphorylation immediately following the exercise (0 min) and after 120 and 240 min was not significantly different from resting (pre-exercise) conditions in the control group ($P = 0.54$, $P = 0.25$ and $P = 0.36$, respectively, vs. rest; Fig. 2a, b). However, compared to pre-exercise values, Thr^{172}-AMPKα phosphorylation was enhanced fivefold 30 min after the sprint exercise (from 100 ± 4 to 531 ± 215%, $P < 0.05$; Fig. 2a, b). This effect was prevented by the ingestion of glucose (Fig. 2a, b). At 30-min post-exercise, the relative change in Thr^{172}-AMPKα phosphorylation was tenfold higher in the control compared to the glucose group ($P < 0.05$; Fig. 2a, b).

Compared to pre-exercise values, Thr^{172}-AMPKα phosphorylation was increased about fourfold 240 min after the Wingate test performed following glucose ingestion ($P < 0.05$; Fig. 2a, b). Thus, at 240-min post-exercise, Thr^{172}-AMPKα phosphorylation was higher in the glucose than in control group (425 ± 176 and 101 ± 46%, respectively, $P = 0.05$, $n = 6$ in each group; Fig. 2a, b).

Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation was 100 ± 6% prior to the start of the control Wingate test and 165 ± 31% immediately after ($P = 0.06$), and decreased to 67 ± 7% ($P < 0.05$) 30 min after the end of the Wingate test (Fig. 2a, c). This reduction in Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation coincided with a fivefold increase in Thr^{172}-AMPKα phosphorylation (Fig. 2a, b).

In contrast, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 was increased immediately and 30 min after the Wingate test performed following glucose ingestion (from 100 ± 8 to 205 ± 37 and 283 ± 41%, respectively, $P < 0.05$; Fig. 2a, c). At 30-min post-exercise, the relative change in Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation was threefold higher in the glucose compared to the control group ($P < 0.05$; Fig. 2a, c).

The level of Ser^{485}-AMPKα1 phosphorylation was not affected by either glucose ingestion or exercise (Fig. 2a, d).

