D. RAFAEL REYES ROMERO, SECRETARIO DEL DEPARTAMENTO DE EDUCACIÓN FÍSICA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA,

Que la Comisión de Investigación del Departamento en su sesión de fecha 26 de septiembre de 2012, tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "*Regulación de la actividad AMPK en músculo esquelético humano por radicales libre e hipoxia"* presentada por el doctorando D. David Morales Álamo, dirigida por los doctores D. José A. López Calbet, Dª. Cecilia Dorado García, D. C. Borja Guerra Hernández.

Y para que así conste, y a efectos de lo previsto en el Art. 73.2 del Reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a veintisiete de septiembre de dos mil doce.

TESIS DOCTORAL

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

Departamento: EDUCACIÓN FÍSICA
Programa de Doctorado: "ACTIVIDAD FÍSICA, SALUD Y RENDIMIENTO DEPORTIVO"

Título de la Tesis:

"REGULACIÓN DE LA ACTIVIDAD AMPK EN MÚSCULO ESQUELÉTICO HUMANO POR RADICALES LIBRES E HIPOXIA."

Tesis Doctoral presentada por D. David Morales Álamo

Dirigida por:

Dr. D. José Antonio López Calbet (C.U.)

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Dra. Dª. C. Borja Guerra Hernández (Profesor Asociado Laboral)

Los Directores ***El Doctorando***

En Las Palmas de Gran Canaria a 20 de Septiembre de 2012.

CONTENIDOS

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LISTADO DE PUBLICACIONES ORIGINALES

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1º/ Increased oxidative stress and anaerobic energy release, but blunted Thr^{172}-AMPKα phosphorylation in response to sprint exercise in severe acute hypoxia in man. Morales-Alamo D, Ponce-Gonzalez JG, Guadalupe-Grau A, Rodriguez-Garcia L, Santana A, Cussó MR Mrs, Guerrero M M, Guerra B, Dorado C, Calbet JA. J Appl Physiol. 2012 Aug 2. PMID: 22858621 [Epub ahead of print]

2º/ Critical role for free radicals on sprint exercise-induced CaMKII and AMPKα phosphorylation in human skeletal muscle. David Morales-Alamo, Ponce-González, J. G., Guadalupe-Grau, A, Rodríguez-García, L., Santana, A., Cusso, R., Guerrero, M., Dorado, C., Guerra, B., Calbet, J. A. L. **(Submitted).**

3º/ Skeletal muscle signaling during sprint exercise in severe acute hypoxia: role of free radicals**.** David Morales-Alamo, Jesús Gustavo Ponce-González, Amelia Guadalupe-Grau, , Alfredo Santana, Roser Cusso, Joan Cadefau, Borja Guerra, Cecilia Dorado, José A.L. Calbet. **(Submitted).**

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LISTADO DE ABREVIATURAS

ACC: (Acetyl coenzyme A carboxylase), acetilcoenzima A carboxilasa.

Akt: (Protein kinase B (PKB)), proteína quinasa B (PKB).

AMP: Adenosín monofosfato.

AMPK: (AMP-activated protein kinase), proteína quinasa activada por adenosin monofosfato.

AS160: (Akt substrate of 160 kDa), substrato de Akt de 160KDa.

ATP: Adenosín trifosfato.

CaMKII: (Ca^{2+}/calmodulin-dependent protein kinase II), Proteína Quinasa II Ca2+/Calmodulina dependiente..

CPTI: Carnitin-palmitoil transferasa I.

DXA: (Dual energy X-ray absorptiometry), absorciometría fotónica dual de rayos X.

eEF2 kinase: (Elongation Factor-2 kinase), proteína quinasa del factor de elongación 2.

ERK: (Extracellular Regulated Kinases), proteínas quinasa reguladas por estímulos extracelulares.

FiO_2: fracción inspiratoria de oxígeno.

GLUT4: (Glucose Transporter 4), Transportador de glucosa 4.

HOMA: (Homeostasis Model Assessment), modelo de valoración homeostático.

JNK: (c-JUN-N-terminal kinase), quinasa c-Jun N-terminal

kDa: Kilodalton.

Lac: (lactate), lactato

LKB1: (Liver Kinase B1), proteína quinasa B1 de hígado.

mA: Miliamperios

MAPK: (Mitogen-Activated Protein Kinase), proteína quinasa activada por mitógenos.

mTOR: mammalian target of rapamycin, proteína quinasa de mamíferos diana de la rapamicina.

MPO: (Wingate Mean Power Output), Potencia media del Wingate.

RPM: revoluciones por minuto.

Ser: (Serine), residuo de aminoácido de serina.

SIRT1: (Sirtuin 1), proteína deacetilasa sirtuína-1.

TAK1: (Transforming Growth Factor-β-Activated Kinase-1), proteína quinasa 1 activada por el factor de crecimiento transformante de tipo β.

Thr: (Threonin), residuo de aminoácido de treonina.

Tyr: (Tyrosine), residuo de aminoácido de tirosina.

p38 MAPK: (p38 Mitogen-Activated Protein Kinases), proteína quinasa activada por mitógenos p38.

PFK2: (Phosphofructokinase 2), fosfofructoquinasa 2.

PGC-1α: (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha), co-activador transcripcional PGC-1α.

PI3K: (Phosphatidylinositol 3-Kinase), fosfatidilinositol-3-quinasa.

PKA: (Protein Kinase A), proteína quinasa A.

PPO: (Peak Power Output), pico de potencia del Wingate.

Pyr: (Pyruvate or Pyruvic acid), piruvato o ácido pirúvico.

Rpm: revoluciones por minuto.

VO_2peak: pico de consumo pico de oxígeno.

Wmax: vatios máximos en el test realizado para determinar el VO_2max.

RESUMEN GENERAL

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La proteína quinasa activada por adenosin monofosfato (AMPK), es un enzima que activa varios procesos catabólicos e inhibe otra serie de procesos anabólicos. La AMPK es una de las principales enzimas involucradas en las adaptaciones al entrenamiento de resistencia. Recientemente se ha demostrado AMPK es activada por fosforilación en el residuo 172 del aminoácido treonina (pThr^{172}-AMPKα) tras ejercicio de sprint en cicloergometro, también se sabe que en cultivos celulares la actividad AMPK aumenta en hipoxia (por acción de los radicales libres). Para comprobar si el estrés oxidativo regula la actividad AMPK en ejercicio de sprint, reclutamos a 10 sujetos sanos (9 en el estudio II y III) que realizaron 4 test de Wingate combinando hipoxia, normoxia antioxidantes y placebo. En el **estudio I** demostramos que la combinación de ejercicio de sprint con hipoxia severa aguda no sólo no aumenta pThr^{172}-AMPKα sino que la inhibe. Los mecanismos que parecen regular dicha inhibición son dos. Por un lado, la disminución de la cantidad de proteína total de SIRT1 (una proteína que regula positivamente la actividad AMPK) y su activación (via NAD^+/$NADH^+$). Por otro lado, el aumento en la fosforilación de residuos de serina de AMPK (un mecanismo inhibitorio ya conocido de pThr^{172}-AMPKα) de manera dependiente de Akt. En el **estudio II** de la presente tesis se demostró que el aumento de la fosforilación de la Thr^{172}-AMPK producido por el ejercicio de sprint no parece ser dependiente del nivel de radicales libres, pues al

realizar el test de Wingate de 30 s tras la ingestión de antioxidantes se produjo una inhibición de la fosforilación en la Thr^{172}-AMPKα. Dicha inhibición se explica por una disminución de la fosforilación en la Thr^{286}-CaMKII (una quinasa activadora de la proteína AMPK). Teniendo en cuenta que la fosforilación de Thr^{172}-AMPK fue bloqueada al realizar el test de Wingate en hipoxia severa aguda, en el **estudio III** comparamos un test de Wingate de 30 s realizado en hipoxia severa aguda con otro en similar condición pero tras la ingesta previa de antioxidantes. La disminución del estrés oxidativo por parte de los antioxidantes no solventó el bloqueo de la fosforilación de la Thr^{172}-AMPK. En ambas condiciones aumentó la fosforilación de de las serinas de la AMPK, coincidente con la fosforilación de Akt. Además, los antioxidantes produjeron una disminución en la fosforilación de CaMKII como en el estudio II. Los resultados demostraron que la activación de AMPK por ejercicio de sprint es altamente dependiente del nivel de estrés oxidativo generado en el músculo y que los mecanismos que la regulan actúan a varios niveles.

SUMMARY

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The enzyme AMP-activated protein kinase (AMPK) is a metabolic master switch that turns on catabolic processes (that produce ATP) and turns of anabolic processes (that consume ATP). Since ATP is the main cell energy source, these actions performed by AMPK have been highly conserved throughout evolution. The human skeletal muscle is the tissue contributing the most to the whole-body energy expenditure. One of the main functions regulated by AMPK is the increase of fatty acid transport into the mitochondria through Carnitin-palmitoil transferasa I (CPT1), by ACCβ inhibition. Moreover, AMPK is also involved in the translocation of the glucose transporter GLUT4 to the sarcolemma, thereby producing an increased glucose transport into the muscle fiber. AMPK activation also stimulates the expression of genes regulating the energy homeostasis, induces mitochondrial biogenesis and a shift of the skeletal muscle fibers towards a more oxidative phenotype. Recent studies have shown that AMPK agonist can mimic some of the muscle adaptations to training (endurance training or high intensity interval training).

The main stimulus that promotes AMPK activity is the increase on AMP/ATP ratio. AMP binds to γ subunit, which changes the conformational structure of AMPK showing the Thr^{172} residue of the α catalytic subunit to be phosphorylated by AMPK protein kinase (AMPKK). In skeletal muscle, it seems that LKB1 is the main AMPKK. The phosphorylation on Thr^{172} on AMPK α subunit produces its activation. One

of the stimuli that produces an increased ratio AMP/ATP and therefore produces the enzyme activation in skeletal muscle is exercise. Specifically, the sprint exercise (a 30 s Wingate test) has been shown to produce an increase of the muscle AMP/ATP ratio and a concomitant increase in the Thr^{172}-AMPKα phosphorylation 30 minutes after the test.

Other factors such as hypoxia, also elicit activation of AMPK through mechanisms influenced by free radicals, although the pathways implicated have not been determined. A synergistic increase in AMPK activity by the combination of sprint exercise and hypoxia is expectable. However, the impact of hypoxia on sprint exercise-induced AMPK phosphorylation remains unknown.

Therefore, the main objective of this Thesis was to determine by which molecular mechanisms is AMPK activated in response to sprint exercise. To this purpose voluntaries performed sprint exercise in four different conditions, with two levels of oxygenation (normoxia and FiO_2=0.10) and under control conditions (after the ingestion of placebo) and after the ingestion of antioxidants. Hypoxia resulted in increased glicolytic rate and oxidative stress. The antioxidants are able to attenuated or abolish skeletal muscle signals mediated by free radicals.

Study I: The AMP-activated protein kinase (AMPK) is a major mediator of the exercise response and a molecular target to improve insulin sensitivity. To determine if the anaerobic component of the exercise, which is exaggerated when the sprint is performed in severe acute

hypoxia, influences the sprint-exercise elicited Thr^{172}-AMPKα phosphorylation, ten voluntaries performed a single 30 s sprint (Wingate test) in normoxia (N) and severe acute hypoxia (H) (P_IO_2=75 mmHg). Vastus lateralis muscle biopsies were obtained before and immediately after, 30 and 120 min post-sprint. Mean power output and VO_2 were, respectively, 6% and 37% lower in H than in N. Oxygen deficit and muscle lactate accumulation was greater in H than N. Carbonylated skeletal muscle and plasma proteins were increased after the sprint in H. Thr^{172}-AMPKα phosphorylation was increased by 3.1 fold 30 min after the sprint in N. This effect was prevented by hypoxia. The NAD^+/$NADH.H^+$ ratio was reduced (x24fold) after the sprints, with greater reduction in H than N ($P<0.05$), concomitant with 53% lower SIRT1 protein levels after the sprint in H ($P<0.05$). This could have led to lower LKB1 activation by SIRT1 and, hence, blunted Thr^{172}-AMPKα phosphorylation. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, a known negative regulating mechanism of Thr^{172}-AMPKα phosphorylation, was increased by 60% immediately after the sprint in H, coincident with increased Thr^{308}-Akt phosphorylation. Collectively, our results indicate that the signaling response to sprint exercise in human skeletal muscle is altered in severe acute hypoxia, which abrogated Thr^{172}-AMPKα phosphorylation likely due to lower LKB1 activation by SIRT1.

Study II: The extremely high energy demand elicited by sprint exercise is satisfied by an increase in oxygen consumption combined with a high glycolytic rate, leading to a marked lactate accumulation, increased AMP/ATP ratio, reduced NAD^+/$NADH.H^+$ and muscle pH, which are accompanied by marked Thr^{172}-AMPKα phosphorylation during the recovery period by a mechanism not fully understood. To determine the role played by free radicals on Thr^{172}-AMPKα phosphorylation in response to sprint exercise, nine voluntaries performed a single 30s sprint (Wingate test) in two occasions: one after the ingestion of placebo (P) and another following the intake of antioxidants (A) (α-lipoic acid, vitamin C, and vitamin E), with a double blind design. Vastus lateralis muscle biopsies were obtained before, immediately after, 30 and 120 min post-sprint. Performance, muscle aerobic and anaerobic metabolism was similar during both sprints. The NAD^+/$NADH.H^+$ ratio was similarly reduced (84%), and the AMP/ATP ratio similarly increased (x21 fold) immediately after the sprints. Thr^{286}-CaMKII and Thr^{172}-AMPKα phosphorylations were increased after the control sprint (P), but not when the sprints were preceded by the ingestion of antioxidants. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, a known inhibitory mechanism of Thr^{172}-AMPKα phosphorylation, was increased only in A. $Thr^{184/187}$-TAK1 phosphorylation was increased by 2.4-fold in A. This study shows that free radicals play a critical role in the skeletal muscle Thr^{172}-AMPKα phosphorylation response to sprint exercise in humans, since the mere

ingestion of antioxidants immediately prior to the sprint blunted this response despite no effects on performance, muscle aerobic and anaerobic metabolism, and the AMP/ATP and NAD^+/$NADH.H^+$ ratios.

Study III: Compared to normoxia, sprint exercise in severe acute hypoxia elicits a much greater glycolytic rate, lower muscle pH at exhaustion and higher oxidative stress leading to a paradoxical blunt of the AMPK Thr^{172} phosphorylation. To determine if free radicals could play a role in blunting Thr^{172}-AMPKα phosphorylation nine voluntaries performed a single 30s sprint (Wingate test) in two occasions while breathing hypoxic gas (P_IO_2=75 mmHg): one after the ingestion of placebo (P) and another following the intake of antioxidants (A) (α-lipoic acid, vitamin C, and vitamin E), with a double blind design. Vastus lateralis muscle biopsies were obtained before and immediately after, 30 and 120 min post-sprint. Compared to the control condition, the ingestion of antioxidants resulted in lower plasma carbonylated proteins, lower elevation of AMP/ATP molar ratio, and reduced glycolytic rate ($P<0.05$) without significant effects on performance or VO_2. The ingestion of antioxidants did not alter the basal muscle signaling. Thr^{172}-AMPKα and $Thr^{184/187}$-TAK1 phosphorylation were not increased after the sprint regardless of the ingestion of antioxidants. Thr^{286}-CaMKII phosphorylation was increased after the sprint but, this response was blunted by the antioxidants. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation increased immediately after the sprints coincident with increased Akt

phosphorylation. In summary, this study shows that antioxidants attenuate the glycolytic response to sprint exercise in severe acute hypoxia and modify the muscle signaling response to exercise. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, a known mechanism of Thr^{172}-AMPKα phosphorylation inhibition, is increased immediately after sprint exercise in hypoxia by a mechanism independent of free radicals.

Globally, these three studies have provided strong evidence for Ser^{485}-AMPKα1/ Ser^{491}-AMPKα2 phosphorylation as an important inhibitory mechanism of Thr^{172}AMPKα phosphorylation during exercise. Ser^{485}-AMPKα1/ Ser^{491}-AMPKα2 phosphorylation appears to be regulated by free radicals. The fact that the ingestion of antioxidants prior to sprint exercise blunts the expected Thr^{172}AMPKα phosphorylation may have important practical implications, since this could prevent some of the pretended adaptations with training.

INTRODUCCIÓN

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Hasta no mucho tiempo atrás, los antecedentes históricos de la fisiología del ejercicio o de la teoría del entrenamiento se basaban en el análisis de resultados, bien de rendimiento o morfológicos, de los seres humanos tras la realización de un ejercicio o programa de entrenamiento. Con el tiempo, se fue prestando cada vez más atención a los procesos de adaptación y a los mecanismos que los regulan. Atrás quedan los primeros pasos desentrañando la matriz de todo, la contracción muscular, y las fuentes de energía o rutas metabólicas que permitían que la contracción se prolongara durante el tiempo, la utilización del oxígeno, etc. En la actualidad, gran parte de los trabajos publicados se centran más en el detalle, es decir, en los mecanismos moleculares responsables de estos fenómenos, tratando de identificar las proteínas implicadas en las adaptaciones, en los estímulos que consiguen modular su actividad y pueden, por tanto, conseguir que antes y mejor los resultados deseados.

Recientemente se ha demostrado que el sedentarismo produce más muertes que el tabaquismo, siendo una de las mayores causas de morbilidad a nivel mundial (Kohl *et al.*, 2012; Lee *et al.*, 2012). De esta forma, actualmente se considera que el sedentarismo es el punto de partida de numerosas patologías, como por ejemplo el síndrome metabólico, la diabetes tipo II, la obesidad y algunos tipos de cáncer. Teniendo en cuenta lo anteriormente comentado, no es de extrañar que muchos de los esfuerzos investigadores desarrollados por la fisiología del

ejercicio en los últimos años se hayan centrado en dilucidar los mecanismos moleculares inducidos por el sedentarismo e implicados en estas patologías, con el objetivo de tratar de prevenirlas y/o curarlas a través de práctica regular de actividad física.

Uno de los principales efectos beneficiosos del ejercicio, en lo que se refiere a la prevención de las patologías anteriormente comentadas, es la estimulación de la oxidación muscular de ácidos grasos. Gracias a los estudios de calorimetría y metabolómica se sabe, desde hace décadas, que durante el ejercicio de intensidades bajas se oxida un mayor porcentaje de grasas (Krogh & Lindhard, 1920b). Sin embargo, también se sabe que después de ejercicios de alta intensidad, la recuperación es predominantemente aeróbica, aunque este tipo de ejercicio sea altamente dependiente de fuentes anaeróbicas (Krogh & Lindhard, 1920a; Hill *et al.*, 1924). En cuanto a la diabetes tipo II, en la actualidad numerosas investigaciones se centran en los mecanismos y estímulos (ejercicio físico entre ellos) que regulan la sensibilidad muscular y del cuerpo entero a la insulina, que se traducen en un aumento de la translocación de GLUT4 hacia el sarcolema de la fibra muscular (Bienso *et al.*, 2012). Por otro lado, algunos trabajos recientes centran su atención en la cantidad de mitocondrias como un factor determinante en la estimulación de la sensibilidad muscular a la insulina a través de

mecanismos moleculares donde los radicales libres podrían jugar un papel clave (Sebastian *et al.*, 2012).

1.1 AMPK

La proteína quinasa activada por adenosin monofosfato (AMPK), fue descubierta en primera instancia en 1973, como una proteína que inactivaba a la acetil coenzima A carboxilasa (ACC) a través de su fosforilación (Carlson & Kim, 1973). A pesar de esto, la caracterización precisa de esta enzima no se logró hasta una década después (Yeh *et al.*, 1980). Hoy en día se sabe que la AMPK es un importante interruptor metabólico. Su principal función es la preservación de los niveles de energía celulares. La prueba biológica de su importancia reside en que es una enzima altamente conservada a lo largo de la evolución, al menos en las células eucariotas (Hardie *et al.*, 2003).

A nivel general, la activación de la AMPK induce la inhibición de varios procesos anabólicos (que consumen ATP) y la activación de procesos catabólicos (producen ATP). Entre los procesos anabólicos inhibidos por la AMPK activa se pueden citar la síntesis proteica, a través de la inhibición de la vía de mammalian target of rapamycin (mTOR) and Elongation Factor-2 kinase (eEF2 kinase) (Horman *et al.*, 2002; Inoki *et al.*, 2003), y la síntesis de glucógeno y ácidos grasos (Henin *et al.*, 1995), procesos que llevan consigo un elevado coste energético para la célula. Por otro lado, entre los principales procesos catabólicos activados por la

AMPK se encuentran la estimulación del transporte de ácidos grasos hacia el interior de la mitocondria a través de la inhibición de la ACC (ACCβ en el músculo esquelético) (Merrill *et al.*, 1997). Además, también se ha demostrado que la activación farmacológica directa de la AMPK inducida por 5-amino-1-β-ribofuranosil-imidazol-4-carboximida (AICAR) produce la translocación de GLUT4 al sarcolema de la fibra muscular (Merrill *et al.*, 1997). Por último, la activación de la AMPK a largo plazo produce un aumento de la biogénesis mitocondrial a través de mecanismos moleculares mediados por el co-activador transcripcional PGC-1α (Winder *et al.*, 2000; Zong *et al.*, 2002).

Lo anteriormente comentado pone de manifiesto que la actividad AMPK es capaz de mediar numerosas adaptaciones en los tejidos tanto a nivel agudo como a nivel crónico. Esta serie de adaptaciones están todas dirigidas a intentar preservar la homeostasis energética celular (niveles de ATP), lo cual demuestra bien a las claras la importancia de dicho sistema y explica su conservación a lo largo de la evolución (Mantovani & Roy, 2011). Además, el hecho de que la activación de la AMPK incremente agudamente el transporte de ácidos grasos al interior de la mitocondria, bloquee la síntesis de ácidos grasos y a largo plazo estimule la biogénesis mitocondrial, ha hecho que actualmente la AMPK sea una importante diana terapéutica para el tratamiento de la obesidad. Con respecto a la diabetes tipo II es tan importante el efecto agudo de translocación de GLUT4 al sarcolema como a nivel crónico la

estimulación de la biogénesis mitocondrial, ya que se ha demostrado que el número de mitocondrias está disminuido en sujetos con diabetes tipo II (Simoneau & Bouchard, 1995; Simoneau *et al.*, 1995). Quizás, la AMPK pueda ser el nexo de unión entre ambas patologías, pues se ha demostrado que la diabetes tipo II está relacionada con el incorrecto funcionamiento de la señalización muscular activada por insulina debido a la mayor cantidad de grasa intramuscular acumulada (lipotoxicidad), y porque también se ha demostrado que la actividad AMPK es menor en sujetos con diabetes tipo II.

1.1.1 Mecanismos de Activación de AMPK

La AMPK es una enzima heterotrímera compuesta de tres subunidades (α, β, γ). La subunidad α es la subunidad catalítica, mientras que las subunidades β y γ son reguladoras (Hardie & Hawley, 2001). La principal función de la AMPK es la conservación del contenido de ATP de la célula, como ya se hizo referencia en el apartado anterior. Por eso no es de extrañar que el principal mecanismo de regulación de la actividad AMPK esté relacionado directamente con los mecanismos modulados por los fosfágenos. El principal mecanismo de activación de la AMPK es dependiente de las concentraciones intracelulares de AMP (activador alostérico) en relación con el nivel de ATP (ratio AMP/ATP) (Richter & Ruderman, 2009). Cuando el AMP aumenta, éste se une a la subunidad γ, lo cual modifica la conformación estructural de la proteína. Dicho

cambio en la conformación estructural deja visible el residuo de aminoácido treonina^{172} localizado en la subunidad α, permitiendo de este modo que pueda ser fosforilado. Dicha fosforilación es imprescindible para producir un aumento en la actividad AMPK, no conociéndose hasta la fecha otro mecanismo independiente (Hardie *et al.*, 2003).

El principal mecanismo que se ha descrito para explicar la fosforilación de la Thr^{172}-AMPKα es el dependiente de la AMPKK denominada LKB1. LKB1 fosforila a la Thr^{172} de la AMPKα cuando este residuo de aminoácido es accesible por la acción alostérica del AMP sobre la subunidad γ (Figura 1) (Hawley *et al.*, 2003). Se sabe que la actividad LKB1 es regulada por la formación del complejo LKB1-STRAD-MO25 (Boudeau *et al.*, 2003), el cual mantiene a LKB1 en el citoplasma. Aun así, no se han encontrado cambios en la actividad LKB1 producidos por factores como el ejercicio físico, por lo que se sospecha que la regulación de AMPK por LKB1 es alostérica (Richter & Ruderman, 2009).