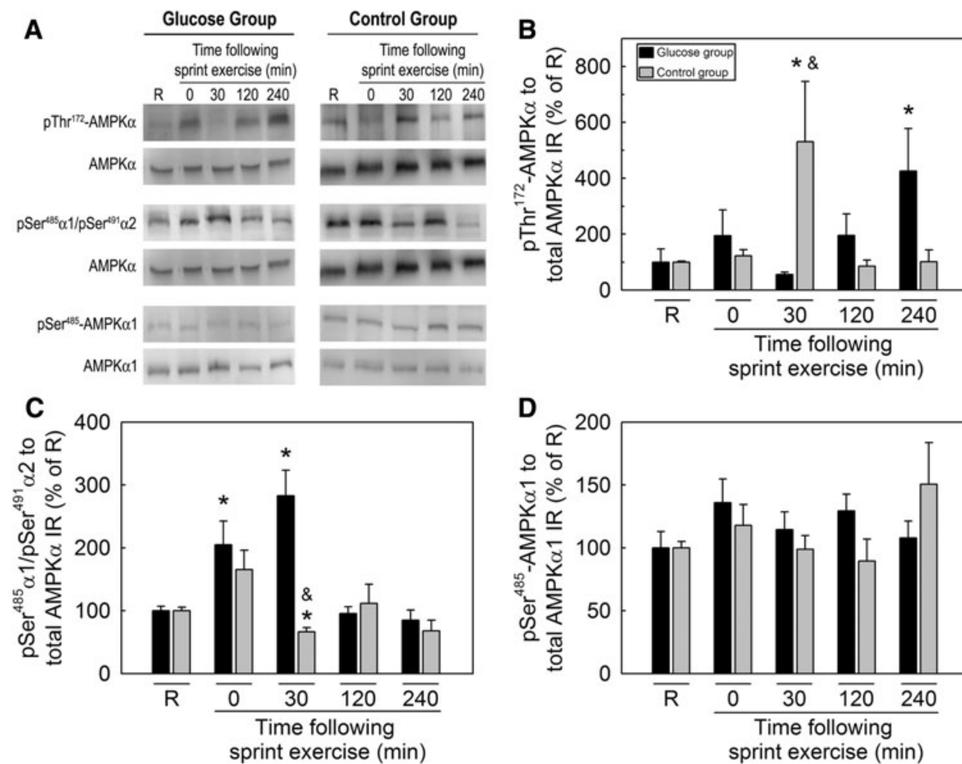


Fig. 2 Levels of Thr^{172}-AMPKα (α, β), Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 (**a**, **c**), and Ser^{485}-AMPKα1 (**a**, **d**) phosphorylation, before and after a Wingate test performed 60 min after the ingestion of 75 g of glucose (*black bars*) or under fasted conditions (*gray bars*). High serum insulin concentrations prior to the sprint exercise reduced Thr^{172}-AMPKα phosphorylation and increased Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 during the recovery period. Values were normalized to the average obtained immediately before the sprint exercise (R), which were assigned a value of 100%. **a** Representative western blots performed with the anti-phospho-Thr^{172}-AMPKα, the anti-phospho-Ser$^{485}\alpha$1/Ser$^{491}\alpha$2 or the Ser^{485}-AMPKα1 antibody show different levels of band densities obtained for both phospho-Thr^{172-}AMPKα and total AMPKα, phospho-Ser$^{485}\alpha$1/Ser$^{491}\alpha$2 and total AMPKα and phospho-Ser^{485}-AMPKα1 and AMPKα1 at the different time points in the glucose (*left column*) and control (*right column*) groups. **b** Densitometric analysis of the phospho-Thr^{172}-AMPKα immunoblots. Values are relative to total AMPKα immunosignals. **c** Densitometric analysis of the phospho-Ser$^{485}\alpha$1/Ser$^{491}\alpha$2 immunoblots. Values are relative to total AMPKα immunosignals. **d** Densitometric analysis of the phospho-Ser^{485}-AMPKα1 immunoblots. Values are relative to total AMPKα1 immunosignals. $^{*}P < 0.05$ versus R; $^{\&}P < 0.05$ versus glucose group. $n = 8$ for all glucose points and $n = 7$ for all control points, except 240 min for which $n = 6$, both in the control and glucose condition

Influence of glucose ingestion on ACCβ Ser^{221} phosphorylation after high-intensity exercise

Under control conditions, Ser^{221}-ACCβ phosphorylation was increased 3.6-fold immediately after the Wingate (from 100 ± 2 to $357 \pm 85\%$, respectively, $P < 0.05$) and remained at this level of phosphorylation 30 min into the recovery period ($330 \pm 57\%$, $P < 0.05$; Fig. 3). A similar response was observed during the recovery period that followed the Wingate test in the glucose group (Fig. 3). Compared to pre-exercise values, Ser^{221}-ACCβ phosphorylation was increased 2.1-fold immediately after the Wingate test (from 100 ± 24 to $213 \pm 33\%$, $P < 0.05$) and 3.1-fold 30 min into the recovery period (to $310 \pm 56\%$, $P < 0.05$; Fig. 3).

Influence of glucose ingestion on Ser^{473}-Akt phosphorylation on after high-intensity exercise

The ANOVA test revealed that Ser^{473}-Akt phosphorylation was significantly increased in response to exercise. Under control conditions, Ser^{473}-Akt phosphorylation level detected immediately following the exercise (0 min) was 63% higher than before the start of exercise ($P < 0.05$; Fig. 4a). Ser^{473}-Akt phosphorylation remained elevated during the first 120 min after the control Wingate test (ANOVA, $P < 0.05$; Fig. 4a). A different response was observed during the recovery period that followed the Wingate test in the glucose group. Compared to pre-exercise values, Ser^{473}-Akt phosphorylation was increased almost fivefold immediately after the Wingate test ($P < 0.05$) and

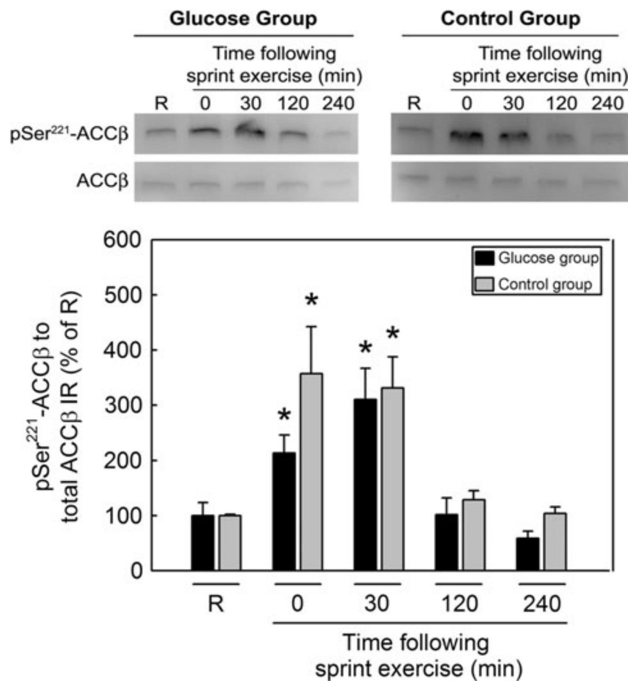


Fig. 3 Levels of Ser^{221}-ACCβ phosphorylation, before and after a Wingate test performed 60 min after the ingestion of 75 g of glucose (*black bars*) or under fasted conditions (*gray bars*). Values were normalized to the average obtained immediately before the sprint exercise, which were assigned a value of 100%. *Top panel* Representative western blot with the anti-phospho-Ser^{221}-ACCβ antibody shows different levels of band densities obtained for both phospho-Ser^{221}-ACCβ and total ACCβ at the different time points in both experimental groups. *Lower panel* Densitometric analysis of immunoblots. Values are relative to total ACCβ immunosignals. *$P < 0.05$ versus R. $n = 8$ for all glucose points and $n = 7$ for all control points, except 240 min for which $n = 6$, both in the control and glucose condition

almost sevenfold 30 min into the recovery period ($P < 0.05$, compared to pre-exercise; Fig. 4a). The 30th minute Ser^{473}-Akt phosphorylation remained twofold above the value observed in basal conditions, prior to the ingestion of glucose ($P < 0.05$; Fig. 4a). Thereafter, Ser^{473}-Akt phosphorylation was reduced to levels similar to those observed prior to the start of the Wingate test 2 and 4 h into the recovery period ($P = 0.13$ and $P = 0.88$, respectively; Fig. 4a).

Influence of glucose ingestion on AS160 Thr^{642} phosphorylation after high-intensity exercise

In the control test, Thr^{642}-AS160 phosphorylation was increased 120 min after the sprint exercise (from 100 ± 1% prior to the Wingate test to 187 ± 18.3% 120 min into the recovery period, $P < 0.05$; Fig. 4b). A different response was observed during the recovery period that followed the Wingate test in the glucose group. Compared to pre-exercise values, Thr^{642}-AS160 phosphorylation was increased almost twofold immediately after the Wingate test (from 100 ± 19.6 to 226 ± 80%, $P < 0.05$) and remained at this level 30 min into the recovery period (199.5 ± 45%, $P < 0.05$ compared to pre-exercise; Fig. 4b). As expected, Thr^{642}-AS160 phosphorylation was closely related to Ser^{473}-Akt phosphorylation ($r = 0.95$, $P < 0.01$, $n = 6$) in the glucose group.

Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation and Ser^{473}-Akt phosphorylation are correlated

As illustrated in Fig. 5, there was a correlation between Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation and Ser^{473}-Akt phosphorylation ($r = 0.84$, $P < 0.05$). No relationship was observed between Ser^{485}-AMPKα1 and Ser^{473}-Akt phosphorylation in either the glucose or control group.

Influence of glucose ingestion on SIRT1 protein expression after high-intensity exercise

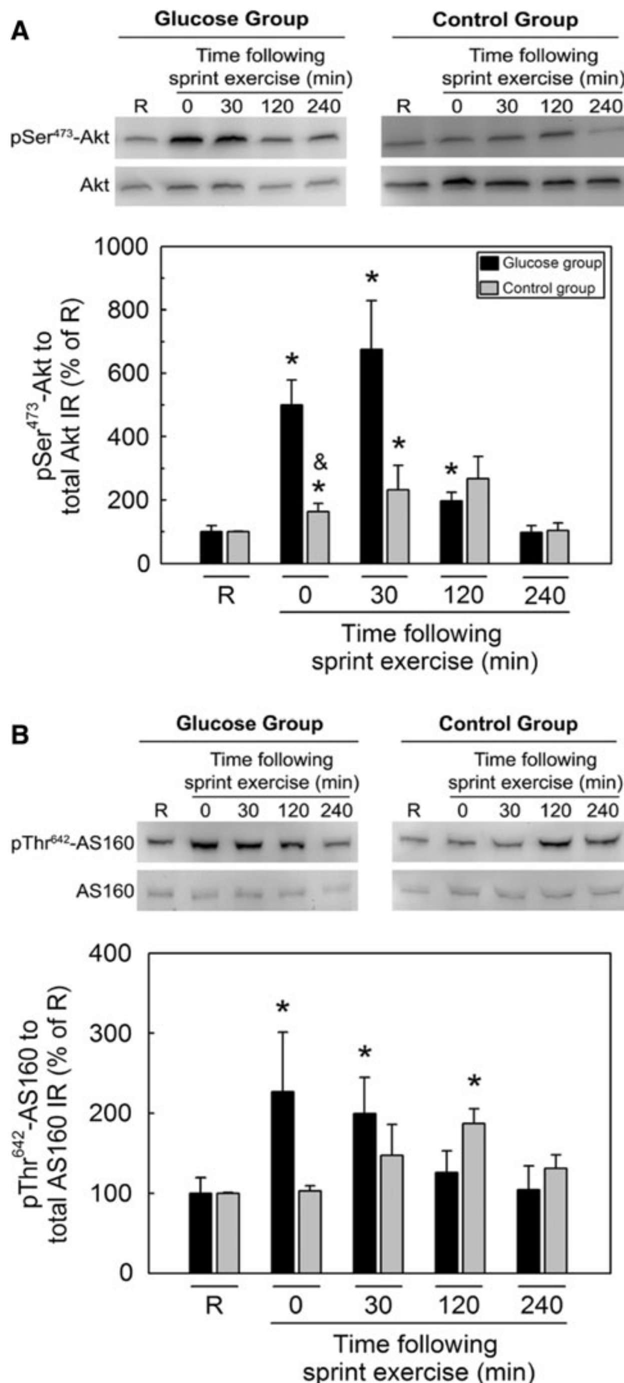
Under control conditions, SIRT1 protein expression was increased by 84%, 120 min into the recovery period (from 100.0 ± 12.0 to 184.9 ± 25.1%, $P < 0.05$; Fig. 6a), but remained unchanged after glucose ingestion. SIRT1 protein expression 120 min after the Wingate was lower during the test performed after glucose ingestion compared to the control conditions (98.9 ± 14.5% 120 min after Wingate in G and 184.9 ± 25.1% 120 min after Wingate in C; $P < 0.05$; Fig. 6a).