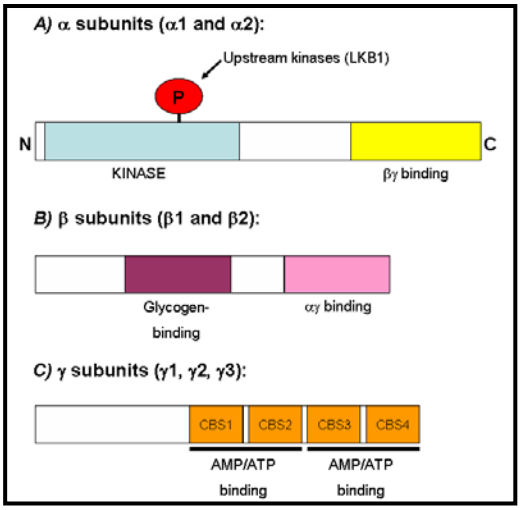

Figura 1. *Figura que muestra las diferentes subunidades de AMPK. La figura ha sido adaptada de Hardie et al. 2001.*

Recientemente, se ha demostrado que la activación de la proteína quinasa CaMKII a través de su fosforilación en Thr^{286} puede inducir un aumento en la fosforilación en la Thr^{172}-AMPKα (Raney & Turcotte, 2008). Más recientemente, se ha observado que la fosforilación de la Thr^{286}-CaMKII tiene gran importancia en la fosforilación de la Thr^{172}-AMPK, de una forma dependiente de la intensidad del esfuerzo físico (Egan *et al.*, 2010). Por otro lado, se sabe que bloqueando la activación de la proteína TAK1, se produce un defecto en la activación de AMPK (Xie *et al.*, 2006). A su vez, la activación de TAK1 produce un aumento de la fosforilación de la Thr^{172}-AMPKα en levaduras (Momcilovic *et al.*, 2006). Teniendo todo esto en consideración, actualmente se piensa que TAK1 puede también actuar como activador directo de AMPK o de LKB1 (Richter & Ruderman, 2009).

1.1.2 Posibles mecanismos de activación de AMPK por hipoxia.

En cultivos celulares se ha demostrado que la hipoxia produce un aumento de la fosforilación de la Thr^{172}-AMPKα (Emerling *et al.*, 2007). Este mismo grupo ha demostrado, también en cultivos celulares, que la activación de dicha fosforilación podría estar mediada por los radicales libres (Figura 2) (Emerling *et al.*, 2009), si bien actualmente se desconoce el mecanismo exacto a través del cual la hipoxia estimularía la producción de estos radicales. Algunos trabajos proponen que la hipoxia (0.3-5% O_2) podría producir una disminución del ratio AMP/ATP, pero lo cierto es que esto sólo ocurre en anoxia (0-0.3% O_2) (Emerling *et al.*, 2009). Esta última afirmación es de gran importancia para el modelo de activación de AMPK que vamos a utilizar en esta tesis doctoral, y será abordado en el siguiente apartado de la introducción. Por otro lado, se sabe que JNK puede activar a TAK1, al menos en miocitos cardiacos (Frazier *et al.*, 2007), y tal como hemos comentando en el apartado anterior, TAK1 puede mediar la activación de AMPK. Entre los mecanismos que pueden producir una mayor activación de JNK se encuentra un aumento de la especies reactivas del oxígeno (ROS) (Frazier *et al.*, 2007)

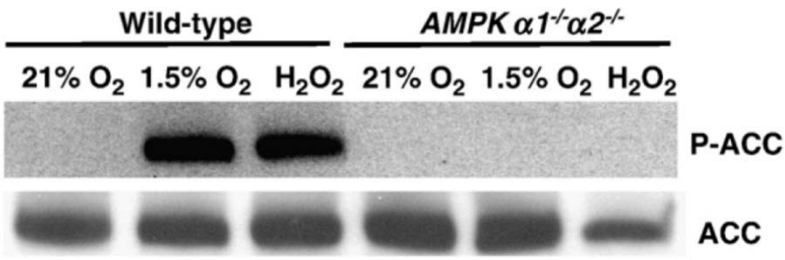

Figura 2. Figura que muestra, *en cultivos celulares (fibroblastos embrionarios de ratón) como existe un aumento en la fosforilación de ACC (enzima downstream de AMPK) en respuesta a la hipoxia (1.5% O2) y a la exposición de las células a peróxido de hidrogeno (H_2O_2), el cual induce un considerable estrés oxidativo. Figura extraída de Emerling et al 2009.*

1.1.3 Activación de AMPK por ejercicio físico.

Como ya se ha comentado en el apartado 1.1, el principal factor que determina un aumento de la actividad AMPK es el incremento del ratio AMP/ATP (Hardie *et al.*, 2003). Además, uno de los principales fenómenos que producen un aumento significativo del ratio AMP/ATP y que por tanto estimulan la actividad AMPK, es el ejercicio físico. Inicialmente, se demostró que la activación de la AMPK muscular se producía en roedores tras ejercicio prolongado en tapiz rodante, sin observar, sin embargo, ningún cambio en la cantidad de AMP intramuscular (Winder & Hardie, 1996). Más recientemente, se demostró en humanos la activación de la AMPKα, en concreto α2, tras la realización de un ejercicio prologado (60 minutos) al 75% del VO_2max en

cicloergometro (Wojtaszewski *et al.*, 2002). Este hallazgo fue doblemente importante pues demostró que la activación de AMPK es además dependiente de la intensidad del esfuerzo, ya que según sea esta intensidad se producirá un mayor o menor aumento del ratio AMP/ATP (Chen *et al.*, 2000). De hecho, otro estudio en el que también se utilizo el ejercicio en cicloergómetro ha demostrado que la fosforilación de la AMPKα en la Thr^{172} es más dependiente de la intensidad del ejercicio físico que de otros factores, como por ejemplo la hipoxia aguda (FiO_2: 0.115) (Wadley *et al.*, 2006). Sin embargo, también es cierto que este estudio además demuestra que la hipoxia combinada con el ejercicio en cicloergómetro produce una estimulación sinérgica de la fosforilación de la Thr^{172}-AMPKα (Wadley *et al.*, 2006).

A una FiO_2 similar al estudio comentado anteriormente (FiO_2: 0.10), el porcentaje de oxígeno al que se encuentra el intersticio existente entre los capilares y las fibras musculares del vasto lateral de la pierna durante un ejercicio de cicloergómetro, es de aproximadamente un 1.5%, según se ha podido calcular por medidas indirectas (Calbet *et al.*, 2009). A ese porcentaje de O_2, un estudio reciente ha demostrado que se produce una activación de la fosforilación Thr^{172}-AMPKα a través de un mecanismo que parece depender del nivel de radicales libres pero que es independiente de un aumento del ratio AMP/ATP (Emerling *et al.*, 2009). Teniendo en cuenta la posible activación de la AMPK en hipoxia por radicales libres, y que en humanos se ha demostrado que el ejercicio en

cicloergometro en hipoxia presenta una actividad mayor de la AMPK (a la misma intensidad absoluta) (Figura 3), este fenómeno puede ser explicado, en parte, por un posible aumento de los radicales libres (a pesar de que el AMP es mayor en ejercicio en condiciones de hipoxia) (Figura 4).

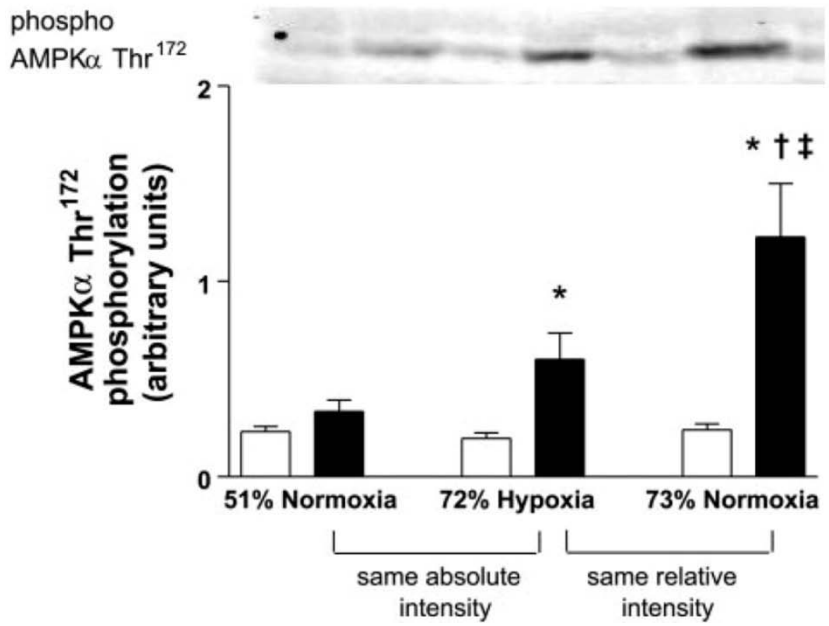

Figura 3. *Esta figura demuestra que la fosforilación Thr^{172}-AMPK inducida por un ejercicio de cicloergómetro es mayor cuanto mayor sea la intensidad absoluta del ejercicio (73% en normoxia). Además, se demuestra que la combinación de ejercicio con hipoxia produce un incremento sinérgico de la fosforilación Thr^{172}-AMPK (si se compara 51% normoxia y 72% hipoxia: ejercicio realizado a la misma intensidad absoluta). Figura tomada de Wadley et al., 2006.*

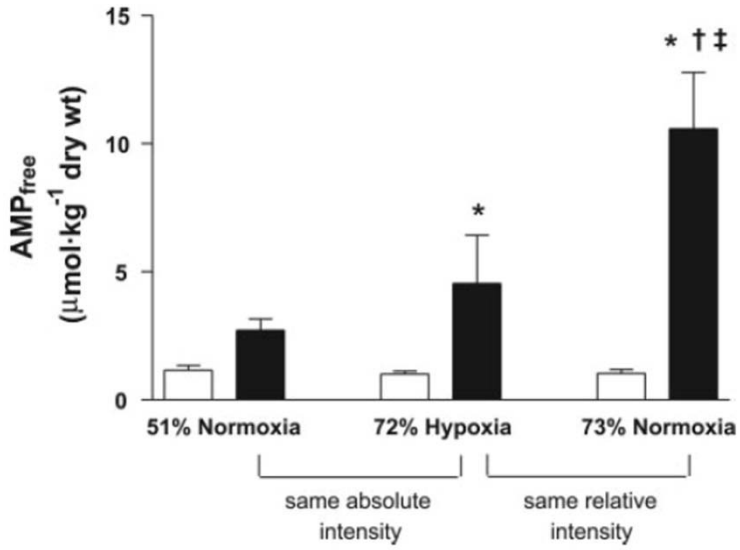

Figura 4. Los resultados mostrados en esta figura sugieren que el incremento de la fosforilación Thr^{172}-AMPKα comentado en la Figura 3 puede ser explicado por un aumento de los niveles intracelulares de AMP. Figura tomada de *Wadley et al., 2006.*

El test de Wingate de 30 segundos en cicloergómetro es un modelo de ejercicio conocido por su elevada intensidad de esfuerzo. En concreto, las características fisiológicas de dicho test serán abordadas más adelante en su correspondiente apartado, de forma que aquí nos centraremos en lo que a la activación de AMPK se refiere. Un test de Wingate de 30 segundos produce un gran incremento (en torno a 3.2 veces) del ratio AMP/ATP en músculo esquelético humano inmediatamente después del ejercicio (Chen *et al.*, 2000). Este importante incremento del ratio AMP/ATP se tradujo además en la estimulación de la actividad AMPK (α1 y α2), si bien los autores no determinaron la fosforilación (por falta de muestra suficiente para realizarlo) de la AMPK

en el residuo de aminoácido Thr^{172} (Chen *et al.*, 2000). Otro estudio posterior demostró que la realización de 4 test de Wingates consecutivos (con descansos de 4 minutos) produce un aumento significativo de la fosforilación de Thr^{172}-AMPK (α1 y α2) justo después del último test (Gibala *et al.*, 2009). El mencionado estudio demuestra además, que se produce una estimulación de la expresión génica del co-activador transcripcional PGC-1α (*downstream* de la AMPK e importante estimulador de la biogénesis mitocondrial) a las 3 horas después del último test y durante la recuperación (Gibala *et al.*, 2009). Más recientemente, un trabajo publicado por nuestro grupo de investigación demostró que la realización de un solo test de Wingate (30s) produce un aumento de 5 veces en la fosforilación de la Thr^{172}-AMPKα a los 30 minutos de la recuperación, siempre y cuando el test se realice en ayunas (Figura 5) (Guerra *et al.*, 2010).

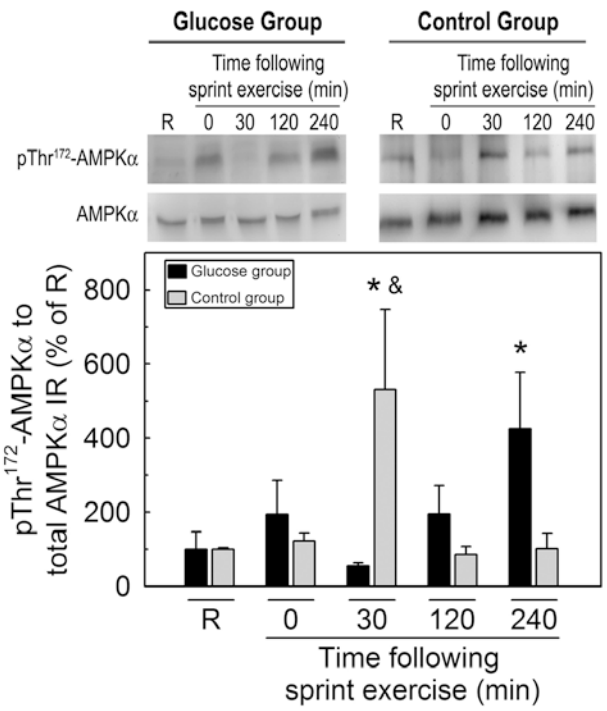

Figura 5. *La figura muestra el aumento de la fosforilación en la Thr^{172}-AMPKα detectado durante la recuperación (30 minutos depués del ejercicio) en respuesta a un test de Wingate de 30s realizado en ayunas (barras grises). Sin embargo, si el test se realiza una hora después de la ingestión de 75g de glucosa se observa que la fosforilación en Thr^{172}-AMPKα no aumenta a los 30 minutos después del ejercicio sino que se ve retrasada hasta las 2 horas post-ejercicio (barras negras). Figura tomada de Guerra et al., 2010.*

Todo lo anteriormente comentado pone de manifiesto que el ejercicio de sprint (test de Wingate) es capaz de producir un aumento en la fosforilación de la Thr^{172}-AMPKα y por consiguiente, un aumento en la actividad de esta enzima. Además, estos estudios han sentado las bases para comprender los mecanismos moleculares que pueden regular las

recuperaciones aeróbicas tras esfuerzos anaeróbicos y las adaptaciones al entrenamiento interválico de alta intensidad (Figura 6). Estos fenómenos se conocían desde tiempo atrás, pero no se habían determinado los mecanismos moleculares intramusculares que los gobiernan.

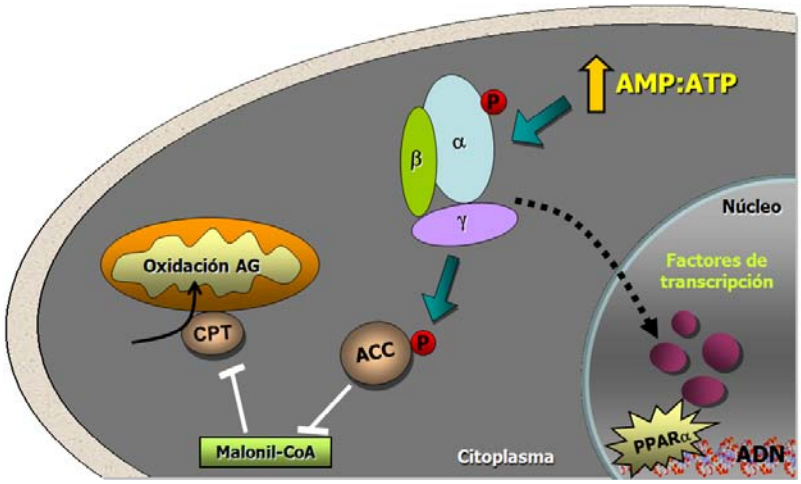

Figura 6. *En esta figura se muestra el mecanismo molecular por medio del cual la AMPK modula (a nivel agudo y crónico) la adaptación de la célula para estimular la oxidación de ácidos grasos. Figura adaptada de Steinberg & Rose 2007.*

1.1.4 La paradoja de ACCβ, ¿actúa o no como *downstream* de AMPK?

Desde que la proteína AMPK fue descubierta en 1973 se demostró que su principal función en músculo esquelético consistía en la estimulación de la oxidación de ácidos grasos a través de la fosforilación e inhibición de la ACC (Carlson & Kim, 1973). Posteriormente, se

publicaron un gran número de trabajos mostrando incremento en la fosforilación de ACCβ, en respuesta a un aumento en la fosforilación (Thr^{172}) o activación de la AMPK (Hutber *et al.*, 1997; Merrill *et al.*, 1997). Tal fue el cuerpo de conocimiento al respecto, que muchos trabajos comenzaron a utilizar la fosforilación en la Ser^{79}-ACCβ (Ser^{221}-ACCβ en humanos) como un marcador directo de la actividad AMPK (Emerling *et al.*, 2009).

En el año 2008, un estudio demostró que la enzima AMPK no es el único regulador de la fosforilación de ACC (Dzamko *et al.*, 2008). Este estudio demostró, a través de un elegante experimento realizado con ratones "*kinase dead*" para AMPK, que si estos ratones eran estimulados farmacológicamente con AICAR o mecánicamente, a través de contracciones musculares inducidas (ambos estímulos capaces de activar la oxidación de grasas a través de la fosforilación e inhibición de la ACC mediada por la AMPK), la fosforilación se mantenía a pesar de no estar presente la actividad AMPK (Dzamko *et al.*, 2008). En humanos, y en respuesta a ejercicio prolongado, también se ha demostrado que existe una disociación entre la fosforilación muscular de ACC y de la AMPK (Wojtaszewski et al., 2002). Sin embargo, esta disociación entre ambas fosforilaciones también ha sido demostrada en músculo esquelético humano en respuesta a modelos de ejercicio de corta duración, como es el test de Wingate. Así por ejemplo, Gibala y colaboradores investigaron la fosforilación de AMPK y ACC en respuesta a 4 test de Wingate

separados por periodos de reposo de 4 minutos (Gibala *et al.*, 2009), poniendo de manifiesto que la fosforilación de la β ACC (Ser^{221}) se estimula ya inmediatamente después del primer sprint, mientras que la de la AMPKα no ocurre hasta la finalización del cuarto (Gibala *et al.*, 2009). Más recientemente, un estudio publicado por nuestro grupo de investigación encontró dos niveles de disociación entre las fosforilaciones de la AMPK y la ACCβ tras la realización de un test de Wingate de 30s en diferentes condiciones (Guerra *et al.*, 2010). En dicho trabajo se demuestra que tras la realización, en ayunas, de un solo test de Wingate la fosforilación de la Ser^{221}-ACCβ aumenta justo después del test, manteniéndose elevada durante los 30 minutos siguientes a la finalización del test, mientras que la fosforilación de la Thr^{172}-AMPKα sólo aumenta a los 30 minutos después del ejercicio, durante la recuperación (Figura 7A) (Guerra *et al.*, 2010). Sin embargo, cuando el mismo test de Wingate fue realizado 1 hora después de la ingestión de 75 gramos de glucosa, la fosforilación de la ACCβ detectada fue muy similar a la observada en respuesta al ejercicio de sprint en ayunas, mientras que la fosforilación de la AMPKα no se vio estimulada hasta transcurridas 2 horas tras la finalización del test (Figura 7B) (Guerra *et al.*, 2010). Los resultados aportados por este estudio ponen de manifiesto que al menos parte de la fosforilación de la ACCβ debe estar modulada por otras vías de señalización independientes de la AMPK en respuesta a este modelo de ejercicio. Sin embargo, se desconoce el efecto de la combinación de

un ejercicio de sprint en cicloergómetro de 30 segundos y la hipoxia, sobre la fosforilación de la ACCβ en músculo esquelético humano. De hecho, recientemente se ha propuesto de manifiesto que la activación de AMPK se traducirá en la modulación de una diana downstream o no, en función del tejido, las condiciones celulares y el estímulo (Mantovani & Roy, 2011).

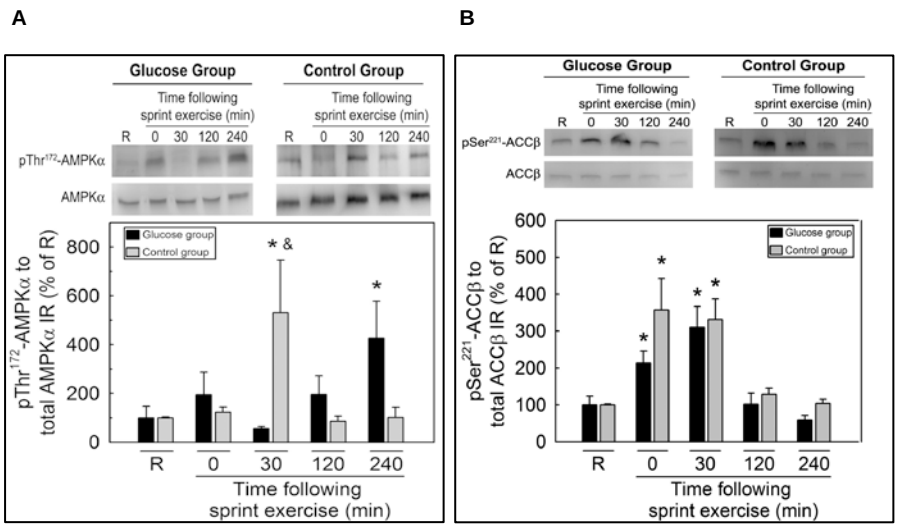

Figura 7. *Niveles de fosforilacion de la Thr^{172}-AMPKα (figura izquierda) y de la Ser^{221}-ACCβ (figura derecha) en respuesta a un ejercicio de sprint de 30 s realizado en ayunas (barras grises) o 1 hora después de la ingestión de 75 g de glucosa (barras negras). Figuras tomadas de Guerra et al., 2010.*

1.1.5 Mecanismos de inhibición de la fosforilación Thr^{172}-AMPKα.

La enzima AMPK regula ciertos mecanismos musculares claves para una mayor utilización de las vías aeróbicas. Dada su tremenda

importancia, es una vía de señalización regulada por diversas señales, redundantes en algunos casos, que la activan o inactivan en función de las necesidades de la célula. El siguiente apartado de la introducción se centrará en desvelar los mecanismos conocidos que puedan tener alguna relación con la inhibición de la actividad AMPK normalmente estimulada en respuesta al ejercicio.

La fosforilación de la Thr^{172}-AMPKα no es la única fosforilación conocida de la subunidad α (catalítica). De hecho, en los últimos años se ha demostrado que existen otros residuos de aminoácido de la subunidad α que se fosforilan, pudiendo jugar un papel importante en la modulación de la actividad AMPK. En concreto, la fosforilación de la AMPKα en residuos alternativos de serina (Ser^{485}-AMPKα1 y Ser^{491}-AMPKα2) parece tener una importante función de regulación negativa de la actividad de la enzima. Estas fosforilaciones en residuos alternativos de serina fueron identificadas, a través de la espectrometría de masas, en el año 2003 (Woods *et al.*, 2003). Tras este hallazgo, se demostró en corazón de rata perfundido con insulina, que la fosforilación de la AMPK en esos residuos alternativos de serina resulta en la inhibición de la enzima AMPK (Horman *et al.*, 2006). Dicho efecto inhibitorio inducido por la insulina sobre la oxidación de ácidos grasos a través de la AMPK había sido demostrado anteriormente (Gamble & Lopaschuk, 1997). En la misma línea, la señalización por insulina en células cardiacas, induce un aumento en la fosforilación de la Akt (Thr^{308} y Ser^{473}), el cual se relaciona

con un bloqueo de la fosforilación de la Thr^{172}-AMPKα (Kovacic *et al.*, 2003). Tomados en su conjunto, los resultados aportados por estos estudios han puesto de manifiesto que la fosforilación de la subunidad α AMPK en estos residuos alternativos de serina puede impedir, probablemente de manera estérica, la fosforilación en el residuo Thr^{172} de la misma subunidad de la enzima (Kovacic *et al.*, 2003; Horman *et al.*, 2006). Recientemente, un estudio publicado por nuestro grupo de investigación ha aportado un importante hallazgo en este sentido. En el mencionado estudio, Guerra y colaboradores demostraron en músculo esquelético humano que la fosforilación de la AMPKα en residuos alternativos de serina (Ser^{485}-AMPKα1/Ser^{491}-AMPKα2), inducida por la ingesta de 75 gramos de glucosa 1 hora antes de un test de Wingate de 30 segundos, bloquea la fosforilación de esta misma subunidad en el residuo de Thr^{172} (Figura 8) (Guerra *et al.*, 2010). Además, el mencionado estudio sugiere que la estimulación de la fosforilación de la AMPKα en los residuos de serina podría estar producida por la activación de la Akt (Ser^{473}-Akt) inducida por la insulina (Guerra *et al.*, 2010).

También se ha demostrado que una regulación negativa de las quinasas de proteína que se encuentran *upstream* de la AMPK, puede producir, como cabría esperar, una disminución de la activación de la AMPK. En concreto, en ratones *knockout* de LKB1, se ha demostrado que no se detecta la activación de AMPK normalmente inducida por el tratamiento farmacológico con AICAR o por la contracción muscular

(Sakamoto *et al.*, 2005). Así que por ello en estos estudios también nos dispondremos a analizar las conocidas upstreams de AMPK (LKB1, Thr^{286}-CaMKII, $Thr^{184/187}$-TAK1) (Sakamoto *et al.*, 2005; Momcilovic *et al.*, 2006; Egan *et al.*, 2010).

Finalmente, evidencias recientes sugieren que un determinado nivel de estrés oxidativo es necesario para la correcta activación de diversas vías intramusculares de señalización (Powers *et al.*, 2011). Se sabe que la contracción muscular produce estrés oxidativo, el cual tiene influencia en la contracción muscular (Reid *et al.*, 1992), y que esta generación de radicales libres es tanto mayor cuanto mayor es la intensidad del ejercicio (Munoz Marin *et al.*, 2010). Aunque en un principio se pensó que los radicales libres eran perjudiciales para la integridad de las células, las nuevas evidencias demuestran que, estos radicales libres intramusculares inducidos por el ejercicio son necesarios en un determinado nivel para una correcta activación de diversas vías de señalización (Powers *et al.*, 2011). De hecho, diversos estudios han puesto de manifiesto que la suplementación con antioxidantes, como por ejemplo la vitamina C (conocido antioxidante), impide determinadas adaptaciones musculares que se producen en respuesta al entrenamiento de resistencia, presumiblemente a través de PGC-1α (Gomez-Cabrera *et al.*, 2008).

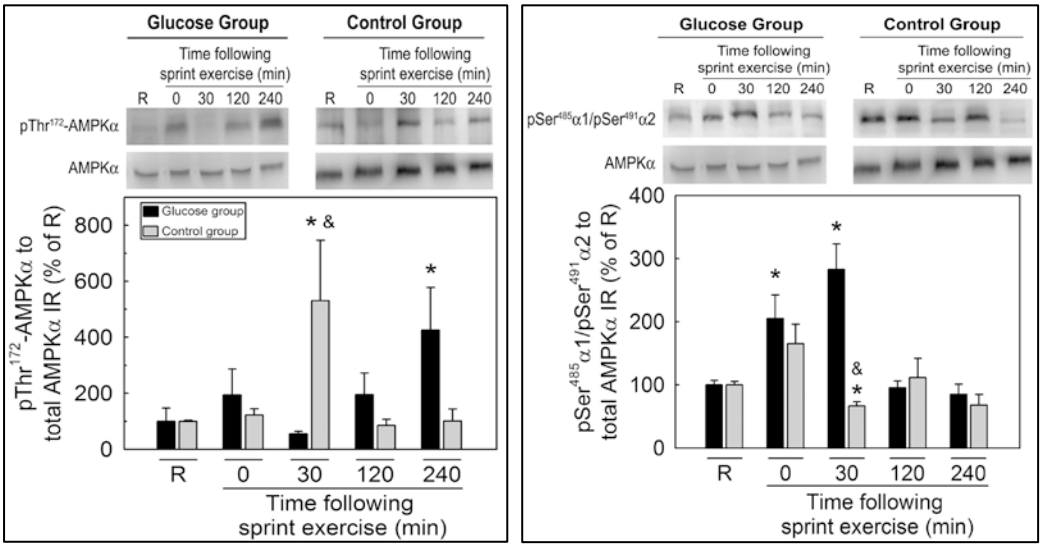

Figura 8. *Niveles de fosforilacion de la Thr^{172}-AMPKα (figura izquierda) y de las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 (figura derecha) en respuesta a un ejercicio de sprint de 30 s realizado en ayunas (barras grises) o 1 hora después de la ingestión de 75 g de glucosa (barras negras). Figuras tomadas de Guerra et al., 2010.*

1.2 Test de Wingate de 30 segundos.

El test de Wingate de 30 s fue creado originariamente en el Instituto Wingate, en Israel. Dicho test consistía en un test en cicloergometro en el que se usaba el 7% del peso corporal del sujeto como resistencia de frenado y cuya duración era de 30 segundos (Bar-Or, 1987). Hay innumerables publicaciones que hablan sobre su enorme dependencia de las vías anaeróbicas debido a las características del esfuerzo, estando la intensidad de este ejercicio por encima del VO_2max (supramáximo). El rendimiento en el test de Wingate ha sido relacionado

con el rendimiento en pruebas con un componente anaeróbico importante como los 400 metros lisos (van Ingen Schenau *et al.*, 1994). De esta forma, se ha determinado que aproximadamente un 80% de la energía utilizada para la realización del test de Wingate de 30 s proviene de la vías anaeróbicas (Calbet *et al.*, 1997). En la misma línea, muchas son las publicaciones que demuestran una alta acumulación de lactato intramuscular tras dicho test (Bogdanis *et al.*, 1995; Parra *et al.*, 2000). Además, la alta intensidad del test provoca un gran incremento del ratio muscular de AMP/ATP (3.2 veces) (Chen *et al.*, 2000). Aun así, a pesar del alto porcentaje de predominio anaeróbico del test de Wingate existe un porcentaje de la energía que proviene de las vías aeróbicas durante el esfuerzo (Calbet *et al.*, 1997; Calbet *et al.*, 2003).

El test de Wingate original presenta un problema. Se sabe que un mismo sujeto pedaleando a una misma intensidad (vatios), pero a diferentes revoluciones no tiene el mismo consumo de oxígeno (Dickinson, 1929; Coast & Welch, 1985; Chavarren & Calbet, 1999). Por ello para la mejor comparación entre sujetos, se ha desarrollado una variante del test original fijando las revoluciones por minuto (rpm), test denominado Wingate isocinético (Jones *et al.*, 1985). La frecuencia de pedaleo óptima para una carga baja se encuentra en ~60 rpm (100 w), para una carga moderada de 300 w se estima que ronda los 80 rpm, mientras que para cargas elevadas se sitúa en unas 120 rpm (600 w) (Coast & Welch, 1985). Aun así, la acumulación de metabolitos y el pico

de potencia (Pmean) son similares en un rango entre 60 y 140 rpm (Jones *et al.*, 1985), siendo éste el rango que se suele alcanzar en un test de Wingate clásico (Calbet *et al.*, 2003). Teniendo en cuenta todo lo anteriormente comentado, y tras experimentos previos realizados en nuestro laboratorio, decidimos fijar las revoluciones óptimas para un test de Wingate isocinético en 100 rpm para los tres estudios que componen esta tesis doctoral.

1.2.1 Test de Wingate de 30s en hipoxia aguda severa.

Debido al alto porcentaje de energía proveniente de las vías anaeróbicas en el test de Wingate (Calbet *et al.*, 1997), se ha intentado en algunos trabajos determinar si la realización de dicho test en hipoxia aguda puede tener alguna incidencia en su rendimiento o en las vías metabólicas que se utilizan durante dicho esfuerzo (McLellan *et al.*, 1990; Calbet *et al.*, 2003). Gracias a esto, actualmente se sabe que la hipoxia leve aguda (FiO_2: 0.13) a pesar de producir un descenso del 16% en la saturación de oxígeno, no produce ningún cambio en el Pico de Potencia del Wingate (PPO) o en la potencia media (MPO) (McLellan *et al.*, 1990). A nivel de acumulación de metabolitos, comparado con el Wingate en normoxia, el test en hipoxia leve aguda produce una mayor acumulación de lactato intramuscular (McLellan *et al.*, 1990), lo que sugiere una mayor dependencia de las vías anaeróbicas (McLellan *et al.*, 1990). En esta misma línea, un trabajo previo de nuestro laboratorio, ha demostrado que

el déficit de oxígeno es mayor en la condiciones de hipoxia severa aguda (FiO_2: 0.10), demostrando también una mayor dependencia de las vías anaeróbicas en el esfuerzo (Calbet *et al.*, 2003). No obstante, sólo los ciclistas de pista especializados en pruebas de sprint experimentaron un deterioro de la potencia media del Wingate (MPO) (Calbet *et al.*, 2003). Esto último demuestra que los deportistas cuyo sistema anaeróbico estaba llevado al límite ya en normoxia (sprinters), no pudieron compensar la ausencia de oxigeno a través de dicho sistema (Calbet *et al.*, 2003). Finalmente, teniendo en cuenta que el test de Wingate es ya de por sí un ejercicio de intensidad muy elevada (lo cual hemos visto que es muy importante para activar la AMPK muscular), que la hipoxia potencia aún más la intensidad del esfuerzo y la dependencia anaeróbica del mismo, y que la hipoxia de por sí misma parece producir un aumento de la actividad AMPK, una de las hipótesis de partida de los estudios que componen esta tesis doctoral es que la combinación de ejercicio de sprint e hipoxia podría potenciar sinérgicamente la fosforilación (activación) de la AMPK en músculo esquelético humano.

OBJETIVOS E HIPÓTESIS

2. OBJETIVOS

1) Determinar si la hipoxia severa aguda potencia sinérgicamente la fosforilación de la Thr^{172}-AMPKα inducida normalmente por un ejercicio de sprint de alta intensidad y corta duración (30s) en músculo esquelético de seres humanos (*Estudio I*).

2) Determinar a nivel proteico y metabólico los mecanismos reguladores de la fosforilación Thr^{172}-AMPKα en respuesta al ejercicio de sprint realizado en normoxia e hipoxia (*Estudios I, II y III)*).

3) Determinar si los radicales libres ejercen algún efecto modulador de la fosforilación en la Thr^{172}-AMPKα o sobre su cascada de señalización, en respuesta al ejercicio de sprint en el músculo esquelético de seres humanos (*Estudios I, II y III)*).

4) Comprobar si los efectos de la hipoxia severa aguda sobre la fosforilación de la Thr^{172}-AMPKα en respuesta a un ejercicio de sprint son debidos a un mayor estrés oxidativo. (*Estudio III*).

3. HIPÓTESIS

1) La combinación del ejercicio de alta intensidad (ejercicio de sprint de 30 s) con la hipoxia severa aguda potencia sinérgicamente la fosforilación de la Thr^{172}-AMPKα, debido a la convergencia de un mayor gasto energético y al mayor estrés oxidativo producido por la hipoxia en comparación con el mismo ejercicio realizado en normoxia (*Estudio I*).

2) La ingestión de antioxidantes previamente a la realización de un sprint, al disminuir el estrés oxidativo, produce una reducción de la fosforilación de la Thr^{172}-AMPKα inducida por el ejercicio de sprint en normoxia (*Estudio II)* e hipoxia *(Estudio III*).

3) La ingestión de antioxidantes previamente a la realización de un sprint bloquea el incremento de fosforilación de la Thr^{172}-AMPKα inducida por el ejercicio de sprint en normoxia (estudio II) e hipoxia (estudio III), a través de un mecanismo molecular mediado por un aumento de la fosforilación de la AMPKα en residuos de serina alternativos (Ser^{485}-AMPKα1/Ser^{491}-AMPKα2).

4) La fosforilación de Ser^{221}-ACCβ no se verá afectada por los cambios inducidos por los antioxidantes y la hipoxia sobre la fosforilación de Thr^{172}-AMPKα en respuesta a un ejercicio de sprint (*Estudio II y Estudio III*).

METODOLOGÍA

4. RESUMEN DE LOS METÓDOS Y PROCEDIMIENTOS UTILIZADOS

4.1 Sujetos y procedimientos generales

Los valores medios de los parámetros edad, talla, peso corporal y porcentaje de grasa de los sujetos que participaron en los estudios realizados se describen en la Tabla 1. Todos los sujetos fueron informados, de forma oral y escrita, acerca de los procedimientos y objetivos del estudio así como de los posibles riesgos y beneficios, tras lo cual firmaron la correspondiente autorización. Además, todos los estudios fueron realizados de acuerdo a la Declaración de Helsinki y fueron aprobados por el Comité Ético de la Universidad de Las Palmas de Gran Canaria (CEIH-2010-01).

	Estudio I	Estudio II y III
	N=10	N=9
	Media ± DE	Media ± DE
Edad (años)	25.1 ± 4.5	25.2 ± 4.7
Altura (cm)	176.7 ± 5.3	176 ± 5.1
Peso (Kg)	80.2 ± 9.9	79.4 ± 10.1
Grasa Corporal (%)	18.2 ± 6.3	18.3 ± 6.7

Tabla 1. Características de los sujetos experimentales que participaron en los estudios I, II y II que componen esta Tesis Doctoral. Inicialmente se reclutaron 10 sujetos pero uno de ellos abandonó los experimentos tras el Estudio I.

Estudio I

En este estudio participaron 10 sujetos sanos estudiantes de educación física. Los sujetos realizaron dos test isocinéticos de Wingate de 30 s (100 rpm), en orden randomizado y en días separados. Los voluntarios llegaron en ayunas al laboratorio y tras la ingesta de píldoras que contenían placebo (similares en textura y forma a las que contenían el coctel de antioxidantes (Estudios II y III)), y que fueron ingeridas en dos tomas, de la siguiente forma: 2 horas antes del ejercicio de sprint la primera toma y 1 hora y media antes del ejercicio la segunda dosis. Al llegar al laboratorio, se les colocó un catéter en una vena del antebrazo, se les extrajo una muestra de sangre y una biopsia del vasto lateral del cuádriceps mediante la técnica de Bergstrom con succión mientras permanecieron tumbados en la camilla. Posteriormente, los sujetos subieron al cicloergómetro *Lode Excaliburt Sport 925900* (Groningen, Holanda) y respiraron durante 4 minutos la mezcla de gas correspondiente al test en normoxia (FiO_2:0.21) o hipoxia (FiO_2:0.10), con una boquilla conectada a un analizador de gases (Vmax N29; Sensormedics, California, USA) mientras respiraban el contenido aire de un saco de *Douglas* (en el caso del test de hipoxia). Tras estos 4 minutos, los sujetos realizaron un test de Wingate isocinético de 30s. Inmediatamente tras la finalización del test, se procedió a extraer una nueva biopsia del vasto lateral del cuádriceps y otra muestra de sangre. Esta toma de muestras fue repetida nuevamente a los 30 minutos y 2

horas tras la finalización del ejercicio y durante la recuperación. Para más detalles consultar la sección de material y métodos del Estudio I anexado.

Estudio II

En este estudio participaron 9 de los 10 sujetos sanos estudiantes de educación física que también tomaron parte en el Estudio I. Los sujetos realizaron dos test de Wingate isocinéticos de 30 s (100 rpm), uno en cada sesión experimental, en orden randomizado y en días separados. Los voluntarios llegaron en ayunas al laboratorio y tras la ingesta de unas píldoras que contenían un coctel de antioxidantes o un placebo (éstas últimas similares en textura y forma a las que contenían el coctel de antioxidantes) y que fueron ingeridas en dos tomas, de la siguiente forma: 2 horas antes del ejercicio de sprint la primera toma (coctel de antioxidantes: ácidoα -lipoico (300 mg), vitamina C (500 mg) y vitamina E (200 IU)) y 1 hora y media antes del ejercicio la segunda dosis. Al llegar al laboratorio, se procedió a colocar a los sujetos un catéter en una vena del antebrazo, se les extrajo una muestra de sangre y una biopsia del vasto lateral del cuádriceps mediante la técnica de Bergstrom con succión mientras permanecieron tumbados en la camilla. Posteriormente, los sujetos subieron al cicloergómetro *Lode Excaliburt Sport 925900* (Groningen, Holanda) y respiraron durante 4 minutos en normoxia (FiO_2:0.21) con una boquilla conectada a un analizador de gases (Vmax N29; Sensormedics, California, USA).Tras estos 4 minutos

de aclimatación, los sujetos realizaron un test de Wingate isocinetico de 30s. Inmediatamente tras la finalización del test, se obtuvo otra biopsia muscular y una nueva muestra de sangre. Esta toma de muestras fue repetida nuevamente a los 30 minutos y 2 horas tras la finalización del ejercicio y durante la recuperación. Para más detalles consultar la sección de material y métodos del Estudio II anexado.

Estudio III

En este estudio participaron los mismos 9 sujetos sanos estudiantes de educación física que participaron en el estudio II. Los sujetos realizaron dos test isocinéticos de Wingate de 30 s (100 rpm), en días diferentes y en orden randomizado. Los voluntarios llegaron en ayunas al laboratorio y tras la ingesta de píldoras que contenían antioxidantes o placebo de manera similar a lo explicado en el estudio II. Al llegar al laboratorio, a los sujetos se les colocó un catéter en una vena del antebrazo y se les extrajo una muestra de sangre y una biopsia del vasto lateral del cuádriceps mediante la técnica de Bergstrom con succión mientras permanecieron tumbados en la camilla. Posteriormente, los sujetos subieron al cicloergómetro *Lode Excaliburt Sport 925900* (Groningen, Holanda) y respiraron durante 4 minutos la mezcla de gas correspondiente al experimento en hipoxia (FiO_2:0.10) con una boquilla conectada a un analizador de gases (Vmax N29; Sensormedics, California, USA) mientras respiraban el contenido aire de un saco de

Douglas. Tras estos 4 minutos de aclimatación, los sujetos realizaron un test de Wingate isocinetico de 30s. Inmediatamente tras la finalización del test, se procedió a extraer una nueva biopsia del vasto lateral del cuádriceps y otra muestra de sangre. Esta toma de muestras fue repetida nuevamente a los 30 minutos y 2 horas tras la finalización del ejercicio y durante la recuperación. Para más detalles consultar la sección de material y métodos del Estudio III anexado.

4.2 Condición Física.

4.2.1 VO_2pico.

Cada uno de los sujetos realizó un test incremental en rampa hasta el agotamiento con una pendiente de (50W/min) empezando desde 0 vatios. En él se midió el VO_2pico, la Frecuencia Cardiaca Máxima y la Potencia Máxima de cada sujeto en Normoxia. El mismo test con idéntica rampa (50W/min) fue repetido en hipoxia (FiO_2:0.10) para medir los mismos parámetros en dicha condición.

4.2.2 Test de Economía de Pedaleo

El test de economía de pedaleo fue realizado en dos días diferentes usando entre 8-11 intensidades de trabajo entre el 50% y 90% del VO_2pico a 100 rpm. Las intensidades de las cargas fueron administradas en orden randomizado y separadas por periodos de descanso de 6 minutos cada uno. Para reducir el estrés térmico y minimizar la pérdida de agua debida a la sudoración, a los sujetos se les

permitió beber agua fresca "*ad libitum*" durante los periodos de recuperación. La duración de cada carga fue de 10 minutos. Se tomó como representativa la media del consumo de oxígeno (VO_2) durante los últimos 2 minutos de cada carga. Para relacionar el VO_2 con la potencia se determinó el mejor ajuste lineal por mínimos cuadrados.

4.3 Composición Corporal.

4.3.1 Antropometría.

La talla se midió en bipedestación con los talones, los glúteos la espalda y la región occipital en contacto con el plano del tallímetro. Estas medidas se efectuaron mediante un tallímetro de 1mm de precisión (Atlántida, Añó Sayol, Barcelona, España), manteniendo la cabeza en el plano de Francfort. La masa corporal se midió mediante una báscula (Atlántida, Añó Sayol, Barcelona, España) de 50 g de precisión, calibrada a 50.0, 70.0 ó 90.0 Kg, mediante masas patrón de la clase M1.

4.3.2 Masa muscular, masa ósea y porcentaje de masa grasa.

La masa magra (masa corporal – [masa grasa + masa ósea]) se determinó mediante absorciometría fotónica dual de rayos X (DXA) (*QDR-1500, Hologic Corp., Software versión 7.10, Waltham, MA*). Los sujetos se escanearon tumbados en posición supina junto a una barra de calibración de diferentes grosores y densidades. A partir del análisis regional y de cuerpo entero se calculó la masa magra (g), la masa grasa (g), el área ósea total (cm^2) y el contenido mineral óseo (BMC) (g) tal

como se ha hecho en trabajos previos de nuestro laboratorio (Ara *et al.*, 2004; Ara *et al.*, 2006).

4.4 Procesamiento de las muestras de sangre.

Los sujetos fueron sometidos a una extracción de sangre periférica en ayunas que se dejó coagular en hielo durante 20 minutos. Las muestras fueron centrifugadas y el suero separado y almacenado en un congelador de -80ºC hasta su posterior análisis. En el suero de determinó: la glucosa a través del método hexoquinasa usando *Glucoquat reagents* (Roche/Hitachi 11876899216, Indianapolis, USA) y la insulina por medio de la técnica inmunoensayo de electroquimioluminiscencia (ECLIA), empleando para ello un analizador *Modular Analytics E170* (Roche/Hitachi, Indianapolis, USA).

4.5 Biopsias musculares.

Las biopsias musculares se obtuvieron por punción bajo anestesia local del vasto lateral del cuádriceps, tal como se ha realizado en el laboratorio de Rendimiento Humano de la ULPGC en numerosas ocasiones usando la técnica de Bergstrom, tras una noche de ayuno. Con esta técnica se pueden obtener 40-60 mg de músculo, pero como fueron realizadas con aspiración se consiguieron alrededor de 200 mg de músculo (Lundby *et al.*, 2006). La biopsia muscular fue limpiada de tejido

conectivo, de grasa visible y sangre usando un microscopio antes de ser guardada a -80ºC.

4.5.1. Análisis bioquímico de metabolitos musculares

De cada biopsia se separó una porción de músculo de 30 mg de peso húmedo que fue tratada con 0.5 M de $HClO_4$ y centrifugada a 15000 g durante 15 min a 4ºC. El sobrenadante obtenido fue neutralizado con $KHCO_3$ 2.1 M y posteriormente se procedió a determinar enzimáticamente los niveles de ATP, fosfocreatina (PCr), creatina (Cr), piruvato (Pyr) y lactato (Lac) mediante análisis fluorimétrico (Lowry & Passonneau, 1972; Gorostiaga *et al.*, 2010). La concentración de metabolitos musculares fue ajustada por la media de la creatina total (PCr + Cr), ya que esta media permanece constante durante el ejercicio (Harris *et al.*, 1976). El ajuste por el contenido de la creatina total evita la variabilidad de los constituyentes sólidos no musculares, los cuales pueden estar presentes en las biopsias (Parra *et al.*, 2000). La velocidad glucolítica (GR) fue calculada según (Rovira *et al.*, 2012) como: GR= 0.5 x (ΔLac + ΔPyr).

El ratio molar de AMP/ATP fue estimado después del cálculo de la concentración de adenosina difosfato (ADP) usando la constante de equilibrio aparente de la creatina quinasa para condiciones basales y de agotamiento después del test de Wingate (Parra *et al.*, 2000), que fue descrita por Sahlin y colaboradores (Sahlin *et al.*, 1975). La concentración

de adenosina monofosfato (AMP) fue calculada usando la constante de equilibrio aparente de la adenilato quinasa para las mismas condiciones (Parra *et al.*, 2000). El ratio $[NAD^+]/[NADH.H^+]$ fue calculado usando la constante de equilibrio de la lactato deshidrogenasa (Bücher & Klingenberg, 1958; Williamson *et al.*, 1967).

4.5.2. Análisis de proteínas mediante la técnica del Western blot

Se trata de una técnica que permite la detección del grado de presencia de una proteína en estudio, mediante la separación diferencial según el peso molecular de esta proteína desnaturalizada, y la posterior exposición a anticuerpos específicos.

4.5.2.1. Obtención de extractos proteicos a partir de biopsias musculares

Para la obtención de los extractos proteicos de músculo esquelético humano, una pieza del tejido congelado fue homogeneizada en Buffer de Lisis de Urea (UREA 6 M- SDS 1% , Inhibidor de proteasas Complete 1X y de fosfatasas PhosStop 1X) ambos inhibidores fueron de *Roche Diagnostics* (*Mannheim, Alemania*). Después de ser centrifugados durante 15 minutos a 20000g, los extractos totales se transfirieron a tubos limpios y una alícuota de cada extracto fue separada para la

cuantificación de proteínas por el método del ácido bicinconínico (Smith *et al.*, 1985).