Influence of glucose ingestion on PGC-1α protein expression after high-intensity exercise

PGC-1α protein expression was not significantly altered by sprint exercise regardless of glucose ingestion (Fig. 6b).

Discussion

In this investigation, we have examined potential in vivo mechanisms of AMPK phosphorylation inhibition in human skeletal muscle and its downstream signaling consequences during the recovery period after a single bout of sprint exercise. We have shown that sprint exercise increases SIRT1 protein expression 120 min after a single all-out sprint of only 30 s. This effect was abrogated by the ingestion of 75 g of glucose prior to the sprint. However, despite the elevation of SIRT1 protein expression, PGC-1α remained unchanged. We have also shown that a sprint exercise 60 min after glucose ingestion elicits an immediate Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation. However, a physiological elevation of insulin (without exercise) does not increase Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation in resting human skeletal muscle. This study



◀ **Fig. 4** Levels of Ser^{473}-Akt (**a**) and Thr^{642}-AS160 (**b**) phosphorylation, before and after a Wingate test performed 60 min after the ingestion of 75 g of glucose (*black bars*) or under fasted conditions (*gray bars*). **a** *Top panel* A representative western blot with the anti-phospho-Ser^{473}-Akt antibody shows different levels of band densities obtained for both phospho-Ser^{473}-Akt and total Akt at the different time points in both experimental groups. *Lower panel* Densitometric analysis of immunoblots. Values are relative to total Akt immunosignals. **b** *Top panel* A representative western blot with the anti-phospho-Thr^{642}-AS160 antibody shows different levels of band densities obtained for both phospho-Ser^{473}-Akt and total Akt at the different time points in both experimental groups. *Lower panel* Densitometric analysis of immunoblots. Values are relative to total AS160 immunosignals. All values were normalized to the average of basal ones obtained prior to the start of the Wingate test which were assigned a value of 100%. $*P < 0.05$ versus R and $^{\&}P < 0.05$ versus glucose group. $n = 8$ for all points except 240 min for which $n = 6$