4.5.2.2. Separación de proteínas a través de electroforesis

Los extractos proteicos fueron diluidos en tampón de carga de electroforesis (Tris-HCl pH 6.8, 62.50 mM, SDS 2.3%, glicerol 10%, β-mercaptoetanol 5%, azul de bromofenol). A continuación, se procedió a la separación electroforética de las proteínas en geles de acrilamida-bisacrilamida (7.5% - 10%) usando el sistema de Laemmli (Laemmli, 1970), con las modificaciones convenientes (Marin et al., 2001). Estos geles permiten separar las proteínas por sus diferentes pesos moleculares.

4.5.2.3 Proceso de detección de proteínas.

Después de la separación electroforética de extractos totales de proteínas, se procedió a la transferencia de las proteínas a membranas de polivinilo (*Hybond-P PVDF, Amersham Biosciences*), la cual se realizó a 400 mA durante 90 minutos a 4ºC.

Para evitar la unión no específica de los anticuerpos, las membranas fueron incubadas con un tampón de bloqueo al menos durante 1 hora a temperatura ambiente:

-*Blotto blocking buffer* (leche desnatada al 5% disuelta en tampón TBS con 0.1% del detergente Tween-20 (TBS-T)),

usado generalmente para diluir los anticuerpos no fosfo-específicos.

-*BSA blocking buffer* (Albúmina de suero bovino (BSA) al 4% disuelta en TBS-T), usado generalmente para diluir los anticuerpos fosfo-específicos.

La inmunodetección comenzó con la incubación de la membrana con el anticuerpo primario correspondiente (ver tabla 2). Las condiciones exactas de incubación para cada anticuerpo pueden ser consultadas en los artículos que componen esta tesis doctoral. Posteriormente a la incubación con los anticuerpos primarios y al lavado de las membranas en tampón TBS-T, se procedió a la incubación con los anticuerpos secundarios correspondientes acoplados a peroxidasa de rábano. Esta incubación se realizó durante 1 hora a temperatura ambiente en *blotto blocking buffer*. La visualización de la reacción inmunológica se llevó a cabo por la reacción enzimática de la peroxidasa con un compuesto que emite luz al oxidarse (*Inmmun-Star*^{TM} *WesternC*^{TM} *de Bio-Rad Laboratories, Hemel Hempstead Hertfordshire, UK*). Las bandas específicas fueron visualizadas con el sistema *Chemidoc XRS* (*Bio-Rad Laboratories*) y analizadas con un programa informático de análisis de imagen (*Quantity One©, Bio-Rad Laboratories*).

4.5.2.4. Detección de proteínas carboniladas.

La carbonilación proteica en músculo esquelético y plasma fue medida a través de la técnica del *Western blo*t, de los grupos carbonilo de las proteínas que son detectables gracias al kit de oxidación proteica "*Oxyblot*" (*Intergen, Purchase, NY*) como se ha sido descrito previamente por Romagnoli y colaboradores (Romagnoli *et al.*, 2010).

Tabla 2. Anticuerpos y diluciones usadas en los ensayos *Western blot*.

ANTICUERPO PRIMARIO	PROVEEDOR	REFERENCIA	PESO MOLECULAR	DILUCIÓN
Monoclonal de ratón anti-**α**-tubulina (Guerra *et al.*, 2010)	Biosigma (Madrid, España)	T-5168-ML	50 KDa	1:50.000
Policlonal de conejo anti-Thr^{172}-**AMPKα** (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 2531	62 KDa	1:1.000
Policlonal de conejo anti-**AMPKα** (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 2532	62 KDa	1:1.000
Policlonal de conejo anti-phospho-AMPK**α**1 (Ser^{485})/AMPK**α**2 (Ser^{491})-**AMPKα** (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 4185	62 KDa	1:500
Policlonal de conejo anti-**AMPKα1** (Ser^{485}) (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 4184	62 KDa	1:250
Policlonal de conejo anti-**AMPKα1** (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 2795	62 KDa	1:250
Policlonal de conejo anti-**ACCβ** (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 3662	280 KDa	1:400
Policlonal de conejo anti-**fosfoACCβ** (Ser^{221}) ((Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 3661	280 KDa	1:400
Policlonal anti-Phospho-AS160(Thr^{642}) (Guerra *et al.*, 2010)	MBL International Corporation (Woburn, MA, USA)	no. AT-7079	160 KDa	1:500
Policlonal anti-AS160 (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 2447	160 KDa	1:250
Policlonal anti-phospho-CaMKII (Thr^{286}) (Egan *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 3361	50 KDa	1:500
Policlonal anti-CaMKII (Egan *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	sc-13082	50 KDa	1:400
Policlonal anti SIRT1 (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 2310	120 KDa	1:250
Policlonal anti-phospho-Akt (Ser^{473}) (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 9271	60 KDa	1:500
Policlonal anti-phospho-Akt (Thr^{308}) (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 9275	60 KDa	1:250
Policlonal anti- Akt (Guerra *et al.*, 2010)	Cell Signalling Technology (Barcelona, España)	no. 9272	60 KDa	1:1000
Policlonal anti-phospho-TAK1(Thr$^{184/187}$) (Blanco *et al.*, 2007)	Cell Signalling Technology (Barcelona, España)	no. 4531	82 KDa	1:250
Policlonal anti-TAK1 (Blanco *et al.*, 2007)	Cell Signalling Technology (Barcelona, España)	no. 4505	82 KDa	1:250

4.5.2.5 Análisis estadístico.

Los datos cuantitativos están expresados como media ± error estándar (S.E.M). La normalidad de las variables fue comprobada mediante el test *Kolmogorov-Smirnov* corregido por *Lilliefors* para el estudio I, y mediante el test *Shapiro Wilks* para los estudios II y III. Cuando fue necesario, el análisis fue realizado con los datos transformados en logaritmos. Para la comparación entre experimentos, la respuesta individual fue normalizada por el nivel de fosforilación justo antes de comenzar el test de Wingate. El ANOVA de medidas repetidas fue realizado sobre tiempo y condición con dos niveles (Estudio I: Normoxia *versus* Hipoxia; Estudio II: Placebo *versus* Antioxidantes; Estudio III: Hipoxia-Placebo *versus* Hipoxia-Antioxidantes) y fue usada para comparar los valores post-ejercicio con respecto a los valores obtenidos en reposo. Cuando la ANOVA de medidas repetidas resultó significativa para el tiempo o para tiempo x condición, se procedió a realizar un ajuste por comparaciónes múltiples mediante el método de *Holm-Bonferroni*. La relación entre variables fue determinada usando análisis de regresión lineal. El área bajo la curva (ABC) fue determinada usando la regla trapezoidal. Las ABC de las distintas condiciones fueron comparadas entre sí mediante pruebas *T de Student*. Un valor de $P \leq 0.05$ fue considerado significativo. El análisis estadístico se llevó a cabo mediante la utilización de la versión 15.0 del programa informático SPSS (SPSS,Chicago,IL).

RESUMEN DE RESULTADOS

5. RESUMEN DE RESULTADOS

En el siguiente apartado se resumen los resultados más relevantes de cada uno de los artículos. La descripción detallada de los resultados se encuentra en las publicaciones anexas que forman parte de esta tesis doctoral.

5.1 Resumen de resultados artículo I (Morales-Alamo, D et al. 2012 JAP).

David Morales-Alamo, Ponce-González, J. G., Guadalupe-Grau, A., Rodríguez-García, L., Santana, A., Cusso, R., Guerrero, M., Guerra, B., Dorado, C., Calbet, J. A. L. **Increased oxidative stress and anaerobic energy release, but blunted Thr^{172}-AMPKα phosphorylation in response to sprint exercise in severe acute hypoxia in man.** J Appl Physiol 2012 Aug 2.

La potencia media y el consumo de oxígeno durante el test de Wingate fueron un 6 y 37%, respectivamente menores en hipoxia ($P \leq 0.05$). Por el contrario, en hipoxia, el déficit de oxígeno y la acumulación de lactato en el músculo fueron mayores que en normoxia ($P \leq 0.05$). En hipoxia también se observó un aumento en las proteínas carboniladas en el músculo esquelético y en plasma después de realizar el test de Wingate ($P \leq 0.05$).

La fosforilación de la Thr^{172}-AMPKα aumentó a los treinta minutos después del ejercicio de sprint y durante la recuperación del test realizado en normoxia, estando ésta bloqueada en hipoxia ($P \leq 0.05$). El ratio $NAD^+/NADH.H^+$ se redujo tras la realización de ambos test de Wingate, pero su descenso fue mucho mayor en hipoxia que en normoxia ($P \leq$

0.05). Del mismo modo, la cantidad total de la proteína SIRT1 se redujo un 53% después del test realizado en hipoxia.

La fosforilación de Ser^{485}-AMPKα1/ Ser^{491}-AMPKα2, la cual es un conocido inhibidor de la fosforilación de la Thr^{172}-AMPKα, se vio aumentada (60%) justo después del test realizado en hipoxia ($P \leq 0.05$). Esto último coincidió, en tiempo y en condición, con un aumento de la fosforilación en la Thr^{308}-Akt.

5.2 Resumen de resultados artículo II (Morales-Alamo, D et al. 2012, Submitted).

David Morales-Alamo, Ponce-González, J. G., Guadalupe-Grau, A, Rodríguez-García, L., Santana, A., Cusso, R., Guerrero, M., Dorado, C., Guerra, B., Calbet, J. A. L. **Critical role for free radicals on sprint exercise-induced CaMKII and AMPKα phosphorylation in human skeletal muscle.** (Submitted).

La ingesta de antioxidantes no alteró el nivel basal de fosforilación de las diferentes proteínas quinasa determinadas en este estudio II. Tanto las variables ergométricas, como el metabolismo aeróbico y anaeróbico fueron similares en las dos condiciones experimentales estudiadas. Los ratios $NAD^+/NADH.H^+$ y AMP/ATP se vieron reducidos de manera independiente a la ingesta de antioxidantes ($P \leq 0.05$). Las fosforilaciones de Thr^{286}-CaMKII y de Thr^{172}-AMPKα se incrementaron tras el sprint control (placebo), pero no tras el sprint de la condición de antioxidantes ($P \leq 0.05$). Mientras que, el nivel de fosforilación de Ser^{485}-AMPKα1/ Ser^{491}-AMPKα2 se vio incrementado justo después del sprint realizado tras ingerir antioxidantes ($P \leq 0.05$). Además, el nivel de fosforilación de TAK1 también fue mayor tras el test realizado después de la ingesta de antioxidantes ($P \leq 0.05$).

5.3 Resumen de resultados artículo III (Morales-Alamo, D et al. Submitted).

David Morales-Alamo, Jesús Gustavo Ponce-González, Amelia Guadalupe-Grau, Alfredo Santana, Roser Cusso, Joan Cadefau, Borja Guerra, Cecilia Dorado, José A.L. Calbet. **Skeletal muscle signaling during sprint exercise in severe acute hypoxia: role of free radicals.** (Submitted).

La ingesta de antioxidantes no alteró el nivel basal de fosforilación de las diferentes proteínas quinasa determinadas en este estudio III. Las fosforilaciones de Thr^{172}-AMPKα y de $Thr^{184/187}$-TAK1 no aumentaron después de los sprints en ninguna condición ($P \leq 0.05$). Mientras que la fosforilación de Thr^{286}-CaMKII aumentó en respuesta al ejercicio de sprint, aunque la ingesta de antioxidantes previa bloqueó este efecto ($P \leq 0.05$). Las fosforilación de Ser^{485}-AMPKα1/ Ser^{491}-AMPKα2 se vio incrementada tras ambos test de Wingate ($P \leq 0.05$). Esto último coincidió con el aumento de la fosforilación de Akt ($P \leq 0.05$).

El descenso del ratio AMP/ATP fue menor después del ejercicio de sprint realizado tras la ingesta de antioxidantes ($P \leq 0.05$). La velocidad glucolítica, la cual aumenta tras la realización de un test de Wingate, pero se exacerba si éste se realiza en condiciones de hipoxia severa aguda (Estudio I), se vio disminuida al realizar el test de Wingate en hipoxia tras la ingestión de antioxidantes ($P \leq 0.05$).

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

Estudio I: Increased oxidative stress and anaerobic energy release, but blunted Thr^{172}-AMPKα phosphorylation in response to sprint exercise in severe acute hypoxia in man. J Appl Physiol 2012 Aug 2.

Este estudio examinó la influencia de la fracción inspiratoria de O_2 en la regulación de la fosforilación de la AMPKα en músculo esquelético humano en respuesta a un ejercicio de sprint de 30 segundos. En contraste con nuestra hipótesis de partida, la hipoxia sumada al ejercicio de sprint, no sólo no potenció el incremento esperado de la fosforilación en la Thr^{172}-AMPKα, y que se produce normalmente durante la recuperación (30 minutos tras el Wingate), sino que por el contrario bloqueó esta respuesta con una estimulación previa de la fosforilación Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, siendo éste un conocido mecanismo inhibitorio de la fosforilación de la Thr^{172}-AMPKα (Horman *et al.*, 2006). La realización del sprint en hipoxia severa aguda provocó una mayor reducción del ratio NAD^+/$NADH.H^+$, combinado con un mayor velocidad glucolítica y estrés oxidativo, como reflejó el incremento en la acumulación de lactato intramuscular y los elevados niveles de proteínas carboniladas en el músculo y plasma, observados durante el periodo de recuperación. En este estudio también demostramos que el sprint en hipoxia reduce los niveles proteicos de SIRT1, lo cual combinado con el menor ratio NAD^+/$NADH.H^+$ observado al final del sprint, podría producir una menor activación de LKB1 por SIRT1 y, de este modo reducir la fosforilación en la Thr^{172}-AMPKα.

Ratios Lac/Pyr y NAD^+/$NADH.H^+$ y señalización muscular

La acumulación de lactato observada fue debida a la enorme demanda de energía generada durante el sprint, que provocó una velocidad glucolítica que sobrepasó la capacidad mitocondrial para oxidar piruvato. En el presente estudio, la hipoxia redujo la potencia media en el test de Wingate pero sólo en un 6%, a pesar de que el VO_2 acumulado se vio reducido en un 37%. Esto último refleja una menor capacidad mitocondrial para oxidar piruvato en hipoxia, debido al menor consumo de O_2. Para compensar la reducción de la producción de energía por oxidación, la velocidad glicolítica fue incrementada un 50% durante el sprint en hipoxia comparado con el sprint en normoxia. Este hallazgo coincide con el incremento del 14% observado en el déficit de O_2 (ml de O_2/w) durante un sprint en hipoxia (Medbo *et al.*, 1988; Calbet *et al.*, 2003).

El incremento de la velocidad de glucólisis en hipoxia provocó una mayor acumulación de lactato y un mayor ratio Lac/Pyr. Por consiguiente, el cálculo del ratio citoplasmático de NAD^+/$NADH.H^+$ (Williamson *et al.*, 1967; Sahlin, 1985) se redujo en mayor medida después del sprint en hipoxia que en normoxia.

Durante el ejercicio, el principal mecanismo que provoca la fosforilación en la Thr^{172}-AMPKα es el incremento de AMP libre (relativo al ATP) (Xiao *et al.*, 2007), el cual actúa alostéricamente a través de la subunidad γ para aumentar la fosforilación de Thr^{172}-AMPKα a través de

la LKB1 (Hawley *et al.*, 2003) y para inhibir *in vitro* la actividad de las fosfatasas PP2A y PP2C, siendo éstas las principales fosfatasas responsables de la de-fosforilación de la Thr^{172} (Davies *et al.*, 1995). A pesar de la mayor producción anaeróbica de energía durante el sprint en hipoxia, el ratio AMP/ATP calculado se incrementó de una manera muy similar inmediatamente después del sprint en ambas condiciones experimentales. Es precisamente por esto último, por lo que nuestros resultados no pueden explicar que el bloqueo de la fosforilación de la Thr^{172}-AMPK se produzca por un mecanismo dependiente de la producción de AMP, en el test en hipoxia. Además del AMP, SIRT1, una de-acetilasa que puede actuar como un sensor metabólico de NAD^+, es capaz de de-acetilar y activar a LKB1 (Hou *et al.*, 2008). La incubación de células HepG2 en un medio enriquecido en piruvato resulta en un incremento de la expresión proteica de SIRT1 y en una fosforilación concomitante de la Thr^{172}-AMPKα (Suchankova *et al.*, 2009), lo cual está probablemente relacionado con la elevación del ratio $NAD^+/NADH.H^+$. La actividad SIRT1 y la pérdida de la cantidad de proteína puede ser explicada por un menor nivel de NAD^+ (Gao *et al.*, 2011) y ambos fenómenos fueron observados en la presente investigación después del sprint en hipoxia. Por eso, la combinación del efecto de la menor cantidad de NAD^+ y de la reducción de la cantidad de proteína SIRT1 podría haber bloqueado la activación de LKB1 en hipoxia y, de este modo, la fosforilación de AMPKα en la Thr^{172}.

En relación con nuestros resultados, la insulina o la glucosa inducen la fosforilación de JNK1 en cultivos celulares, la cual una vez activa es capaz de mediar a su vez la fosforilación en Ser^{47}-SIRT1 (Gao *et al.*, 2011). La fosforilación de SIRT1 produce su translocación al núcleo y un incremento de su actividad de-acetilasa, estimulando además la degradación proteolítica de SIRT1 en el proteasoma (Gao *et al.*, 2011), lo que reduce los niveles proteicos de SIRT1 unos 30 minutos aproximadamente (Gao *et al.*, 2011). Aunque la respuesta de la insulina y la glucosa plasmática a los sprints no pudo explicar en el presente estudio los cambios observados en los niveles proteicos de SIRT1, es bien sabido que el H_2O_2 (inductor de estrés oxidativo) produce en cultivos celulares la fosforilación de JNK1, la cual es capaz de mediar la fosforilación de SIRT1, tal como comentamos anteriormente (Nasrin *et al.*, 2009). De esta forma, el gran estrés oxidativo producido por el ejercicio de sprint en hipoxia podría conducir a una reducción de la cantidad proteica de SIRT1, a través de un mecanismo que implicaría la fosforilación de JNK1 y SIRT1, lo que conduciría a la degradación proteolítica de esta última.

La comentada reducción de los niveles de la proteína SIRT1 después de sprint en hipoxia se vio acompañada por un incremento de la fosforilación de Thr^{308}-Akt. En concordancia, el resveratrol (un agente antioxidante activador de SIRT1) reduce la activación de fosfoinosol-3-quinasa (PI3K) en cultivos celulares de músculo (Frojdo *et al.*, 2007).

Puesto que PI3K es una proteína quinasa que se encuentra *upstream* de la fosforilación en la Thr^{308}-Akt (Hers *et al.*, 2011), la reducción de los niveles de proteína de SIRT1 después del sprint en hipoxia podría explicarse, al menos en parte, por un aumento en la fosforilación de la Thr^{308}-Akt inducida por la mencionada condición experimental.

Radicales Libres

Se ha demostrado que los radicales libres pueden ser inductores de la fosforilación de la Thr^{172}-AMPKα por varios mecanismos (Song & Zou, 2012b). Además, algunos trabajos también han demostrado que la hipoxia puede producir la fosforilación en la Thr^{172}-AMPKα por un mecanismo independiente del ratio AMP/ATP en el que las especies reactivas del oxígeno producidas en la mitocondria jugarían un papel clave (Emerling *et al.*, 2009). En el presente estudio, los niveles plasmáticos y musculares de proteínas carboniladas se vieron incrementados en la recuperación del sprint en hipoxia, indicando un mayor estrés oxidativo después de este test en hipoxia. El hecho de que se encontrara un aumento de las proteínas carboniladas también en el plasma indica que algunas especies reactivas del oxígeno fueron liberadas a la circulación sanguínea tras el ejercicio (Bailey *et al.*, 2007). La carbonilación proteica es un buen marcador de la existencia de estrés oxidativo (Shacter, 2000; Beal, 2002), sin embargo, los radicales libres también pueden reaccionar con otras moléculas (Bailey *et al.*, 2007), lo

cual implicaría que el estrés oxidativo observado en hipoxia con respecto al test en normoxia podría ser aún mayor.

Los radicales libres pueden activar CaMKII a través de la modificación del par Met-$^{281/282}$ localizado en el dominio regulatorio de la quinasa, lo cual bloquea la reasociación con el dominio catalítico, preservando la actividad quinasa, a través de un mecanismo similar pero paralelo a la auto-fosforilación del residuo de aminoácido Thr^{286} (Erickson *et al.*, 2008). Por otra parte, la capacidad que posee la CaMKII de responder a incrementos en la concentración de Ca^{+} está aumentada en condiciones pro-oxidantes (Erickson *et al.*, 2008). Sin embargo, en nuestro estudio no encontramos diferencias significativas entre ambas condiciones experimentales en la respuesta de la CaMKII al ejercicio. De esta forma, la ausencia de fosforilación en la Thr^{172}-AMPKα en respuesta al sprint en hipoxia no puede ser atribuida a una menor fosforilación de la Thr^{286}-CaMKII.

La fosforilación de las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 esta incrementada después del ejercicio de sprint: potencial mecanismo inhibitorio de la fosforilación de la Thr^{172}-AMPKα.

La proteína quinasa B (Akt) (Horman *et al.*, 2006) y proteína quinasa dependiente de AMP cíclico (PKA) (Hurley *et al.*, 2006) pueden fosforilar AMPKα1/α2 en los residuos de serina alternativos, ejerciendo esta fosforilación un efecto inhibitorio de la fosforilación en la Thr^{172}-

AMPKα. El ejercicio de sprint en hipoxia realizado en el presente estudio impidió el esperado aumento de la fosforilación de la Thr^{172}-AMPKα 30 minutos después del ejercicio (Guerra *et al.*, 2010; Fuentes *et al.*, 2011). Sin embargo, sí que se observó un incremento en la fosforilación de Thr^{308}-Akt inmediatamente después del sprint en hipoxia. Algunos estudios previos han relacionado la fosforilación de las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 con un aumento previo de Akt (Horman *et al.*, 2006; Guerra *et al.*, 2010). Consecuentemente, en el presente estudio, también observamos un incremento significativo de la fosforilación en las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 después de test de Wingate en hipoxia. Todo lo anteriormente comentado confirmó un trabajo previo de nuestro laboratorio en el que se observó el bloqueo de la fosforilación de la Thr^{172}-AMPKα por un aumento de la fosforilación en Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 inducido por la ingestión de 75 g de glucosa 1 hora antes de un ejercicio de sprint de 30 segundos, a través de un mecanismo que implica la activación de la Akt (Guerra *et al.*, 2010). Como en Guerra y colaboradores, el ejercicio provocó un incremento de los niveles plasmáticos de insulina 30 minutos después del mismo, durante la recuperación, el cual podría explicar la estimulación de la fosforilación de Akt (Ser^{473}) y AS160 (Thr^{642}) detectada en ese mismo punto temporal (Guerra *et al.*, 2010). Sin embargo, inmediatamente después del ejercicio de sprint en hipoxia se observó una estimulación de la fosforilación de la Thr^{308}-Akt (significativamente mayor que en el test de normoxia) que

ocurrió en presencia de cambios menores en los niveles plasmáticos de insulina. Por todo ello, los resultados aportados por el presente estudio sugieren que el efecto de la FiO_2 sobre la fosforilación de la AMPK en los residuos alternativos de serina no puede ser atribuido a la insulina (aunque la fosforilación de la Thr^{308}-Akt sí se vio aumentada) y requiere de futuras investigaciones.

El aumento de los niveles intracelulares de AMP cíclico (AMPc) pueden producir un bloqueo de fosforilación de la AMPK en la Thr^{172}-AMPKα, a través de la inhibición de la CaMKKβ (una AMPK quinasa) (Hawley *et al.*, 2005), pero no de la LKB1 (Hurley *et al.*, 2006). Por otra parte, Hurley y colaboradores demostraron que la fosforilación Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 parece ser necesaria, pero no suficiente, para inhibir AMPK en condiciones donde los niveles de AMPc están elevados (Hurley *et al.*, 2006). Sin embargo, se desconoce aún si los niveles de AMPc son modificados por el ejercicio de sprint de manera dependiente de la FiO_2.

TAK1 puede actuar como una AMPK quinasa (Xie *et al.*, 2006) y su actividad puede ser estimulada por la hipoxia (Blanco *et al.*, 2007; Melvin *et al.*, 2011). Sin embargo, en el presente trabajo no se observó ningún cambio en los niveles de fosforilación de TAK1 en respuesta al ejercicio o a la FiO_2. Por tanto, nuestros resultados no apoyan la idea de que TAK1 pueda haber influido en la activación de AMPK en el presente estudio.

Estudio II: Critical role for free radicals on sprint exercise-induced CaMKII and AMPKα phosphorylation in human skeletal muscle. (Submitted).