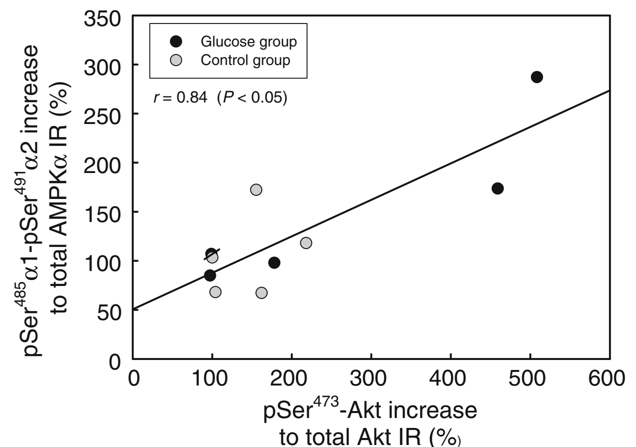
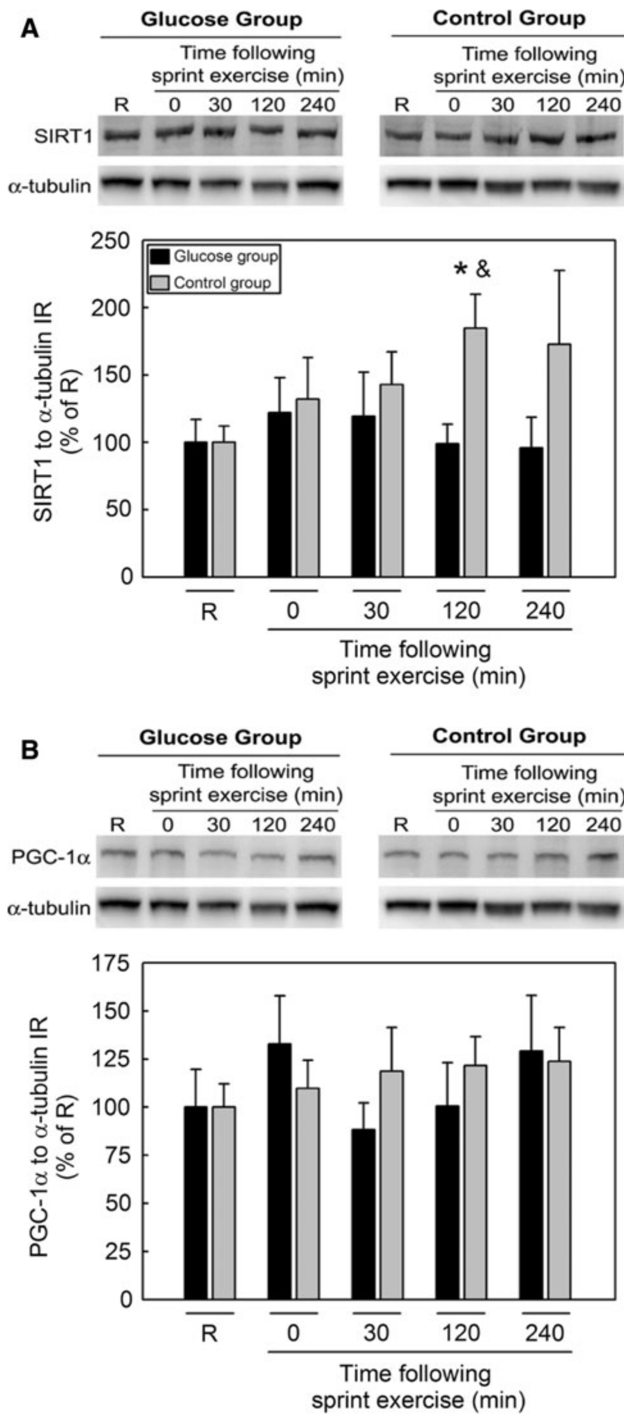


Fig. 5 Relationship between Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 and Ser^{473}-Akt phosphorylation. The glucose group (*black symbols*) ingested 75 g of glucose 60 min prior to the start of the Wingate test. The control group (*gray circles*) performed the test under fasting conditions. Each point represents of the mean of six subjects

provides also some evidence for a new mechanism that could regulate Thr^{172}-AMPKα2 phosphorylation in human skeletal muscle in response to exercise. By pre-exposing skeletal muscles to high (but physiological) levels of insulin prior to very-high-intensity exercise, we have blunted the Thr^{172}-AMPKα phosphorylation normally observed 30 min after the sprint exercise. This effect was accompanied by increased phosphorylation of Ser^{485}-AMPKα1/Ser^{491}-AMPKαa known mechanism of Thr^{172}-AMPKα phosphorylation inhibition in rodent cardiac myocytes (Horman et al. 2006; Kovacic et al. 2003). In addition, we have shown that sprint exercise alone, regardless of glucose ingestion, elicits almost instantaneously Ser^{473}-Akt phosphorylation and this effect last for 30–120 min, depending on the ingestion or not of glucose 60 min prior to the start of the sprint. Although we observed a correlation between Ser^{473}-Akt phosphorylation and Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation in the experiments after glucose ingestion, this was not the case when the exercise was performed under fasting conditions. Altogether, these results indicate that Ser^{473}-Akt phosphorylation alone cannot explain the exercise-induced Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation observed after glucose ingestion.