En este estudio se examinó el papel de las especies reactivas del oxígeno (ROS) sobre la regulación de la fosforilación de la AMPK en músculo esquelético humano en respuesta a un ejercicio de sprint isocinético de 30 segundos. Los resultados aportados por la presente investigación demuestran que la suplementación con antioxidantes previa al ejercicio previene la fosforilación de la Thr^{172}-AMPKα a través de dos potenciales mecanismos. Primero, la ingesta de antioxidantes inhibió la fosforilación de la Thr^{286}-CaMKII, proteína quinasa que se encuentra *upstream* de la AMPK en esta vía de señalización muscular. En segundo lugar, la suplementación previa con antioxidantes produjo un aumento de la fosforilación de las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, inmediatamente después del sprint. Estos resultados sugieren que los ROS pueden jugar un papel clave en la modulación de la fosforilación de la Thr^{172}-AMPKα en respuesta al ejercicio de sprint. Previamente, se ha demostrado que el coctel de antioxidantes empleado en el presente estudio, reduce el estrés oxidativo en reposo y en respuesta al ejercicio en humanos (Richardson *et al.*, 2007). A pesar de ello, en la presente investigación los antioxidantes no produjeron una alteración del rendimiento, del metabolismo muscular aerobio o anaerobio, de los ratios AMP/ATP o NAD^+/$NADH.H^+$, o de la carbonilación proteica en musculo esquelético o en plasma. La carbonilación de proteínas es a la vez, irreversible e

irreparable (Barreiro & Hussain, 2010). Por lo tanto, el presente estudio II de esta tesis doctoral demuestra que un sprint de 30 s en humanos sanos no induce un incremento detectable de carbonilación proteica, posiblemente porque el nivel de carbonilación proteica de ambos sprints fue reducido o modesto. Lo cual no significa que no exista un aumento del estrés oxidativo ya que muchos radicales libres han podido ser captados por sistemas antioxidantes o por otras estructuras celulares.

Regulación de AMPK por la fosforilación de la Thr^{286}-CaMKII: posible papel de los radicales libres.

Como ya comentamos en el estudio I, se ha demostrado que los radicales libres son inductores de la fosforilación en la Thr^{172}-AMPKα por varios mecanismos (Song & Zou, 2012a), y que la hipoxia puede incrementar la fosforilación de Thr^{172}-AMPKα por un mecanismo que requiere de las especies reactivas del oxígeno de origen mitocondrial, pero que es independiente del ratio AMP/ATP (Emerling *et al.*, 2009). Por ello, la hipótesis de partida de este estudio II fue la siguiente: la ingestión de antioxidantes previa a la realización de un ejercicio de sprint puede mitigar la esperada fosforilación en Thr^{172}-AMPKα normalmente observada en el vasto lateral del cuádriceps durante la recuperación tras único sprint (Guerra *et al.*, 2010; Fuentes *et al.*, 2012b). En relación directa con nuestros resultados, Gómez-Cabrera y colaboradores (2008) demostraron en ratas y humanos que la suplementación con vitamina C

bloquea las determinadas adaptaciones musculares al entrenamiento de resistencia, las cuales son en parte mediadas por AMPK (Narkar *et al.*, 2008). Otros estudios han puesto de manifiesto que la administración de vitamina C y E produce, en humanos, un descenso de la sensibilidad a la insulina y bloquea determinadas adaptaciones musculares mediadas por PGC-1α (Ristow *et al.*, 2009). En la presente investigación usamos el coctel de antioxidantes referido en el estudio I, para el cual se ha demostrado su efectividad reduciendo el estrés oxidativo (Richardson *et al.*, 2007). Aunque en la discusión del estudio I ya hemos comentado que la carbonilación proteica es un marcador estable de estrés oxidativo, la ausencia de cambios en la carbonilación proteica, observada en el presente estudio en respuesta a un ejercicio de sprint, no implica la no producción de radicales libres. Esto puede indicar simplemente, que los radicales libres han sido capturados por otras moléculas y sistemas antioxidantes. De hecho, en el estudio I de la presente tesis doctoral se demuestra que el ejercicio de sprint en hipoxia severa aguda produce un incremento de las proteínas carboniladas en plasma y músculo (Morales-Alamo *et al.*, 2012). Sin embargo, el nivel de proteínas carboniladas observado tras el ejercicio de sprint en normoxia podría ser demasiado bajo para que éstas puedan ser detectadas por medio de la técnica del *Western blot* en las muestras de plasma o músculo (Shacter, 2000). Además, el hecho de que la suplementación con antioxidantes empleada en el presente estudio II haya podido ser relacionada con una inhibición

de la las fosforilaciones Thr^{286}-CaMKII /Thr^{172}-AMPKα, sugiere que nuestra intervención disminuyó efectivamente la respuesta de señalización intramuscular activada por el ejercicio de sprint y mediada por los ROS.

La activación de la CaMKII a través de su fosforilación en la Thr^{286}, puede inducir la fosforilación de la AMPK en la Thr^{172}. Este mecanismo ha demostrado tener relevancia en respuesta al ejercicio de alta intensidad (Egan *et al.*, 2010). La fosforilación de la Thr^{286}-CaMKII es regulada de una manera dependiente de la liberación de Ca^{2+} (Raney & Turcotte, 2008). Además, el incremento en la liberación del Ca^{2+} sarcoplasmático que se produce durante el ejercicio de sprint depende en gran medida del receptor de rianodina, el cual es activado por ROS y por cambios en el *estatus* antioxidante (Zima & Blatter, 2006). Sin embargo, el hecho de que el pico de potencia del Wingate no se viera afectado por la ingestión de antioxidantes, sugiere que, al menos en este modelo, la liberación de Ca^{2+} sarcoplasmático no está regulada de una manera muy importante por los ROS.

Como se ha mencionado en la discusión del estudio I, los ROS pueden activar CaMKII a través de la modificación del par $Met^{281/282}$ del dominio regulatorio de la enzima, de manera similar pero paralela a la autofosforilación de la Thr^{286} (Erickson *et al.*, 2008). Esta evidencia sugiere que se pueden producir cambios en el nivel de actividad de la

CaMKII por mecanismos independientes de la liberación sarcoplasmática de Ca^{2+} y regulados de manera directa por el nivel del estrés oxidativo.

En un estudio reciente de Wright y colaboradores (2009) se ha demostrado que las fosfatasas de serina/treonina pueden ser inhibidas por los ROS en músculo esquelético humano, de forma que la suplementación con antioxidantes puede bloquear este fenómeno. Si ese hubiera sido el principal mecanismo que hubiera modulado la fosforilaciones en Thr^{286}-CaMKII /Thr^{172}-AMPKα en el presente estudio II, la suplementación por antioxidantes debería haber producido una estimulación de las mencionadas fosforilaciones en respuesta al sprint. Sin embargo, los resultados aportados por la presente investigación apuntan en el sentido contrario, lo que sugiere que el efecto estimulador inducido por los ROS sobre las fosforilaciones Thr^{286}-CaMKII /Thr^{172}-AMPKα en respuesta al ejercicio de sprint es mayor que su potencial efecto inhibitorio sobre las correspondientes fosfatasas.

Las fosforilaciónes en las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 pueden mediar el efecto inhibidor que la ingestión de antioxidantes induce sobre la fosforilación de la Thr^{172}-AMPKα en músculo esquelético.

En este estudio observamos como la fosforilación de las Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 aumenta inmediatamente después del ejercicio de sprint, cuando éste fue realizado tras la ingesta del coctel de antioxidantes. Un mecanismo similar fue descrito en el estudio I tras la

realización del test en hipoxia (Morales-Alamo *et al.*, 2012). Tal como comentamos en la discusión de dicho estudio, la estimulación de la fosforilación de la AMPK en los residuos alternativos de serina que se detecta a los 10 segundos después de la finalización del sprint, debe estar mediado por un mecanismo intracelular rápido no regulado por la insulina. Tal como también hemos comentado anteriormente, el ejercicio de sprint en hipoxia severa aguda produce una mayor velocidad glucolítica, un menor pH intramuscular y un mayor grado de carbonilación proteica, lo cual sugiere la existencia de un mayor estrés oxidativo en hipoxia en comparación con el mismo test realizado en normoxia (Morales-Alamo *et al.*, 2012). Sin embargo, en el presente estudio II, hemos demostrado que si se reduce el estrés oxidativo causado por el ejercicio de sprint, mediante la suplementación con antioxidantes, se produce un aumento de los niveles de fosforilación de la AMPK en los residuos de serina alternativos. Por lo tanto, parece que un nivel de ROS excesivamente alto o bajo produce de igual manera una estimulación de la fosforilación de la AMPK en residuos de serina alternativos y una concomitante reducción de la fosforilación de la Thr^{172} de esta misma quinasa. Este efecto dual de los ROS no es nuevo. De hecho, se ha demostrado que tanto los niveles altos de ROS (Rudich *et al.*, 1998; Houstis *et al.*, 2006), como la reducción del estrés oxidativo causado por el ejercicio (Ristow *et al.*, 2009), están asociados con la resistencia a la insulina. Por lo tanto, parece que un nivel óptimo de ROS es necesario

para que la fosforilación en la Thr^{172}-AMPKα se estimule en respuesta a un ejercicio de sprint.

Tal como comentamos en la discusión del estudio I, la fosforilación de la AMPK en los residuos de serina alternativos puede ser explicada por un aumento de la fosforilación de la Akt en la Thr^{308}, fenómeno que ocurre con cambios ligeros en los niveles de insulina. En el presente estudio II, se observa que la fosforilación de la AMPK en los residuos de serina alternativos ocurre inmediatamente después del sprint realizado tras la ingesta del coctel de antioxidantes. Sin embargo, este fenómeno no parece estar mediado por la fosforilación de la Akt en la Thr^{308}, ya que aunque ésta ocurre inmediatamente después del ejercicio de sprint, no se vio afectada por la ingestión previa del coctel de antioxidantes. Un mecanismo alternativo que podría explicar este fenómeno podría estar mediado por la PKA, ya que existen estudios que han mostrado que esta proteína quinasa puede fosforilar a la AMPK en los residuos de serina alternativos, bloqueando así su fosforilación en la Thr^{172} (Hurley *et al.*, 2006). Además, el AMPc puede bloquear la fosforilación en la Thr^{172}-AMPKα por inhibición de la CaMKKβ, pero no de la LKB1 (Hurley *et al.*, 2006). Sin embargo, se desconoce si los niveles musculares de AMPc responden de manera diferente al ejercicio de sprint dependiendo del *status antioxidante* ofreciendo una respuesta diferente al ejercicio de sprint con diferentes niveles de estrés oxidativo.

Kinasa activada por el factor de crecimiento transformante β 1 (TAK1)

Anteriormente hemos comentado la posibilidad de que TAK1 pudiera actuar como una proteína quinasa *upstream* de AMPK (Xie *et al.*, 2006), si bien hasta que el estudio I de esta tesis doctoral no fue publicado, la respuesta de TAK1 muscular al ejercicio de sprint no había sido estudiada en humanos (Morales-Alamo *et al.*, 2012). En cualquier caso, el ejercicio de sprint produce una rápida producción de radicales libres (Cuevas *et al.*, 2005; Morales-Alamo *et al.*, 2012), los cuales pueden influir en diferentes procesos de señalización (Powers *et al.*, 2011). Entre las vías que pueden ser activadas por un aumento en los niveles de radicales libres se encuentran NF-κB, AP-1, y Nrf2, las cuales regulan la expresión de numerosas enzimas antioxidantes (Barbieri & Sestili, 2012). TAK1 es una de las principales quinasas activadoras de los factores de transcripción NF-κB, AP-1, y Nrf2, lo cual le confiere un papel clave en las respuestas adaptativas en respuesta a los incrementos de la producción de ROS (Omori *et al.*, 2012). La fosforilación de TAK1 es un requisito indispensable para su actividad (Singhirunnusorn *et al.*, 2005). De esta forma, en el presente estudio II la fosforilación de la $Thr^{184/187}$- TAK1 aumentó en respuesta al ejercicio de sprint realizado tras la ingesta de antioxidantes. Tal como hemos comentado en la discusión del estudio I, existen estudios que han sugerido que los ROS podrían inducir la inhibición de determinadas fosfatasas de tirosina y

serina/treonina, lo cual se podría traducir en la estimulación de la fosforilación de diversas quinasas (Wright *et al.*, 2009), como por ejemplo TAK1. Sin embargo, este mecanismo parece no operar en este estudio II, ya que si lo hubiera hecho, debería haberse observado una reducción de la fosforilación de TAK1 inducida por la suplementación con antioxidantes. Del mismo modo, a pesar de que potencialmente TAK1 podría actuar como una AMPK quinasa, la ingestión de antioxidantes produjo una menor fosforilación de CaMKII y AMPK, lo cual sugiere que los cambios detectados en la fosforilación de TAK1 no median los efectos inhibitorios de los antioxidantes sobre el esperado incremento de la fosforilación de CaMKII y AMPK inducido por el ejercicio de sprint. El mecanismo de activación de AMPK por TAK1 necesariamente ha de ser objeto de estudio en futuras investigaciones para su mejor entendimiento.

Los ratios Lac/Pyr y NAD^+/$NADH.H^+$ y la señalización activada por SIRT1/LKB1/AMPK

Diversos estudios previos han obtenido, durante el test de Wingate, una velocidad de la glucólisis y cambios metabólicos similares a los observados en la presente investigación (Bogdanis *et al.*, 1995; Parra *et al.*, 2000). Como novedad, este estudio muestra que la administración previa de antioxidantes no parece modificar el metabolismo durante un sprint (30 s) y no tiene ningún impacto sobre la fatiga muscular. Este puede que no sea el caso durante un ejercicio submáximo, donde hay

mayor sensibilidad a la fatiga a través de mecanismos en los que los ROS jugarían un papel importante (Westerblad & Allen, 2011). En las dos condiciones experimentales investigadas en este estudio II, la contribución del metabolismo anaeróbico fue comparable, como reflejó el similar déficit de oxígeno y la similar acumulación intramuscular de lactato.

Tal como quedó demostrado en el anterior estudio I, un test de Wingate en hipoxia severa aguda produce un notable aumento de la velocidad glucolítica para así compensar la reducción en el rendimiento de energía oxidativa, lo cual conduce a una mayor velocidad de la glucólisis y del ratio Lac/Pyr y a una reducción concomitante de la relación $NAD^+/NADH.H^+$ (Williamson *et al.*, 1967; Sahlin, 1985). Esto último produjo, además, una disminución de la cantidad de proteína total de SIRT1 y un bloqueo en la fosforilación de la Thr^{172}-AMPKα durante la recuperación del Wingate en hipoxia comparado con el test en normoxia. Ya que SIRT1 puede activar LKB1, el principal mecanismo de activación de AMPK, la disminución en hipoxia de la fosforilación de la Thr^{172}-AMPKα pudo ser explicada de esta manera. Sin embargo, en el presente estudio, los ratios de Lac/Pyr y $NAD^+/NADH.H^+$ fueron similares después de ambas condiciones experimentales. Por lo tanto, el efecto de SIRT1 sobre la actividad LKB1 debió ser también similar en ambas condiciones, lo que significa que es muy poco probable que el bloqueo de la fosforilación de la Thr^{172}-AMPKα inducido por los antioxidantes, fuera

producido por una reducción en la actividad de la LKB1 inducida por éstos.

Existen evidencias experimentales previas que han demostrado que la combinación de vitamina C (1000 mg/día) y vitamina E (400IU/día) previene los efectos beneficiosos del ejercicio (20 sesiones de 85 minutos, x 5 días a la semana x 4 semanas) sobre la concentración de insulina en plasma, la sensibilidad a la insulina, los niveles de adiponectina en plasma, reduciendo a su vez la inducción de la expresión de PGC1α, PGC1β, PPARγ y la expresión génica de las enzimas antioxidantes en el músculo esquelético (Ristow *et al.*, 2009). Lo que añade el presente estudio II a este cuerpo de conocimiento es que la ingestión de antioxidantes previa a un ejercicio de sprint, el cual se ha demostrado que puede inducir la expresión de PGC1α (Guerra *et al.*, 2011), previene la fosforilación en Thr^{172}-AMPKα, jugando esta actividad un papel crítico en la estimulación de la expresión génica de PGC1α (Jager *et al.*, 2007; Dasgupta *et al.*, 2012). Por tanto, la suplementación con antioxidantes podría prevenir también el esperado efecto positivo del ejercicio sobre las adaptaciones musculares relacionadas con la sensibilidad a la insulina y la capacidad antioxidante.

Disociación entre la fosforilación de la Ser^{221}-ACCβ y la fosforilación de la Thr^{172}-AMPKα.

La fosforilación de Ser^{221}-ACCβ ha sido comúnmente usada como marcador de la actividad AMPK (Emerling *et al.*, 2009). Sin embargo, en los últimos años se han acumulado evidencias que han puesto de manifiesto que, al menos en respuesta al ejercicio de sprint, la fosforilación de Thr^{172}-AMPKα no es necesaria para que se produzca la de la ACCβ en la Ser^{221} (Guerra *et al.*, 2010; Fuentes *et al.*, 2012a; Morales-Alamo *et al.*, 2012). En relación con esto último, el presente estudio II demuestra claramente que la inhibición de la fosforilación de laThr^{172}- AMPK inducida por la ingestión de antioxidantes previa al ejercicio de sprint, no afecta a la respuesta de la fosforilación de la Ser^{221}-ACCβ observada normalmente en respuesta a este modelo de ejercicio.

Estudio III: Skeletal muscle signaling during sprint exercise in severe acute hypoxia: role of free radicals. (Submitted).

Este estudio fue diseñado para determinar si el incremento en los radicales libres podría explicar el efecto inhibitorio inducido por la hipoxia severa aguda sobre la fosforilación de la Thr^{172}-AMPKα en respuesta a un ejercicio de sprint en humanos. Los sujetos que participaron en este estudio (los mismos que en el estudio II) ingirieron el mismo coctel de antioxidantes utilizado en el estudio II (Richardson *et al.*, 2007), para de esta forma atenuar el efecto de los radicales libres generados en respuesta al ejercicio de sprint en hipoxia sobre la señalización

intramuscular. De hecho, los resultados aportados por este estudio III demuestran que la suplementación con antioxidantes atenúa la cabonilación de proteínas en plasma normalmente producida ejercicio de sprint en hipoxia. Este efecto sugiere que la ingesta de antioxidantes previa al ejercicio de sprint en hipoxia mitiga el elevado estrés oxidativo generado por éste. En el presente estudio hemos demostrado por primera vez que la administración de antioxidantes previa a un ejercicio de sprint modifica el metabolismo energético durante el ejercicio, reduciendo la velocidad de la glucólisis sin alterar negativamente el rendimiento del metabolismo aerobio. Del mismo modo, la señalización muscular normalmente producida en respuesta al ejercicio de sprint es modificada por la ingestión previa de antioxidantes. Sin embargo, en contraste con nuestra hipótesis de partida, la reducción del estrés oxidativo generado por el ejercicio de sprint en hipoxia, mediante la suplementación con antioxidantes, no fue capaz de revertir la inhibición de la fosforilación de la Thr^{172}-AMPK observada en el estudio I en respuesta al ejercicio de sprint en hipoxia. Este hallazgo sugiere el estrés oxidativo generado por el ejercicio de sprint en hipoxia no es el principal mecanismo responsable de la inhibición de la fosforilación de la Thr^{172}-AMPK observada en respuesta a esa condición experimental. Por otro lado, el presente estudio III, junto con los resultados del estudio II, sí que ha demostrado que los ROS juegan un papel clave en la modulación de la fosforilación de la Thr^{286}-CaMKII en el músculo esquelético, la cual es completamente

bloqueada por la ingestión de antioxidantes. El hecho de que el bloqueo en la fosforilación de Thr^{286}-CaMKII inducido por la suplementación con antioxidantes no se tradujera en una menor fosforilación de la Thr^{172}-AMPKα, podría indicar que la fosforilación en la Thr^{286}-CaMKII no es requerida para mantener la fosforilación de la Thr^{172}-AMPKα a niveles basales.

Independientemente de la ingestión del coctel de antioxidantes, la fosforilación de TAK1 no se vio alterada por el ejercicio de sprint. Este hallazgo coincide con nuestro estudio anterior (Estudio I), en el que no se observó ningún cambio en los niveles de fosforilación de TAK1 en respuesta a ejercicios de sprint realizados en normoxia o hipoxia (Morales-Alamo *et al.*, 2012). Del mismo modo, los resultados del estudio II de la presente tesis, demuestran que aunque la fosforilación de $Thr^{184/187}$-TAK1 aumentó en el experimento con antioxidantes, no produjo un aumento en la fosforilación de la Thr^{172}-AMPKα (Morales-Alamo, D Submitted) . Estos resultados analizados en conjunto sugieren que TAK1 no parece tener un papel relevante en la modulación de la fosforilación de la Thr^{172}-AMPKα inducida por diferentes niveles de estrés oxidativo.

En el presente estudio III investigamos, además, el efecto de la ingestión de antioxidantes sobre la estimulación fosforilación de la AMPK en residuos de serina alternativos inducida por el ejercicio de sprint en hipoxia. Tal como hemos comentado en las discusiones de los estudios I y II, la fosforilación de las Ser^{485}-AMPKα1/Ser^{491}- AMPKα2 es un

conocido mecanismo inhibidor de la fosforilación en la Thr^{172}-AMPKα (Kovacic *et al.*, 2003; Horman *et al.*, 2006; Pulinilkunnil *et al.*, 2011). De esta forma, tal como ocurrió en el estudio I (Morales-Alamo *et al.*, 2012), el ejercicio de sprint realizado en hipoxia produjo un aumento en la fosforilación de Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, el cual no fue alterado por la ingesta previa de antioxidantes. Por lo tanto, el principal mecanismo que podría producir un bloqueo en la fosforilación de la Thr^{172}-AMPKα durante el ejercicio de sprint realizado en hipoxia, es el mediado por la fosforilación en Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, el cual no fue prevenido por la ingesta de antioxidantes previa al ejercicio. Un mecanismo similar había sido descrito por nuestro grupo al realizar un test de Wingate de 30 segundos tras la ingesta previa de 75 g de glucosa (Guerra *et al.*, 2010).

Tal como hemos comentado en las discusiones de los estudios I y II, la fosforilación de la AMPK en los residuos de serina alternativos es inducida por la Akt. En el presente estudio III, demostramos que el ejercicio de sprint en hipoxia produce un incremento de la fosforilación de la Akt (Ser^{473} y Thr^{308}), inmediatamente después del ejercicio, independientemente de la ingesta de antioxidantes. La activación de la Akt fue confirmada por el concomitante aumento de la fosforilación (activación) de su substrato AS160. Además, los resultados aportados por el presente estudio III sugieren que la fosforilación de la Akt en la Thr^{308} es necesaria para que la AMPK pueda ser fosforilada en los

residuos de serina alternativos. La fosforilación de Thr^{308}-Akt , pero no de Ser^{473}-Akt, volvieron a niveles basales a los 30 minutos de la recuperación. La fosforilación de Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 volvió a niveles basales también a los 30 minutos de la recuperación, lo cual es compatible con que la fosforilación de la Thr^{308}-Akt es necesaria para producir la fosforilación en Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, lo cual confirma los resultados del estudio número I (Morales-Alamo *et al.*, 2012).

En el estudio I, demostramos que el ejercicio de sprint en hipoxia, comparado con el mismo test realizado en normoxia, produce un bloqueo en la fosforilación de Thr^{172}-AMPKα, regulado a través de una reducción del ratio NAD^+/$NADH.H^+$ y de una disminución de la cantidad proteica total de SIRT1, todo ello parece que mediado por un mecanismo que dependería de un elevado estrés oxidativo (Morales-Alamo *et al.*, 2012). Para profundizar en este aspecto, procedimos, en el presente estudio III, a determinar los niveles proteicos de SIRT1 con el objetivo de investigar los potenciales efectos del nivel de estrés oxidativo sobre los niveles intramusculares de esta importante de-acetilasa. El área bajo la curva de los niveles de expresión proteica de SIRT1 durante la recuperación después del ejercicio de sprint realizado tras la ingesta de antioxidantes, fue aproximadamente 2 veces mayor que el observado para el test realizado tras la ingesta de un placebo. Sin embargo, estas diferencias no alcanzaron significación estadística (P = 0.22), lo cual pudo deberse a un

error estadístico de tipo II debido a la elevada variabilidad observada en los ensayos de *Western blot* realizados para cuantificar la expresión proteica de SIRT1. Por otro lado, la ingesta de antioxidantes atenuó la reducción del ratio $NAD^+/NADH.H^+$ inducida por el ejercicio de sprint realizado en hipoxia, lo cual pudo haber facilitado una mayor actividad LKB1. Sin embargo, los resultados aportados por este estudio III sugieren que este fenómeno no se produjo, ya que esa menor reducción del ratio $NAD^+/NADH.H^+$ inducida por los antioxidantes no restableció la normal fosforilación de la Thr^{172}-AMPKα en respuesta al ejercicio de sprint. Esto último podría ser explicado ya que el ratio AMP/ATP, el cual es considerado el principal mecanismo que regula la fosforilación de Thr^{172}-AMPKα vía LKB1, se vio menos incrementado tras la ingestión de los antioxidantes. Por todo ello, es muy posible que el efecto combinado de la menor reducción de ratio $NAD^+/NADH.H^+$ y el menor incremento en el ratio AMP/ATP, podría haber cancelado mutuamente su efecto sobre la actividad de la LKB1.

Los antioxidantes atenúan la respuesta glucolítica al ejercicio de sprint en hipoxia.

Diversos estudios han demostrado en cultivos celulares que la glucólisis puede ser activada por los ROS (Shi *et al.*, 2009; Wu & Wei, 2012) y que esta respuesta puede ser bloqueada por el ácidoα -lipoico y otros antioxidantes (Shi *et al.*, 2009). En el presente estudio hemos

demostrado que el coctel de antioxidantes que contiene ácido α-lipico atenúa la respuesta glucolítica durante el ejercicio de sprint en hipoxia. Este fenómeno puede haber estado mediado por el bajo incremento en el ratio AMP/ATP después de la ingestión de antioxidantes. Un mecanismo alternativo que podría explicar la baja velocidad de la glucólisis observada después de la ingestión de antioxidantes, podría ser debido a una reducción en la producción de fructosa 2,6 bifosfato por parte de la fosfofructoquinasa 2 (PFK2) (dicho metabolito es el activador más potente de la fosfofructoquinasa 1, la cual es la principal enzima reguladora de la velocidad de la glucólisis). La isoforma de hígado PFK2, la cual también se expresa en el músculo esquelético, es una enzima bifuncional que cuando está fosforilada en la Ser^{32} cambia su actividad de quinasa a fosfatasa reduciendo la fructosa 2,6-bifosfato (Kurland *et al.*, 1992; Rovira *et al.*, 2012). Sin embargo, actualmente se desconoce si los antioxidantes son capaces de mediar dicha fosforilación en músculo esquelético humano.

Aunque el ion lactato es antioxidante (Groussard *et al.*, 2000), la producción de lactato durante el ejercicio está siempre asociada con acidosis, la cual es una condición potencialmente oxidativa (Siesjo *et al.*, 1985; Rehncrona *et al.*, 1989). Al respecto cabe destacar, que los resultados aportados por el presente estudio III demuestran que la suplementación con antioxidantes reduce la acumulación de lactato normalmente detectada tras el ejercicio de sprint en hipoxia, atenuando

así la acidificación del pH intramuscular. De esta forma, podemos afirmar que la ingesta de antioxidantes previa al ejercicio de sprint produce una reducción del estrés oxidativo (Richardson *et al.*, 2007), de la velocidad de la glucólisis y de la caída del pH (Siesjo *et al.*, 1985; Rehncrona *et al.*, 1989).

La fosforilación de la Ser^{221}-ACCβ se ve incrementada en respuesta al ejercicio de sprint hipoxia, a pesar de la ausencia de fosforilación en la Thr^{172}-AMPKα

Tal como hemos comentado a lo largo de esta tesis doctoral, la ACCβ es una quinasa que se encuentra *downstream* de la AMPK en esta cascada de señalización (Steinberg *et al.*, 2006). Sin embargo, el presente estudio III confirma los resultados obtenidos en estudios previos realizados en nuestro laboratorio que demuestran una disociación entre la fosforilación de la Thr^{172}-AMPKα y la de la Ser^{221}-ACCβ, inmediatamente después del ejercicio de sprint (Guerra *et al.*, 2010; Fuentes *et al.*, 2012a; Morales-Alamo *et al.*, 2012). Del mismo modo, las evidencias parecen indicar que la fosforilación de Ser^{221}-ACCβ no está regulada tampoco a los 30 minutos de la recuperación por la AMPK, ya que está perfectamente conservada en todas las condiciones en la que la fosforilación de la Thr^{172}-AMPKα está inhibida. Por tanto, debe existir un mecanismo alternativo que explique por qué la fosforilación de la Ser^{221}-ACCβ inducida por el ejercicio de sprint puede ocurrir aunque la AMPK no

esté fosforilada (Dzamko *et al.*, 2008). Como novedad, este estudio III demuestra que la fosforilación de la Ser^{221}-ACCβ inducida por el ejercicio de sprint en hipoxia es independiente del nivel de estrés oxidativo, ya que no se ve afectada por la suplementación con antioxidantes.

CONCLUSIONES

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Las siguientes conclusiones han sido extraídas de los resultados de los estudios experimentales incluidos en la tesis.

1/ El ejercicio de sprint en hipoxia severa aguda produce una mayor velocidad de la glucólisis, una mayor acumulación de lactato muscular, un aumento en el ratio Lac/Pyr y una reducción concomitante del ratio $NAD^+/NADH.H^+$. Además, la señalización activada en respuesta al ejercicio de sprint se ve modificada por la hipoxia severa aguda en el músculo esquelético humano

2/ La hipoxia severa aguda bloquea el esperado aumento de la fosforilación de la Thr^{172}-AMPKα en respuesta al ejercicio de sprint a través de dos posibles mecanismos. Primero, el descenso del ratio $NAD^+/NADH.H^+$ combinado con la reducción de la cantidad de la proteína SIRT1 observada en hipoxia, podrían haber bloqueado la fosforilación de la Thr^{172}-AMPKα mediada por SIRT1/LKB1. Segundo, el bloqueo de la fosforilación de la Thr^{172}-AMPKα pudo haberse debido al incremento de la fosforilación de la AMPK en residuos alternativos de serina, siendo éste un conocido mecanismo inhibitorio de la fosforilación de la Thr^{172}-AMPKα mediado por la Akt.

3/ La fosforilación de la proteína substrato de Akt, AS160, se ve incrementada durante la recuperación tras el ejercicio de sprint,

independientemente de la FiO_2 y a pesar de los cambios observados en los niveles de fosforilación de la AMPK.

4/ La ingesta de un coctel de antioxidantes antes de un ejercicio de sprint realizado en normoxia bloquea el esperado incremento de la fosforilación de la Thr^{172}-AMPKα observado durante la recuperación, sin afectar al rendimiento, al metabolismo aerobio y anaerobio, ni a los ratios AMP/ATP y NAD^+/$NADH.H^+$. Este fenómeno sugiere que la activación de la AMPKα, que se produce durante la recuperación tras un solo ejercicio de sprint, depende del nivel de radicales libres producidos durante el ejercicio.

5/ El efecto inhibitorio de la suplementación con antioxidantes sobre la fosforilación de la Thr^{172}-AMPKα detectada durante la recuperación tras un ejercicio de sprint, puede deberse al bloqueo de la fosforilación de la Thr^{286}-CaMKII (una proteína quinasa upstream de la AMPK) inducida por los antioxidantes y/o al efecto estimulante de éstos sobre los niveles de fosforilación de la AMPK en residuos alternativos de serina.

6/ La ingesta de antioxidantes reduce la velocidad de la glucólisis provocada normalmente en respuesta a un ejercicio de sprint realizado en

hipoxia severa aguda, atenuando el incremento en el ratio AMP/ATP, pero sin afectar al rendimiento.

7/ El bloqueo de la fosforilación en la Thr^{172}-AMPKα producido por la hipoxia severa aguda en respuesta a un ejercicio de sprint, se produce a través de un mecanismo molecular independiente del estrés oxidativo, que posiblemente implica la fosforilación de la AMPK en residuos alternativos de serina de la AMPK mediada por la Akt, y que parece no estar gobernado ni por CaMKII ni por TAK1.

8/ El incremento de la fosforilación de la Thr^{286}-CaMKII observado en respuesta al ejercicio de sprint parece ser dependiente, al menos en parte, de la producción de radicales libres durante el ejercicio.

CONCLUSIONS

CONCLUSIONS

The following conclusions have been extracted from the results of the experimental studies includes in this thesis.

1/ Sprint exercise in severe acute hypoxia produces a higher glycolytic rate, intramuscular lactate accumulation, a increase on Lac/Pyr ratio and a concomitant decrease on $NAD^+/NADH.H^+$ ratio. Moreover, the activated signaling in response to sprint has modified by severe acute hypoxia in human skeletal muscle.

2/ Severe acute hypoxia blunts the expected increase of Thr^{172}-AMPKα phosphorylation in response to sprint exercise through two possible mechanisms. Firstly, it decreases the $NAD^+/NADH.H^+$ ratio combined with the decrease of SIRT1 total protein in hypoxia, could have blunted the Thr^{172}-AMPKα phosphorylation by SIRT1/LKB1. Secondly, the blunt of Thr^{172}-AMPKα phosphorylation could have been due to the increase in alternative serines residues of AMPK, which is a known inhibitory mechanism of Thr^{172}-AMPKα phosphorylation regulated by Akt.

3/ The phosphorylation of Akt protein substrate, AS160, is increased during the recovery period of the sprint, regardless of FiO_2 despite the changes observed on AMPK and Akt.

4/ The ingestion of antioxidants before the exercise in normoxia blunts the expected increase of Thr^{172}-AMPKα phosphorylation, without changes in performance, aerobic or anaerobic metabolism, AMP/ATP and $NAD^+/NADH.H^+$ ratios. The latter suggest that Thr^{172}-AMPKα phosphorylation during the recovery of a single sprint exercise, in modulated by the free radicals produced during the exercise.

5/ The inhibitory effect of antioxidants on the Thr^{172}-AMPKα phosphorylation detected during the recovery period of the sprint exercise, could be due to a blunted Thr^{286}-CaMKII phosphorylation induced by antioxidants and/or the stimulating effect of these on the levels of phosphorylation of AMPK in alternative serine residues.

6/ The ingestion of antioxidants reduces the glycolytic rate achieved normally in response to sprint exercise performed under severe acute hypoxia, attenuating the increase on AMP/ATP ratio, but without changes in the performance.

7/ The blunt on Thr^{172}-AMPKα phosphorylation by severe acute hypoxia in response to sprint exercise, is produced by an independent molecular mechanism to the oxidative stress, possibly involving phosphorylation of AMPK in alternative serine residues of AMPK mediated Akt, and appears to be regulated either by CaMKII or by TAK1.

8/ The increase on Thr^{286}-CaMKII phosphorylation in response to a sprint exercise may be regulated by free radicals.

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ANEXO

ESTUDIO I

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

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[1]*Department of Physical Education, University of Las Palmas de Gran Canaria (Campus Universitario de Tafira), Las Palmas de Gran Canaria, Spain;* [2]*Genetic Unit, Chilhood Hospital-Materno Infantil de Las Palmas, Avenida Marítima, Las Palmas de Gran Canaria, Spain;* [3]*Research Unit, Hospital de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria, Spain; and* [4] *Department of Physiological Sciences I, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain*

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

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

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

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

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

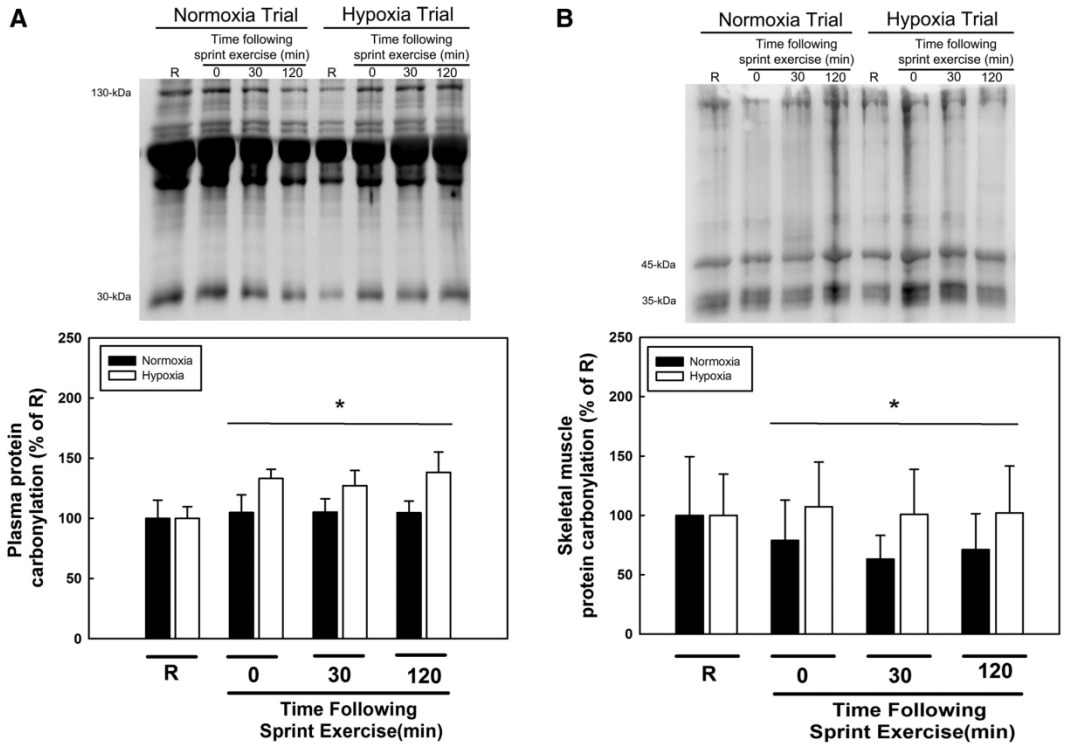


Fig. 1. Levels of carbonylated proteins in plasma (*A*) and skeletal muscle (*B*) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to those observed in the biopsies obtained immediately before the sprint exercise [resting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

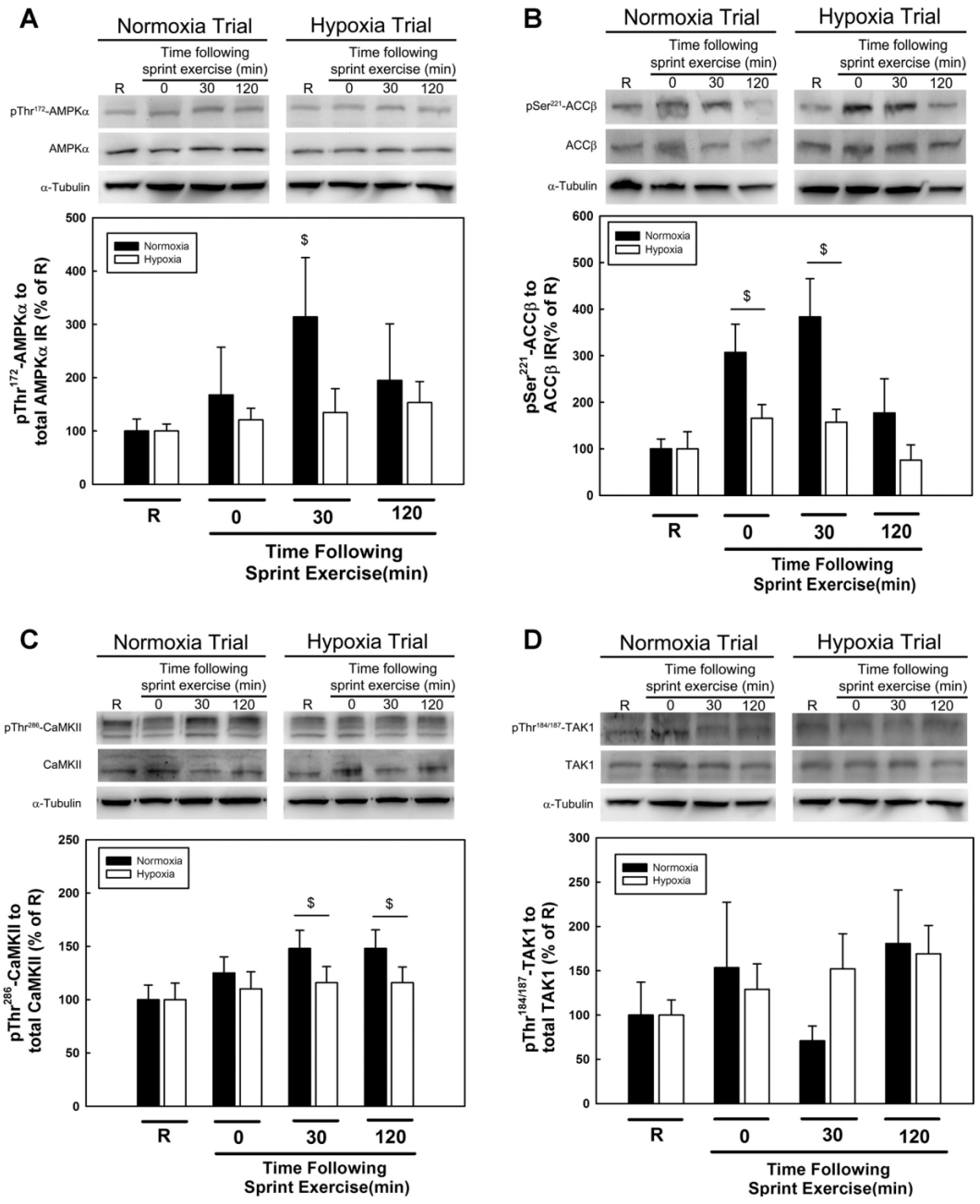
(Thr^{308}), no. 9275 (molecular mass: 60 kDa); anti-Akt, no. 9272 (molecular mass: 60 kDa); anti-phospho-TAK-1 (Thr$^{184/187}$), no. 4531 (molecular mass: 82 kDa); anti-TAK-1, no. 4505 (molecular mass: 82 kDa); anti-phospho-CaMKII (Thr^{286}), no. 3361 (molecular mass: 50 kDa); and AS160, no. 2447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

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

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

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

Values are mean ± SD. The AMP-to-ATP ratio was calculated from the creatine kinase and adenilate kinase apparent equilibrium constants for free AMP and ADP. Statistical analyses for lactate/pyruvate and $NAD^+/NADH.H^+$ were performed with normalized values, taking the resting values as 100%. $*P < 0.05$, postsprint vs. resting values (same condition); $\dagger P < 0.05$, postsprint in normoxia vs. hypoxia; $\ddagger P < 0.05$ for $F_{IO_2} \times$ time interaction.


A
Normoxia Trial
Hypoxia Trial
Time following sprint exercise (min)
R 0 30 120
pThr172-AMPKα
AMPKα
α-Tubulin
Normoxia
Hypoxia
pThr172-AMPKα to total AMPKα IR (% of R)
$
R 0 30 120
Time Following Sprint Exercise(min)
B
Normoxia Trial
Hypoxia Trial
Time following sprint exercise (min)
R 0 30 120
pSer221-ACCβ
ACCβ
α-Tubulin
Normoxia
Hypoxia
pSer221-ACCβ to ACCβ IR(% of R)
$
R 0 30 120
Time Following Sprint Exercise(min)
C
Normoxia Trial
Hypoxia Trial
Time following sprint exercise (min)
R 0 30 120
pThr286-CaMKII
CaMKII
α-Tubulin
Normoxia
Hypoxia
pThr286-CaMKII to total CaMKII (% of R)
$
R 0 30 120
Time Following Sprint Exercise(min)
D
Normoxia Trial
Hypoxia Trial
Time following sprint exercise (min)
R 0 30 120
pThr184/187-TAK1
TAK1
α-Tubulin
Normoxia
Hypoxia
pThr184/187-TAK1 to total TAK1 (% of R)
R 0 30 120
Time Following Sprint Exercise(min)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

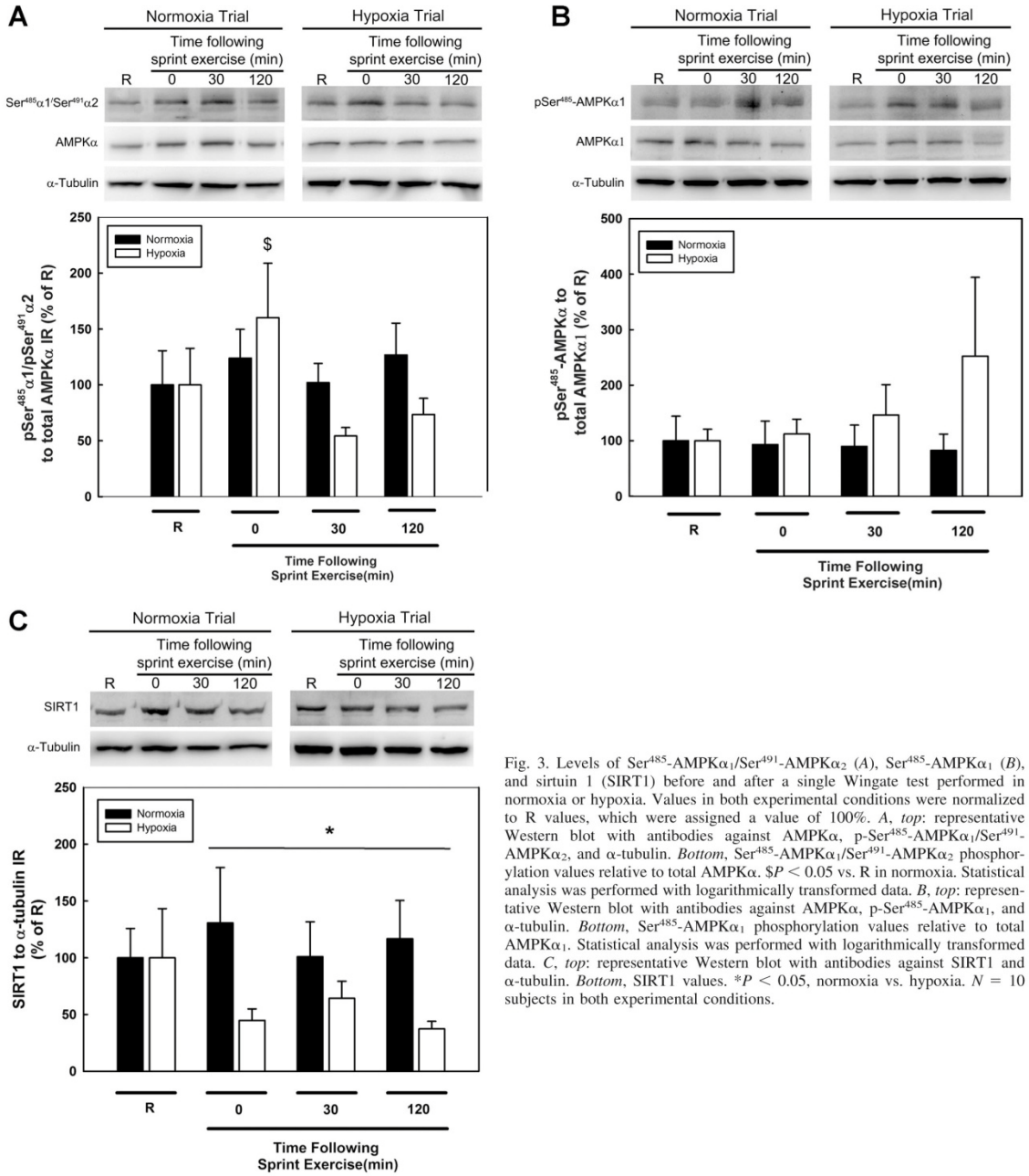


Fig. 3. Levels of Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 (*A*), Ser^{485}-AMPKα_1 (*B*), and sirtuin 1 (SIRT1) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to R values, which were assigned a value of 100%. *A*, *top*: representative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

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

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

DISCUSSION

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

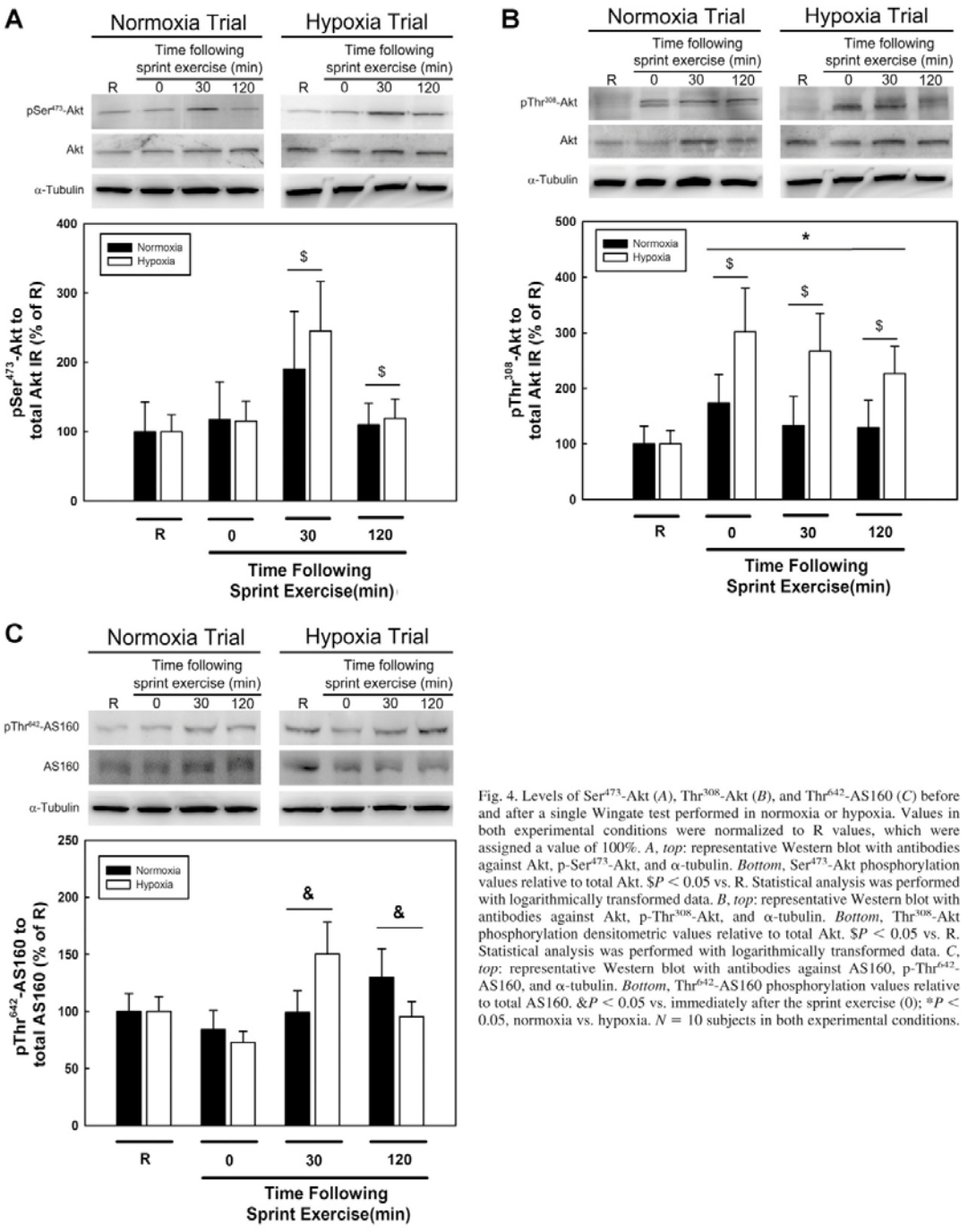


Fig. 4. Levels of Ser^{473}-Akt (*A*), Thr^{308}-Akt (*B*), and Thr^{642}-AS160 (*C*) before and after a single Wingate test performed in normoxia or hypoxia. Values in both experimental conditions were normalized to R values, which were assigned a value of 100%. *A*, *top*: representative Western blot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

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

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

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

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

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

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

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

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

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

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

Increased levels of cellular cAMP may blunt Thr^{172}-AMPKα phosphorylation by inhibition of Ca^{2+}/calmodulin-dependent kinase kinase-β [an AMPK kinase (29)] but not LKB1 (33). Moreover, Hurley et al. (33) have shown that phosphorylation of the Ser$^{485/491}$ site appears to be required but not sufficient to inhibit AMPK under conditions of elevated cAMP. It remains to be determined if muscle cAMP levels are differently altered by sprint exercise depending on $F_{I_{O_2}}$.