Effects of sprint exercise on SIRT1 and PGC-1α protein expression

PGC-1α stimulates mitochondrial biogenesis and promotes transcription of genes involved in fatty acid transport, lipid



◀ **Fig. 6** SIRT1 (**a**) and PGC-1α (**b**) protein expression before and after a Wingate test performed 60 min after the ingestion of 75 g of glucose (*black bars*) or under fasted conditions (*gray bars*). **a** *Top panel* A representative western blot with the anti-SIRT1 and anti-alpha-tubulin antibodies shows different levels of band densities obtained for both SIRT1 and alpha-tubulin at the different time points in both experimental groups. *Lower panel* Densitometric analysis of immunoblots. Values are relative to alpha-tubulin immunosignals. **b** *Top panel* A representative western blot with the anti-PGC-1α and anti-alpha-tubulin antibodies shows different levels of band densities obtained for both PGC-1α and alpha-tubulin at the different time points in both experimental groups. *Lower panel* Densitometric analysis of immunoblots. Values are relative to alpha-tubulin immunosignals. All values were normalized to the average of basal ones obtained prior to the start of the Wingate test which were assigned a value of 100%. $^{*}P < 0.05$ versus R and $^{\&}P < 0.05$ versus glucose group. $n = 8$ for all points except 240 min for which $n = 6$

oxidation and oxidative phosphorylation (Leick et al. 2008; Puigserver and Spiegelman 2003). PGC-1α transcriptional activity is also increased by SIRT1-mediated deacetylation (Gerhart-Hines et al. 2007). Previous studies have found that PGC-1α and SIRT1 expression are increased in rat skeletal muscle in response to endurance exercise (Suwa et al. 2008). The present investigation shows for the first time that SIRT1 protein expression is augmented in human skeletal muscle 2 h after a single bout of sprint exercise (just 30 s). Little is known about the regulation of SIRT1 protein levels (Milner 2009; Zschoernig and Mahlknecht 2008). It has been shown that AMPK can increase SIRT1 activity by increasing mitochondrial respiration causing an elevation of the $NAD^{+}/NADH.H^{+}$ quotient (Canto et al. 2009). In the present investigation, glucose administration 1 h prior to the sprint blunted Thr^{172}-AMPKα phosphorylation (at 30 min) and the subsequent increase of SIRT1 protein expression (at 120 min). The latter is compatible with a role of AMPK in the regulation of SIRT1 protein expression in human skeletal muscle. In agreement with our hypothesis, glucose administration was associated with a slightly lower mean blood lactate concentration during the recovery period, compatible with increased PDH activity (Putman et al. 1995). Despite the latter, the glycolytic rate was very high in the sprints performed after the ingestion of glucose, as indicate by the high blood lactate levels observed during the recovery period. Thus, in both sprints, there was a very high glycolytic rate, which should have elicited a marked elevation of the $NAD^{+}/NADH.H^{+}$ ratio (Putman et al. 1995). However, SIRT1 protein expression was only increased when Thr^{172}-AMPKα phosphorylation was elevated.

Another interesting finding from this study is that 120 min after a 30-s all-out sprint, PGC-1α protein content is not altered by exercise, in agreement with Gibala et al. (2009). This implies that 120 min after the Wingate test under fast conditions, there is a dissociation between SIRT1 protein expression and PGC-1α protein expression. Similar findings have been reported in rodents (Chabi et al. 2009; Gurd et al. 2009). Our results also suggest that exercise-induced AMPK phosphorylation alone is not sufficient to increase PGC-1α protein expression into recovery period.

AMPKα phosphorylation after high-intensity exercise

The main mechanism regulating the activity of AMPK is the ratio AMP/ATP (Hardie et al. 1998). Accordingly,

AMPK activation in human skeletal muscle has been reported during moderate (above, 50% of VO_2max; Chen et al. 2003; Fujii et al. 2000; Roepstorff et al. 2006; Wojtaszewski et al. 2000) and high-intensity exercise (Chen et al. 2000; Gibala et al. 2009), when the AMP/ATP ratio is also increased (Birk and Wojtaszewski 2006; Chen et al. 2000, 2003; Norman et al. 2001). At lower exercise intensities, AMPK activity only increases if the effort is prolonged to exhaustion (Wojtaszewski et al. 2002), when the AMP/ATP ratio may also be increased at least in the most fatigued muscle fibers (Sahlin et al. 1997). In agreement with Birk and Wojtaszewski (2006) and with Gibala et al. (2009), we did not observe AMPKα phosphorylation immediately after a 30-s sprint.