TAK-1 acts like an AMPK kinase (68), which may be stimulated by hypoxia (5, 40). However, in the present work, Thr$^{184/187}$-TAK-1 phosphorylation did not change after the sprint, regardless of $F_{I_{O_2}}$, i.e., our results cannot be attributed to differences in TAK-1 phosphorylation.

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

Conclusions. In summary, we have shown that sprint exercise in severe acute hypoxia elicits a greater glycolytic rate with a higher accumulation of muscle Lac, increased Lac-to-Pyr ratios, and, hence, reduced NAD^+-to-$NADH.H^+$ ratios. Consequently, the human skeletal muscle signaling response to sprint exercise is modified in hypoxia. Acute hypoxia blunted the expected increase in Thr^{172}-AMPKα phosphorylation, and this article provides results with two potential mechanisms to explain this response. First, the lower NAD^+-to-$NADH.H^+$ ratio after the sprint in hypoxia combined with the reduced SIRT1protein levels may have blunted the SIRT1/LKB1-mediated phosphorylation of AMPKα. Second, Thr^{172}-AMPKα phosphorylation may have been blunted due to increased Ser^{485}-AMPKα_1/Ser^{491}-AMPKα_2 phosphorylation, a known mechanism of Thr^{172}-AMPKα phosphorylation inhibition mediated by Akt. The latter concords with the greater increase of Akt phosphorylation observed immediately after the sprint in hypoxia. Finally, we have shown that AS160 phosphorylation was elevated 30–120 min after the sprints, regardless of $F_{I_{O_2}}$ and despite the differences in AMPK and Akt phosphorylation responses. These findings may have important implications in terms of better understanding the potential differences in acute and adaptive responses to exercise in humans with chronic hypoxia or increased oxidative stress, such as in patients with chronic lung diseases or in chemotherapy.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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ESTUDIO II

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

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Running title: Antioxidants blunt AMPK phosphorylation.

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Abstract

The extremely high energy demand elicited by sprint exercise is satisfied by an increase in oxygen consumption combined with a high glycolytic rate, leading to a marked lactate accumulation, increased AMP/ATP ratio, reduced NAD^+/$NADH.H^+$ and muscle pH, which are accompanied by marked Thr^{172}-AMPKα phosphorylation during the recovery period by a mechanism not fully understood. To determine the role played by free radicals on Thr^{172}-AMPKα phosphorylation in response to sprint exercise, nine voluntaries performed a single 30s sprint (Wingate test) in two occasions: one after the ingestion of placebo (P) and another following the intake of antioxidants (A) (α-lipoic acid, vitamin C, and vitamin E), with a double blind design. Vastus lateralis muscle biopsies were obtained before, immediately after, 30 and 120 min post-sprint. Performance, muscle aerobic and anaerobic metabolism was similar during both sprints. The NAD^+/$NADH.H^+$ ratio was similarly reduced (84%), and the AMP/ATP ratio similarly increased (x21 fold) immediately after the sprints. Thr^{286}-CaMKII and Thr^{172}-AMPKα phosphorylations were increased after the control sprint (P), but not when the sprints were preceded by the

ingestion of antioxidants. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, a known inhibitory mechanism of Thr^{172}-AMPKα phosphorylation, was increased only in A. $Thr^{184/187}$-TAK1 phosphorylation was increased by 2.4-fold in A. This study shows that free radicals play a critical role in the skeletal muscle Thr^{172}-AMPKα phosphorylation response to sprint exercise in humans, since the mere ingestion of antioxidants immediately prior to the sprint blunted this response despite no effects on performance, muscle aerobic and anaerobic metabolism, and the AMP/ATP and NAD^+/$NADH.H^+$ ratios.

Introduction

In skeletal muscle, AMPK intervenes in the regulation of fat oxidation (Steinberg *et al.*, 2006), glucose transport (Merrill *et al.*, 1997), mitochondrial biogenesis (Zong *et al.*, 2002; O'Neill *et al.*, 2011), and Na,K-ATPase activity (Ingwersen *et al.*, 2011), among other functions (Viollet *et al.*, 2010). Thr^{172}-AMPKα phosphorylation is required for activation of AMPK (Hardie *et al.*, 1998). This can be elicited by several AMPK kinases among which the liver kinase B1 (LKB1) plays an important role in skeletal muscle, since it responds to increases of AMP/ATP ratio (Hardie, 2007) and can be also activated through deacetylation by SIRT1 in response to the increase in $NAD^+/NADH.H^+$ ratio (Hou *et al.*, 2008). Whether free radicals may also play a role in contraction-mediated Thr^{172}-AMPKα phosphorylation in skeletal muscle remains controversial (Sandstrom *et al.*, 2006; Merry *et al.*, 2010). Moreover, the influence that free radicals may have on the regulation of Thr^{172}-AMPKα phosphorylation in response to sprint exercise has not been studied in humans, despite that this exercise mode elicits marked oxidative stress (Cuevas *et al.*, 2005).

Free radicals may modulate Thr^{172}-AMPKα phosphorylation by several mechanisms which may involve activation/inhibition of AMPK kinases and phosphatases. AMPK kinases which may be regulated by free radicals are CaMKII (Erickson *et al.*, 2008; Egan *et al.*, 2010; Wagner *et al.*, 2011), Ca^{2+}/calmodulin-dependent protein kinase kinase beta (CaMKKβ) (Mungai *et al.*, 2011) and transforming growth factor β-activated kinase (TAK-1) (Xie *et al.*, 2006; Egan *et al.*, 2010; Omori *et al.*, 2010). However, the impact of sprint exercise-induced oxidative stress on the activity of these kinases remains unknown.

Recent studies have shown that AMPK phosphorylation in Ser^{485} of the α1 and Ser^{491} of the α2 subunits mitigates or completely blunts Thr^{172}-AMPKα phosphorylation. For example, insulin antagonizes anoxia or ischemia-induced AMPKα phosphorylation through Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation (Horman *et al.*, 2006), which may be produced by Akt (Kovacic *et al.*, 2003; Soltys *et al.*, 2006). Phenylephrine induces Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation in cardiomyocytes, preventing Thr^{172}-AMPKα phosphorylation in response to adenosine agonists (Pang *et al.*, 2010). In brown

adipose tissue, reducing both α- and β- adrenergic signaling in vivo activates Akt (also known as protein kinase B) and protein kinase A (PKA), which in turn increase Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation to reduce AMPK activity (Pulinilkunnil *et al.*, 2011). 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 of glucose (Guerra *et al.*, 2010) or when the sprint is performed in severe acute hypoxia (F_IO_2:0.105) (Morales-Alamo *et al.*, 2012). In both circumstances Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation was increased with a time pattern adequate to inhibit Thr^{172}-AMPKα phosphorylation. Since, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 may be regulated via changes in free radical production (Pulinilkunnil *et al.*, 2011; Morales-Alamo *et al.*, 2012), we hypothesized that antioxidant administration would increase Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, blunting the normal skeletal muscle Thr^{172}-AMPKα phosphorylation response to a single sprint (Guerra *et al.*, 2010; Fuentes *et al.*, 2012a; Fuentes *et al.*, 2012b; Morales-Alamo *et al.*, 2012).

Immediately after and during the first 30 min following a single bout of sprint exercise Acetyl CoA Carboxylase (ACC) is phosphorylated in Ser^{221} (Guerra *et al.*, 2010; Fuentes *et al.*, 2012a) enabling fat oxidation during recovery. Ser^{221}-ACCβ phosphorylation may be elicited by AMPK (Carling *et al.*, 1987) and unknown AMPK-independent mechanisms (Raney *et al.*, 2005; Dzamko *et al.*, 2008). In cell cultures, treatment with H_2O_2 elicits Ser^{221}ACCβ phosphorylation in AMPK dependent manner (Emerling *et al.*, 2009). However, whether Ser^{221}ACCβ phosphorylation in response to sprint exercise is modulated depending on oxidative stress remains unknown. We hypothesized that antioxidant ingestion prior to sprint exercise will attenuate or blunt the expected Ser^{221}ACCβ phosphorylation due to inhibition of Thr^{172}-AMPKα phosphorylation, the main upstream kinase for ACCβ.

Therefore, the main aim of this study was to determine the role that free radicals may have on Thr^{172}-AMPKα phosphorylation in response to sprint exercise. For this purpose antioxidants were administered prior to 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. More specifically, by determining the glucose and plasma insulin responses, combined with the assessment of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 and Ser^{473}/Thr^{308}-Akt phosphorylation, we expected to determine if Thr^{172}-AMPKα phosphorylation is modulated by free radicals through a Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation-dependent mechanism.

Materials and methods

Materials

The Complete protease inhibitor cocktail and the PhosSTOP phosphatase inhibitor were obtained from Roche Diagnostics (Mannheim, Germany). All the primary antibodies used were from Cell Signaling Technology (Denvers, MA, USA) except the anti-CaMKII antibody (no. sc-13082, MW: 50 KDa) that was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the monoclonal mouse anti-alpha-tubulin antibody (no. T-5168-ML, MW: 50 KDa) that was obtained from Biosigma (Sigma, St .Louis, MO, USA). The corresponding catalogue number of the antibodies from Cell Signaling were: anti-Phospho-AMPKα (Thr^{172}) no. 2531

(MW: 62 KDa), anti-AMPKα no. 2532 (MW: 62 KDa), anti-phospho-AMPKα1 (Ser^{485})/AMPKα2 (Ser^{491}) no. 4185 (MW:62 KDa), anti-phospho-AMPKα1 (Ser^{485}) no. 4184 (MW: 62 KDa), anti-AMPKα1 no. 2795 (MW: 62 KDa), anti-Phospho-Acetyl-CoA Carboxylase (Ser^{221}) no. 3661 (MW: 280 KDa), anti-Acetyl CoA Carboxylase (ACC) no. 3662 (MW: 280 KDa), anti-SIRT1 no. 2310 (MW: 120 KDa), anti-Phospho-Akt (Ser^{473}) antibody no. 9271 (MW: 60 KDa), anti-Phospho-Akt (Thr^{308}) antibody no. 9275 (MW: 60 KDa), anti-Akt antibody no. 9272 (MW: 60 KDa), anti-Phospho-TAK1 ($Thr^{184/187}$) no. 4531 (MW: 82 KDa), anti- TAK1 no. 4505 (MW: 82 KDa), anti-Phospho-CaMKII (Thr^{286}) no. 3361 (MW: 50 KDa), and AS160 no. 2447 (MW: 160 KDa). The secondaries HRP-conjugated goat anti-rabbit (no. 111-035-144) and the HRP-conjugated donkey anti-mouse (no. 715-035-150) antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). The Immun-Blot™ PVDF Membranes, and the Immun-Star™ WesternC™ were from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK). The ChemiDoc XRS System and the image analysis software Quantity One© were obtained from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK).

Subjects

Nine healthy male physical education students (age = 25 ± 5 yr, height = 176.0 ± 5.1 cm, body mass = 79.4 ± 10.1 kg, body fat = 18.3 ± 6.7%) agreed to participate in this investigation (Table 1). 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. 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 (CEIH-2010-01).

General Procedures

The subjects' body composition 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.*, 2008). Subjects reported to the laboratory to complete different test on separate days. First, their maximal VO_2 (VO_2max), HRmax and maximal power output (Wmax), 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, the subjects were familiarized with the experimental protocol (a single 30s-isokinetic Wingate tests at 100 RPM). On separate days, the nine subjects performed one 30s-isokinetic Wingate test at 100 RPM after the ingestion of either placebo (P) or antioxidants (A) with a double-blind design. Antioxidants were administered split into two doses with the first dose ingested 2 h before the sprint (at 07:00 a.m.) followed by a second dose 30 min later, i.e. 90 min prior to the sprint. The first dose consisted of 300 mg of α-lipoic acid, 500 mg vitamin C, and 200 IU vitamin E, whereas the second 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 radicals levels at rest and in response to exercise (Richardson *et al.*, 2007). Placebo microcrystalline cellulose capsules were of similar taste, color, and appearance and were likewise consumed in two similarly timed doses (Richardson *et al.*, 2007).On each trial, 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, insulin and plasma carbonylated proteins. Right after, a muscle biopsy was obtained from the middle portion of the vastus lateralis muscle using the Bergstrom's technique with suction.

During both sprints, the subjects attempted to pedal as fast and hard as possible (i.e., all-out) from the start to the end of the exercise. Since the cycle Ergometer (Excalibur Sport, Lode, Groningen, NL) 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.

Peak power output was calculated as the highest work output performed during 1 s interval, and mean power output from the average work performed during the 30 s. Warm up was not allowed prior to 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 (Calbet *et al.*, 1997; Calbet *et al.*, 2003; Guerra *et al.*, 2011b).

Within 10 seconds from the end of the sprint a second muscle biopsy was taken, and then another blood sample was obtained. During the following two hours the subjects were fasting, 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 minutes. For the last two biopsies a new incision was performed in the contralateral leg. To avoid injury-triggered activation of signaling cascades the muscle biopsies were obtained at least 3 cm apart, using the procedures described by Guerra et al. (Guerra *et al.*, 2011a). 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. The time needed to obtain and freeze the muscle biopsies was below 30 s.

Cycling economy tests.

Cycling economy was determined on two different days using 8-11 submaximal workloads at intensities between 50 to 90% of VO_2peak, at 100 RPM (Morales-Alamo *et al.*, 2012). 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 VO_2 registered during the last two minutes was taken as representative of each submaximal exercise intensity. To relate VO_2 to power, linear regression equations were calculated by least square linear fit.

Oxygen uptake and respiratory variables

Oxygen uptake was measured with a metabolic cart (Vmax N29; Sensormedics, California, USA), calibrated immediately before each test according to the manufacturer instructions. Respiratory variables were analyzed breath-by-breath and averaged every 5 seconds during the Wingate test and every 20 second during the incremental and cycling economy tests. The highest 20-s averaged VO_2 recorded in normoxia was taken as the VO_2max.

Muscle metabolites

From each muscle biopsy, 30 mg of wet tissue were treated with 0.5 M $HClO_4$ and centrifuged at 15000 g at 4 °C for 15 min. The

supernatant was neutralized with $KHCO_3$ 2.1M. ATP, phosphocreatine (PCr), creatine (Cr), pyruvate (Pyr) and lactate (Lac) were enzymatically determined in neutralized extracts by fluorometric analysis (Lowry & Passonneau, 1972; Gorostiaga *et al.*, 2010). Muscle metabolite concentrations were adjusted to the individual mean total creatine (PCr + Cr) because this mean should remain constant during the exercise (Harris *et al.*, 1976). The adjustment to the total creatine content accounts for the variability in solid non-muscle constituents, which may be present in the biopsies (Parra *et al.*, 2000). The glycolytic rate (GR) was calculated as $GR= 0.5 \times (\Delta Lac + \Delta Pyr)$ (Spriet *et al.*, 1987). The free AMP/ATP molar ratio was estimated after calculating the adenosine diphosphate (ADP) concentration using the creatine kinase equilibrium apparent constant for resting conditions and exhaustion after a Wingate test (Parra *et al.*, 2000), as described in Sahlin et al. (Sahlin *et al.*, 1975). Subsequently, adenosine monophosphate (AMP) concentration was calculated using the adenilate kinase apparent equilibrium constant for the same conditions (Parra *et al.*, 2000). The $[NAD^+]/[NADH.H^+]$ was calculated using the equilibrium constant for the lactate

dehydrogenase (Bücher & Klingenberg, 1958; Williamson *et al.*, 1967).

Total protein extraction, electrophoresis, and 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). Briefly, proteins were solubilized in sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 2.3% (wt/vol) sodium dodecyl sulfate (SDS) polyacrylamide gels, 10% (vol/vol) glycerol, 5% (vol/vol) beta-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. Equal amounts (50 μg) of each sample were electrophoresed on 7.5–10% SDS-PAGE using the system of Laemmli (1970) and transferred to Immun-Blot PVDF Membranes. To determine Thr^{172}-AMPKα, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, Ser^{485}-AMPKα1, Ser^{221}-ACCβ, Ser^{473}-Akt, Thr^{308}-Akt, $Thr^{184/187}$-TAK1, and Thr^{286}-CaMKII phosphorylation levels, antibodies directed against the phosphorylated and total form of these kinases were diluted in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20

(TBS-T) (BSA-blocking buffer). SIRT1 was assessed in membranes incubated with a SIRT1 antibody (diluted in BSA-blocking buffer). To control for differences in loading and transfer efficiency across membranes, membranes were incubated with a monoclonal mouse antialpha-tubulin antibody diluted in TBS-T with 5% blotting grade blocker non-fat dry milk (blotto-blocking buffer). No significant changes were observed in alpha-tubulin protein levels during the experiments (data not shown). Antibody-specific labeling was revealed by incubation with an HRP-conjugated goat anti-rabbit antibody (1:20,000) or an HRP-conjugated donkey anti-mouse (1:10,000) antibody both diluted in 5% blotto blocking buffer and visualized with the Immun-Star™ WesternC™ kit (Bio-Rad Laboratories, Hemel Hempstead Hertfordshire, UK). The densitometry analyses were carried out immediately before saturation of the immunosignals. Specific bands were visualized with the Immun-Star™ WesternC™ kit, using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with the image analysis program Quantity one© (Bio-Rad laboratories, Hercules, CA, USA). 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 (antioxidants 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 inter-gel variability.

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 µIU/ml.

Serum Glucose

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

Protein carbonylation

Protein carbonylation in skeletal muscle and plasma was assessed by immunobloting detection of protein carbonyl groups using

"OxyBlot" protein oxidation kit (Intergen Company, Purchase,NY) as previously described (Romagnoli *et al.*, 2010). Protein carbonylation data were represented as a percentage of immunostaining values.

Statistics

Variables were checked for normal distribution by using the Shapiro-Wilks test with the Lilliefors correction. When necessary, the analysis was carried out on logarithmically transformed data. First, the pre-exercise values were compared between the two conditions using a Student's t-test. Since not significant differences between conditions were observed prior to the start of the sprint, the individual responses were normalized to the band densities or level of phosphorylation observed just before the start of the Wingate test. A repeated-measures ANOVA over time and antioxidant condition with two levels (placebo vs. antioxidants) was used to compare the responses with the value prior to the start of the Wingate test. When there was a significant condition effect or condition x 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 analysis. The areas under the curve (AUC) were determined using the trapezoidal rule and compared between conditions with paired Student t-tests. 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

Performance and respiratory variables

Exercise performance, oxygen deficit and respiratory variables were not significantly affected by the ingestion of antioxidants prior to the Wingate test (Table 1).

Serum glucose and insulin

Basal serum glucose and insulin concentration were not altered by the ingestion of antioxidants. Compared to rest, serum glucose concentration was increased by 9% just after the exercise and it remained elevated until 30 minutes into recovery period (time effect

P<0.05) (Table 2). Serum insulin was increased by 50% and 2.2 fold, respectively, just after the test and 30 minutes into the recovery (time effect P<0.05) (Table 2). Both variables decrease to values similar to those observed prior to the sprint 120 min after the exercise.

Muscle metabolites

The changes observed in muscle metabolites are depicted in Table 3. Basal muscle metabolites were not altered by the ingestion of antioxidants. Antioxidants did not change the metabolic response to sprint exercise. The AMP/ATP molar ratio was similarly increased (x21 fold) immediately after the sprints (time effect, P<0.05). Compared to resting, immediately after the sprint, muscle pyruvate and lactate concentrations were increased by 3 and 14-fold, respectively (time effect, P<0.05). After both sprints, the Lac/Pyr ratio was increased similarly by 4.1-fold (time effect, P<0.05), and consequently the NAD^+/$NADH.H^+$ ratio was reduced similarly reduced by 84% (time effect, P<0.05).

Plasma and skeletal muscle carbonylated proteins

Antioxidants did not alter the basal levels of plasma nor 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 (Figure 1).

Muscle signaling

The ingestion of antioxidants did not alter the basal levels of Thr^{172}-AMPKα phosphorylation, ACCβ phosphorylation, Thr^{286}-CaMKII phosphorylation, $Thr^{184/187}$-TAK1 phosphorylation, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, Ser^{485}-AMPKα1 phosphorylation, Ser^{473}-Akt phosphorylation, and Thr^{308}-Akt phosphorylation, nor total SIRT1 protein content. As illustrated in Figure 2, Thr^{172}-AMPKα phosphorylation was increased by 3.2 fold 30 min after the control Wingate. This effect was not observed when the sprint was performed after the ingestion of antioxidants (condition x time interaction, $P<0.05$) (Fig. 2A). However, Ser^{221}-ACCβ phosphorylation was enhanced similarly (3.9 and-3.4 fold) (Fig. 2B), 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 minutes after the control sprints, while it remained unchanged following the ingestion of antioxidants (condition x time interaction, $P<0.05$) (Fig. 2C). During the recovery period, the AUC of Thr$^{184/187}$-TAK1 phosphorylation was 2.4-fold greater in antioxidants than in placebo trial ($P<0.05$) (Fig. 2D).

Compared to rest, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation levels were increased by 57% but only immediately after the sprint with antioxidants (time effect $P<0.05$), returning to pre-exercise levels 30 min later (Fig. 3A). Subsequently, 120 min after the sprints with antioxidants Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation levels were increased by 76% (time effect $P<0.05$). Ser^{485}-AMPKα1 phosphorylation and SIRT1 protein levels were unchanged after the sprints (Fig. 3B and C).