AMPKα activity during the recovery period after exercise has been measured 30 min after 1 h of exercise at 70% of VO_2max on the cycle ergometer and despite a 50% reduction in muscle glycogen AMPKα2 activity was not significantly elevated compared to the pre-exercise values (Fujii et al. 2000). In another study, AMPKα phosphorylation remained 3.3-fold above resting values 150 min after the cessation of a 40-min cycling at 70% of VO_2max (Sriwijitkamol et al. 2007). Our investigation shows that 30 min after a 30 s all-out exercise bout Thr^{172}-AMPKα phosphorylation is fivefold above the pre-exercise resting value; however, 120 min after the end of the Wingate test Thr^{172}-AMPKα phosphorylation levels were similar to pre-exercise values. This finding agrees with the recent study by Gibala et al. (2009) who have shown increased Thr^{172}-AMPKα phosphorylation immediately after four bouts of sprint exercise interspersed with 4 min of rest, i.e., about 20 min after the first sprint. By combining the present investigation with that by Gibala et al., it becomes clear that a single bout of exercise is as efficient as four bouts to elicit Thr^{172}-AMPKα phosphorylation, and that 2–4 h after the exercise Thr^{172}-AMPKα phosphorylation levels are similar to pre-exercise values.

Inhibition of AMPKα phosphorylation by glucose ingestion

To test if Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation induced by high insulin levels followed by exercise blunts Thr^{172}-AMPKα phosphorylation through an increase in Akt phosphorylation, we exposed the skeletal muscle to increased levels of insulin during 60 min before an exercise stimulus able to elicit a fivefold increase in Thr^{172}-AMPKα phosphorylation 30 min after the exercise. Our study shows that this intervention completely abolishes the 30-min post-exercise Thr^{172}-AMPKα phosphorylation. The blunted Thr^{172}-AMPKα phosphorylation was accompanied by a marked increase of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, supporting our hypothesis, i.e., Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation attenuates Thr^{172}-AMPKα phosphorylation in human skeletal muscle.

In the control group, the reduction of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation 30 min after exercise occurred despite elevated Ser^{473}-Akt phosphorylation. The latter implies that under fasting conditions the reduction in Ser^{491}-AMPKα2 phosphorylation observed 30 min after sprint exercise cannot be accounted for by reduced Akt activity, and should be explained by alternative mechanisms such as an increased phosphatase activity.

Bandyopadhyay et al. (2006) reported a reduction in basal Thr^{172} AMPKα phosphorylation after a 300-min long hyperinsulinemic–euglycemic clamp which elicits supraphysiological insulin plasma concentrations (insulin set at 1–1.2 nmol/l and glycemia at 5 mmol/l) in healthy humans. However, there were no significant effects on Thr^{172}-AMPKα phosphorylation and activity in healthy obese humans after a 240-min long hyperinsulinemic-euglycemic clamp eliciting physiological levels of hyperinsulinemia (~400 pmol/l; Hojlund et al. 2004). In agreement with the latter, no changes in basal Thr^{172}-AMPKα phosphorylation were observed in the present investigation 60 min after the ingestion of 75 g of glucose. However, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation was reduced by 18% and Ser^{473}-Akt phosphorylation by 70%. Thus, a mild elevation of insulin concentration via glucose ingestion does not elicit Ser^{473}-Akt phosphorylation in the resting human skeletal muscle. According to rodent heart in vitro experiments (Horman et al. 2006; Kovacic et al. 2003), the increase in insulin triggered by the ingestion of glucose should have elicit Ser^{473}-Akt phosphorylation; however, the opposed effect was actually observed. This result is in agreement with studies showing that Akt phosphorylation in skeletal muscle peaks soon after an increase in insulin concentration, declining thereafter rather fast, despite elevated levels of insulin (Turinsky and Damrau-Abney 1999; Walker et al. 1998). As expected, with lesser Ser^{473}-Akt phosphorylation, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation was also reduced (Horman et al. 2006; Kovacic et al. 2003). Our Akt results are further supported by the close relationship observed between Ser^{473}-Akt phosphorylation and AS160 phosphorylation, a downstream kinase phosphorylated by Akt (Howlett et al. 2008). However, exposure to higher levels of insulin during hyperinsulinemic–euglycemic clamps results in increased Ser^{473}-Akt phosphorylation 30 min after the start of the clamp (Christ-Roberts et al. 2003). In agreement with Christ-Roberts et al. (2003), sprint exercise after the ingestion of glucose elicited a higher level Ser^{473}-Akt phosphorylation than the exercise without previous ingestion of glucose.

We have also observed a close correlation between Ser^{473}-Akt phosphorylation and Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, indicating that Akt activity is likely responsible for the increased Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation (Horman et al. 2006; Soltys

et al. 2006). The latter could account for the blunted Thr^{172}-AMPKα phosphorylation in the recovery period for the Wingate tests performed after the ingestion of glucose. In agreement, Ser^{473}-Akt phosphorylation and Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation were correlated in the experiments after glucose ingestion. Under fasting conditions, Ser^{473}-Akt phosphorylation levels were similar to those observed in the glucose experiment but were not accompanied by enhanced Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation. The combination of both experiments leads to the conclusion that Ser^{473}-Akt phosphorylation alone cannot explain the exercise-induced Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation.

AMPK phosphorylation is not mandatory for exercise-induced ACCβ phosphorylation

An increase in ACC phosphorylation has been reported immediately after submaximal exercise at intensities ranging from 50 to 85% of VO_2max (Sriwijitkamol et al. 2007; Yu et al. 2003) and also after 30- and 120-s sprints (Birk and Wojtaszewski 2006; Chen et al. 2000; Gibala et al. 2009). In agreement with these studies, we observed an increase in Ser^{221}-ACCβ phosphorylation immediately after a 30-s Wingate test. What our study adds to the established knowledge is that we have shown that this response is not inhibited by the ingestion of 75 g glucose 60 min before the start of the sprint. In addition, we report the first data available on the evolution of Ser^{221}-ACCβ phosphorylation during the recovery process after a single bout of 30-s sprint exercise, showing that not only Ser^{221}-ACCβ phosphorylation is an immediate response to sprint exercise but it is also a response that remains for at least 30 min into the recovery period. Interestingly, we observed dissociation between Thr^{172}-AMPKα phosphorylation and ACCβ phosphorylation immediately after exercise, regardless of glucose ingestion. This phenomenon was also observed during the recovery process.

A dissociation between ACCβ and AMPKα phosphorylation has also been observed in skeletal muscle of knockout (KO) mice for the upstream AMPK kinase (LKB1), and in human skeletal muscle during prolonged exercise (Sakamoto et al. 2005; Wojtaszewski et al. 2002). In LKB1-deficient mouse muscle, AICAR-induced, but not contraction-induced, phosphorylation of ACC was reduced (Sakamoto et al. 2005). Furthermore, studies with AMPKα1 or AMPKα2 KO mice have shown that in both, α2 and α1 KO, ACC phosphorylation is increased during contraction but not during AICAR stimulation, suggesting that ACC phosphorylation in response to exercise is, at least in part, independent of AMPK activation (Jorgensen et al. 2004).

This dissociation between Thr^{172}-AMPKα phosphorylation and ACCβ phosphorylation could be explained by inhibition of ACCβ dephosphorylation by an unidentified protein phosphatase (Sakamoto et al. 2005). In addition, AMPK-independent ACCβ phosphorylation is possible in skeletal muscle (Dzamko et al. 2008).

In summary, this study shows that a single bout of sprint exercise elicits an immediate phosphorylation of Ser^{473}-Akt phosphorylation and Ser^{221}-ACCβ. Under fasting conditions, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation is reduced and Thr^{172}-AMPKα phosphorylation is markedly increased 30 min after a 30-s sprint exercise, while reciprocal changes are observed when the sprint is preceded by glucose ingestion. Thus, like previously reported in rodent cardiac myocytes, pre-exposure of human skeletal muscle to elevated (but physiological) levels of insulin prevents Thr^{172}-AMPKα phosphorylation in response to stress (very intense exercise), likely by eliciting Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 which may blunt the action of LKB1 (the upstream kinase responsible for Thr^{172}-AMPKα phosphorylation) or other unidentified AMPK kinases. We have also shown that sprint exercise enhances SIRT1 protein content in skeletal human muscle, a response likely mediated by Thr^{172}-AMPKα phosphorylation. Abrogating the exercise-induced Thr^{172}-AMPKα phosphorylation abolishes the increase in SIRT1 protein expression. Finally, we have shown that the evolution of PGC-1α protein expression after sprint exercise appears to be independent of SIRT1 protein expression. A practical implication from this study is that AMPK signaling in response to sprint exercise is modulated by the feed/fasting state. The effects of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation on specific AMPK heterotrimers deserve to be examined in future studies.

Acknowledgments This study was supported by grants from the Ministerio de Educación y Ciencia (BFI2003-09638, BFU2006-13784 and FEDER) and the Gobierno de Canarias (PI2005/177) and FUNCIS (PI/10/07). Borja Guerra is a fellow of the "Recursos Humanos y Difusión de la Investigación" Programe (ISCIII, MSC, Spain). Special thanks are given to José Navarro de Tuero and Oscar Bernales for his excellent technical assistance. The specialized advice from Tony Webster in editing the English version of the manuscript is also acknowledged. Special thanks are given to all subjects who volunteered for these experiments.

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