Ser^{473}-Akt phosphorylation was increased by 2.1-fold and 33%, respectively 30 and 120 minutes after the sprints (time effect, $P<0.05$) (Fig. 4A). Thr^{308}-Akt phosphorylation was increased by 75 and 16% just after the exercise and 30 minutes into the recovery

period, respectively (time effect, $P<0.05$), without significant differences between conditions (Fig. 4B).

Discussion

This study examined the role of ROS on the regulation of skeletal muscle AMPKα phosphorylation in response to a single 30-seconds sprint exercise in humans. The ingestion of antioxidants prior to the sprint prevented Thr^{172}-AMPKα phosphorylation by two potential mechanisms. Firstly, antioxidant ingestion blunted Thr^{286}-CaMKII phosphorylation, an upstream kinase for AMPK in skeletal muscle. Secondly, antioxidants increased Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation immediately after the sprint. These findings indicate that reactive oxygen species (ROS) may play a critical role in the Thr^{172}-AMPKα phosphorylation response to sprint exercise in humans. Despite the fact that the antioxidant cocktail administered in this study has been shown to effectively reduce oxidative stress (Richardson *et al.*, 2007), in the present investigation antioxidants did not alter exercise performance, muscle aerobic and anaerobic metabolism, the AMP/ATP and NAD^+/$NADH.H^+$ ratios, or the degree of protein carbonylation in plasma and skeletal muscle.

Protein carbonylation is both, irreversible and irreparable (Barreiro & Hussain, 2010). This study shows that in healthy humans a 30 s sprint does not induce a detectable increase of carbonylated proteins, possibly because the level of protein carbonylation induced by both sprints was mild or modest. Mildly oxidized proteins are committed to direct degradation by the 20S proteasome without ubiquitination, whilst modestly oxidized proteins are degraded rapidly by proteases, and highly carbonylated proteins cannot be degraded and tend to aggregate (Fedorova *et al.*, 2010).

AMPK regulation by Thr^{286}-CaMKII phosphorylation: role of free radicals

Free radicals have been found to be a inducers Thr^{172}-AMPKα phosphorylation by several mechanisms (Song & Zou, 2012), and hypoxia have been shown to increase Thr^{172}-AMPKα phosphorylation by a mechanism involving mitochondrial reactive oxygen species independent of the AMP:ATP ratio (Emerling *et al.*, 2009). Because of this, we hypothesized that antioxidant ingestion prior to sprint exercise could blunt or mitigate the expected Thr^{172}-AMPKα phosphorylation normally observed in the vastus lateralis

muscle after a single sprint (Guerra *et al.*, 2010; Fuentes *et al.*, 2012b). In agreement with our results, in rats and humans, Gomez-Cabrera et al. (2008) have shown that vitamin C supplementation hampered endurance training adaptations, which are in part mediated by AMPK (Narkar *et al.*, 2008). Vitamin C and E administration decreases insulin sensitivity and PGC-1α adaptations in humans (Ristow *et al.*, 2009). In the present investigation we administered an antioxidant cocktail containing α-lipoic acid, vitamin C and E which have been shown to decrease in humans circulating free radicals at rest (~98%) and during exercise (~85%) (Richardson *et al.*, 2007). These changes were assessed by electron parametric resonance (EPR) a high sensitive technique. Although protein carbonylation is a relatively stable marker of oxidative stress, the lack of change in protein carbonylation in response to sprint exercise does not implies lack of free radicals production, simply it may indicate that free radicals have been captured by other molecules and antioxidant systems. We have previously observed an increase in protein carbonylation after sprint exercise in severe hypoxia (Morales-Alamo *et al.*, 2012). However, the level of protein carbonylation induced by a single sprint in normoxia may be

too low to be detected by western blotting in plasma and muscle (Shacter, 2000). Moreover, the fact that the ingestion of antioxidants was associated with blunted Thr^{286}-CaMKII /Thr^{172}-AMPKα phosphorylations indicates that our intervention effectively prevented a free radical-mediated signaling response to sprint exercise.

Thr^{286}-CaMKII can phosphorylate AMPK on Thr^{172}, and this mechanism has been shown to be physiologically relevant during high intensity exercise (Egan *et al.*, 2010). Thr^{286}-CaMKII phosphorylation is regulated in Ca^{2+} dependent manner (Raney & Turcotte, 2008) 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 ROS and changes of the antioxidant status (Zima & Blatter, 2006). However, the fact that peak power output was not affected by the ingestion of antioxidants is against a major change in the sarcoplasmic Ca^{2+} transients.

ROS may activate CaMKII through modification of the $Met^{281/282}$ pair within the regulatory domain, blocking reassociation with the catalytic domain and preserving kinase activity via a similar but parallel mechanism to Thr^{286} autophosphorylation

(Erickson *et al.*, 2008). Thus, reducing free radicals by antioxidant administration could result in lower CaMKII activity by a mechanism unrelated to sarcoplasmic Ca^{2+}.

Wright et al. (2009) have shown that tyrosine and Ser/Thr phosphatases may be inhibited by exposure to ROS in skeletal muscle. Antioxidant administration prior to exercise may attenuate the inhibitory ROS influence on protein phosphatases. Had this been the predominating mechanisms controlling Thr^{286}-CaMKII /Thr^{172}-AMPKα phosphorylations, antioxidant should have increased Thr^{286}-CaMKII /Thr^{172}-AMPKα phosphorylations after the sprint. The fact that these two phosphorylations were actually inhibited shows that the stimulating effect of free radicals on Thr^{286}-CaMKII /Thr^{172}-AMPKα phosphorylations outweighs the potential inhibitory effect on their corresponding phosphatases.

Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation may mediate the blunting effect of antioxidant ingestion on sprint-induced Thr^{172}-AMPKα phosphorylation in skeletal muscle.

Here we show that Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation is increased immediately after a sprint exercise

when preceded by the ingestion of antioxidants. A similar response was observed after a similar sprint performed with severe acute hypoxia (Morales-Alamo *et al.*, 2012). A fast intracellular mechanism should account for Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, since this phosphorylation was present 10s after the sprint. Sprint exercise in severe acute hypoxia elicits higher glycolytic rate, with lower muscle pH and greater degree of protein carbonylation than a similar sprint in normoxia, suggesting increased oxidative stress in hypoxia (Morales-Alamo *et al.*, 2012). The present study indicates that reducing the oxidative stress caused by the sprint exercise is associated with increased Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation. Thus, it seems that an excessively high or low level of ROS during sprint exercise may end causing the same outcome, i.e. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation and subsequent blunting of the expected Thr^{172}-AMPKα phosphorylation. This dual effect of ROS is not new, in fact, it has been reported that high ROS (Rudich *et al.*, 1998; Houstis *et al.*, 2006), as well as preventing the exercise-elicited oxidative stress (Ristow *et al.*, 2009), are associated with insulin resistance. Thus, it seems that an optimal level of ROS-

mediated signaling during sprint exercise is required to elicit Thr^{172}-AMPKα phosphorylation.

Protein kinase B (Akt) (Horman *et al.*, 2006) and cAMP-dependent protein kinase (PKA) (Hurley *et al.*, 2006) can phosphorylate AMPKα1/α2 at Ser^{485}/^{491}, which inhibits AMPK phosphorylation at Thr^{172} (Horman *et al.*, 2006; Hurley *et al.*, 2006; Soltys *et al.*, 2006; Pang *et al.*, 2010). In the present investigation, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation occurred immediately after the sprint, while Ser^{473}-Akt phosphorylation was increased 30 min after the end of the sprint, suggesting that alternative mechanisms should account for the immediate post-exercise Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation. In our previous study the temporal pattern of Thr^{308}-Akt after a sprint performed in hypoxia coincided with AMPKα1/Ser^{491}-AMPKα2 phosphorylation (Morales-Alamo *et al.*, 2012). In the current study, Thr^{308}-Akt 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}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation could involve the cAMP-dependent protein kinase (PKA), but this is uncertain

(Viollet *et al.*, 2010). cAMP may blunt Thr^{172}-AMPKα phosphorylation through inhibition of CaMKKβ (an AMPK kinase (Hawley *et al.*, 2005)), but not LKB1 (Hurley *et al.*, 2006). It remains to be determined if muscle cAMP levels respond differently to sprint exercise depending on antioxidant status.

Transforming growth factor β-activated kinase 1 (TAK1)

Although TAK1 acts like an AMPK kinase (Xie *et al.*, 2006), skeletal muscle TAK1 response to exercise has not been studied in humans. Sprint exercise due to the combination of a high oxygen consumption with full activation of the anaerobic pathways leads to a fast production of free radicals (Cuevas *et al.*, 2005; Morales-Alamo *et al.*, 2012). These free radicals trigger a number of signaling processes (Powers *et al.*, 2011), which included transcription factors like NF-κB, AP-1, and Nrf2 regulating antioxidant enzymes (Barbieri & Sestili, 2012). TAK1 is one of the major upstream activators of NF-κB, AP-1, and Nrf2, having a crucial role in the adaptive response to increased ROS production (Omori *et al.*, 2012). TAK1 phosphorylation is absolutely required for TAK1 activity (Singhirunnusorn *et al.*, 2005). In the present

investigation, $Thr^{184/187}$- TAK1 phosphorylation was enhanced after the sprint exercise preceded by antioxidants. It seems that tyrosine and Ser/Thr phosphatases may be inhibited by exposure to ROS (Wright *et al.*, 2009). Had this been the case in human skeletal muscle after sprint exercise then the increase of $Thr^{184/187}$- TAK1 phosphorylation following the administration of antioxidant may indicate that in skeletal muscle free radicals have inhibitory influences on $Thr^{184/187}$- TAK1 phosphorylation. Despite the potential AMPK kinase activity of TAK1, antioxidant ingestion resulted in lower CaMKII and AMPK phosphorylation. Thus, the changes observed in TAK1 phosphorylation cannot account for the antioxidant-mediated blunting of the expected CaMKII and AMPK phosphorylation after sprint exercise.

Lac/Pyr and NAD^+/$NADH.H^+$ ratios and SIRT1/LKB1/AMPK signaling

Previous studies have also reported similar glycolytic rates and metabolite changes during Wingate tests (Bogdanis *et al.*, 1995; Parra *et al.*, 2000). As a novelty, this study shows that antioxidant administration prior to 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 ROS-mediated fatigue mechanisms (Westerblad & Allen, 2011). In both trials, the contribution of the anaerobic metabolism was comparable, as reflected by the similar oxygen deficits and intra-muscular accumulation of lactate.

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 lactate accumulation and Lac/Pyr ratios (2012), and hence, large reduction of the $NAD^+/NADH.H^+$ ratio (Williamson *et al.*, 1967; Sahlin, 1985). Consequently, the sprint exercise in hypoxia was associated with reduced SIRT1-mediated signaling and blunted Thr^{172}-AMPKα phosphorylation during the recovery period compared to a similar sprint in normoxia. One of the most important upstream kinases for Thr^{172}-AMPKα phosphorylation is LKB1, which activity parallels the changes of the $NAD^+/NADH.H^+$ ratio. A larger reduction of SIRT1 activity is expected when the $NAD^+/NADH.H^+$ ratio is lowered. Since SIRT1 deacetylates (and

activates) LKB1 (Hou *et al.*, 2008), a lower LKB1 activity is expectable when the $NAD^+/NADH.H^+$ is reduced, and this mechanism could explain the blunted Thr^{172}-AMPKα phosphorylation after the sprint exercise in hypoxia. However, in the present investigation, *Lac/Pyr* and cytoplasmatic $NAD^+/NADH.H^+$ ratios were similar after both sprints. Consequently, the stimulus provided by SIRT1 to activate LKB1 should have been similar, what means that the blunting Thr^{172}-AMPKα phosphorylation by antioxidants is unlikely caused by lower LKB1 activity after the ingestion of antioxidants.

The ingestion of a combination of vitamin C (1000 mg/day) and vitamin E (400 IU/day) prevents the positive effects of exercise (twenty 85 min sessions x 5 days per week x 4 weeks) on fasting plasma insulin concentrations, insulin sensitivity, plasma adiponectin levels, and attenuates the induction of PGC1α, PGC1β, PPARγ and antioxidant enzymes gene expression in skeletal muscle (Ristow *et al.*, 2009). What the present investigation adds is that we have shown that the ingestion of antioxidants prior to a single sprint, which we have shown is able to induce PGC1α (Guerra *et*

al., 2011c), prevents Thr^{172}-AMPKα phosphorylation, a critical step for the induction of PGC1α gene expression (Jager *et al.*, 2007; Dasgupta *et al.*, 2012). Therefore, ingestion of antioxidants prior to sprint exercise may also prevent some of the expected positive muscle adaptations on insulin sensitivity and antioxidant capacity.

Ser^{221}-ACCβ phosphorylation is dissociated from Thr^{172}-AMPKα phosphorylation.

Ser^{221}-ACCβ phosphorylation has been often used as an indicator AMPK activity. However, evidence is accumulating showing that, at least during sprint exercise Thr^{172}-AMPKα phosphorylation is dispensable for Ser^{221}-ACCβ phosphorylation (Guerra *et al.*, 2010; Fuentes *et al.*, 2012a; Morales-Alamo *et al.*, 2012). 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 (Merry *et al.*, 2010).

In conclusion, we have shown that a single short sprint exercise elicits Thr^{172}-AMPKα phosphorylation in the human skeletal muscle and this response depends on free radical produced during the sprint or first min into the recovery period, since antioxidant ingestion two hours prior to sprint abrogates the Thr^{172}-AMPKα phosphorylation response observed after the ingestion of placebo. This might have been caused by the suppression of Thr^{286}-CaMKII phosphorylation, an upstream kinase for AMPK in skeletal muscle after the ingestion of antioxidants. In addition, we have shown that the ingestion of antioxidants was associated with increased Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation immediately after the sprint, a known inhibitory mechanism of Thr^{172}-AMPKα phosphorylation. These findings indicate that free radicals play a critical role on the Thr^{172}-AMPKα phosphorylation response to sprint exercise in human skeletal muscle. Interestingly these effects were observed despite the fact that the ingestion of antioxidants did not influence exercise performance, muscle aerobic and anaerobic metabolism, and the AMP/ATP and NAD^+/$NADH.H^+$ ratios, further emphasizing a direct role of free radicals in the signaling response to sprint exercise in human

skeletal muscle. Recapitulating, ingestion of antioxidants prior to high intensity training may blunt some of the training adaptations and, hence, some of the potential benefits of training.

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Table 1. Physical characteristics and ergoespirometric variables during sprint in control conditions (placebo) and after the ingestion of antioxidants (mean±SD).

	Placebo	Antioxidants
Age (years)	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	19.5 ± 2.4	
HRmax (Beats/min)	188 ± 6	
VO_2max (L/min)	3.99 ± 0.25	
Wmax (W)	359 ± 34	
Wpeak (W)	999 ± 129	979 ± 114
Wpeak/Kg LLM (W/kg)	51.6 ± 6.2	50.5 ± 4.3
Wmean (W)	575 ± 61	572 ± 61
Wmean/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 VO_2 (L)	1.192 ± 0.406	1.207 ± 0.391
O_2 deficit (L)	3.003 ± 0.498	2.921 ± 0.513
O_2 deficit/Wmean	5.24 ± 0.83	5.11 ± 0.72
Wingate $P_{ET}O_2$ (mmHg)	114 ± 7	115 ± 5

Wmax: maximal intensity during the incremental exercise test to exhaustion; Wpeak: peak power output during the Wingate test; LLM: lean mass of the lower extremities; Wmean: mean power output during the Wingate test; Accumulated VO_2: amount of O_2 consumed during the 30s Wingate test; (pulsoximetry); $P_{ET}O_2$: end tidal O_2 pressure. N=9 for all variables and conditions.

Table 2. Serum glucose and insulin concentrations before and after sprint exercise in control conditions (placebo) and after the ingestion of antioxidants (mean±SD).

		R	0 min	30 min	120 min	
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Condition x time interaction
Glucose (mg/dL)	Placebo	89.2 ± 6.4	97.1 ± 6.7 $	96.8 ± 8.7 $	87.7 ± 6.5	P=0.98
	Antioxidants	92.4 ± 9.6	100.2 ± 6.7 $	100.8 ± 8.4 $	89.7± 9.9	
Insulin (μU/mL)	Placebo	4.6 ± 2.1	7.8 ± 3.1 $	10.7 ± 3.9 $	4.6 ± 2.6	P= 0.70
	Antioxidants	5.5 ± 2.4	7.5 ± 3.3	11.4 ± 8.3 $	4.6 ± 1.7	

0 min corresponds to immediately after the Wingate test. $ P< 0.05 versus resting (R) values. N=9 for all variables and conditions.

Table 3. Muscle metabolites before and immediately after a 30-second sprint in control conditions (placebo) and after the ingestion of antioxidants (mean ± SD).

	Placebo				Antioxidants			
	Resting		Post-sprint		Resting		Post-sprint	
	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
ATP (mmol/Kg)	5.08 ± 1.88		2.43 ± 0.81 [$]		4.96 ± 1.59		3.51 ± 1.40 [$]	
AMP/ATP (mmol/mol) [c]	7.9 ± 7.5		170.7 ± 316.0 [$]		4.2 ± 2.4		67.5 ± 55.7 [$]	
PCr (mmol/Kg)	15.97 ± 2.78		4.95 ± 2.23 [$]		17.77 ± 2.35		6.04 ± 2.95 [$]	
Cr (mmol/Kg)	12.44 ± 2.78		23.46 ± 2.23 [$]		10.64 ± 2.35		22.37 ± 2.95 [$]	
Pyruvate (mmol/Kg) [b]	0.09 ± 0.04		0.28 ± 0.11 [$]		0.10 ± 0.04		0.28 ± 0.09 [$]	
Lactate (mmol/Kg) [b]	2.6 ± 2.2		38.5 ± 13.1[$]		2.5 ± 2.4		35.5 ± 15.3 [$]	
Lac/Pyr [b d]	30.1 ± 27.8		145.3 ± 36.9[$]		37.5 ± 36.4		129.8 ± 53.6 [$]	
[NAD+]/([NADH.H+]) x 10^{7} [b]	450.4 ± 223.9		65.5 ± 16.5 [$]		496.6 ± 387.9		87.9 ± 54.4 [$]	

[$] Post-sprint vs. resting (same condition); [b] n=8; [c] AMP/ATP ratio calculated from the CK and AK apparent equilibrium constants for free AMP and ADP. N=9 for all variables and conditions, except for pyruvate and lactate.

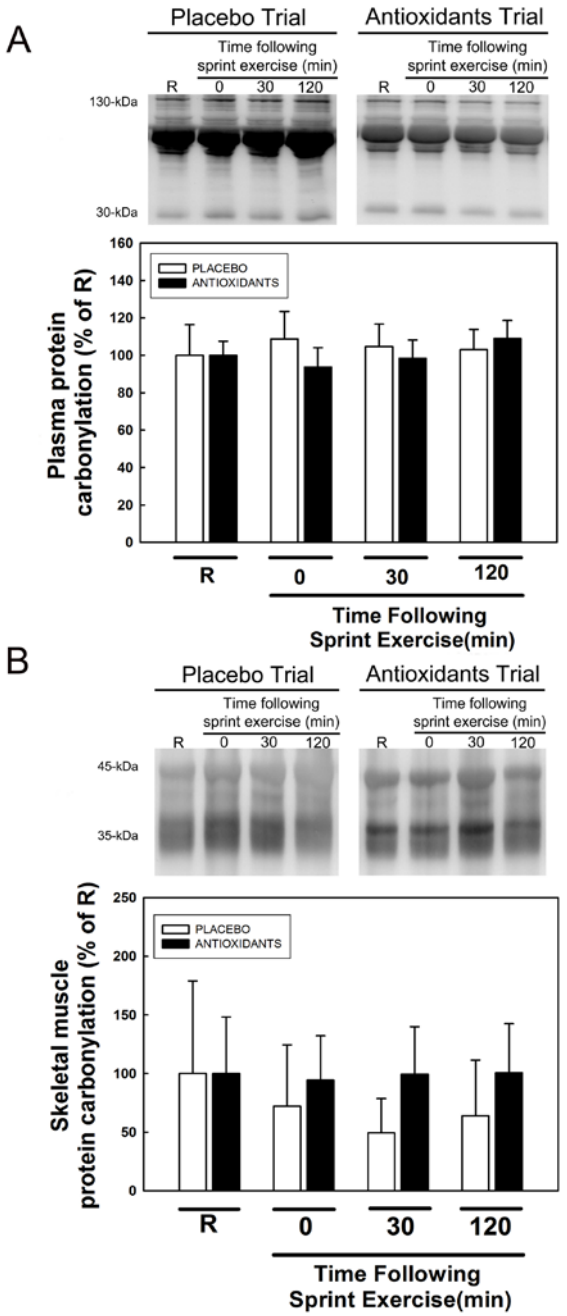
A
Placebo Trial
Antioxidants Trial
Time following sprint exercise (min)
R
0
30
120
130-kDa
30-kDa
PLACEBO
ANTIOXIDANTS
Plasma protein carbonylation (% of R)
Time Following Sprint Exercise(min)
B
45-kDa
35-kDa
Skeletal muscle protein carbonylation (% of R)

Figure 1.

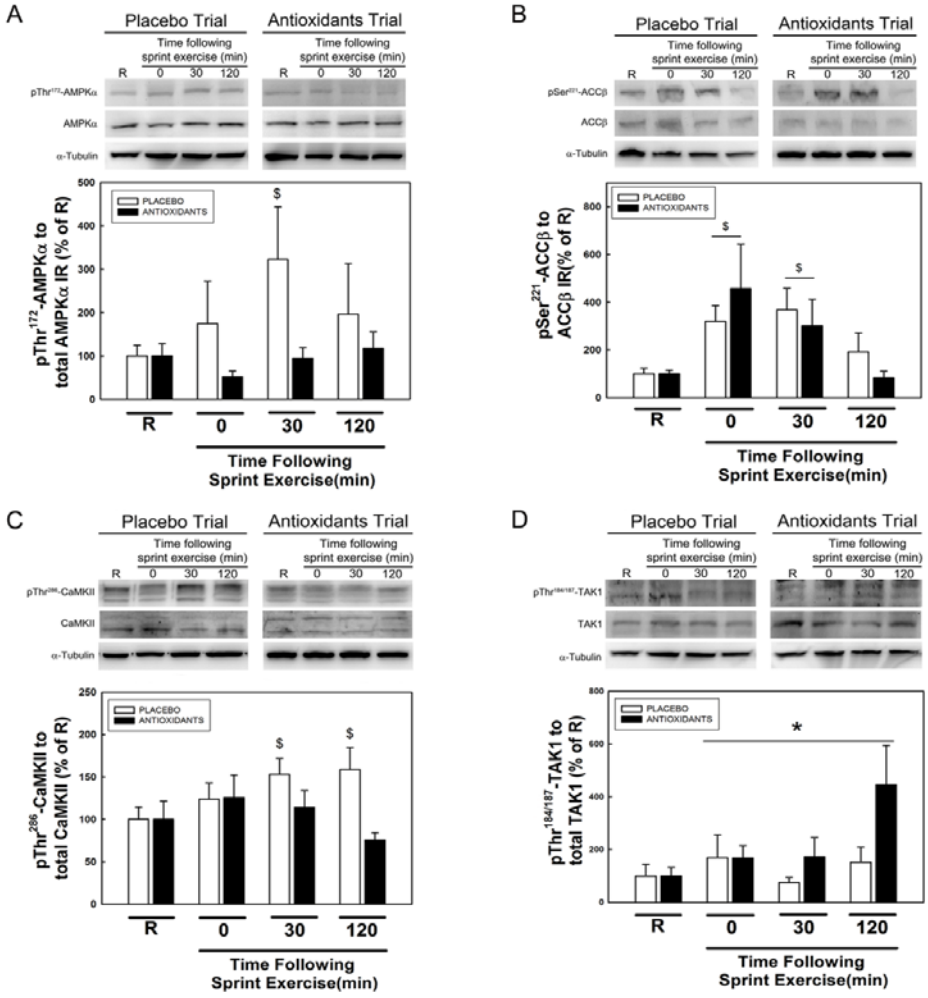
A
Placebo Trial
Antioxidants Trial
Time following sprint exercise (min)
R 0 30 120
pThr172-AMPKα
AMPKα
α-Tubulin
PLACEBO
ANTIOXIDANTS
pThr172-AMPKα to total AMPKα IR (% of R)
Time Following Sprint Exercise(min)
B
pSer221-ACCβ
ACCβ
pSer221-ACCβ to ACCβ IR(% of R)
C
pThr286-CaMKII
CaMKII
pThr286-CaMKII to total CaMKII (% of R)
D
pThr184/187-TAK1
TAK1
pThr184/187-TAK1 to total TAK1 (% of R)

Figure 2.

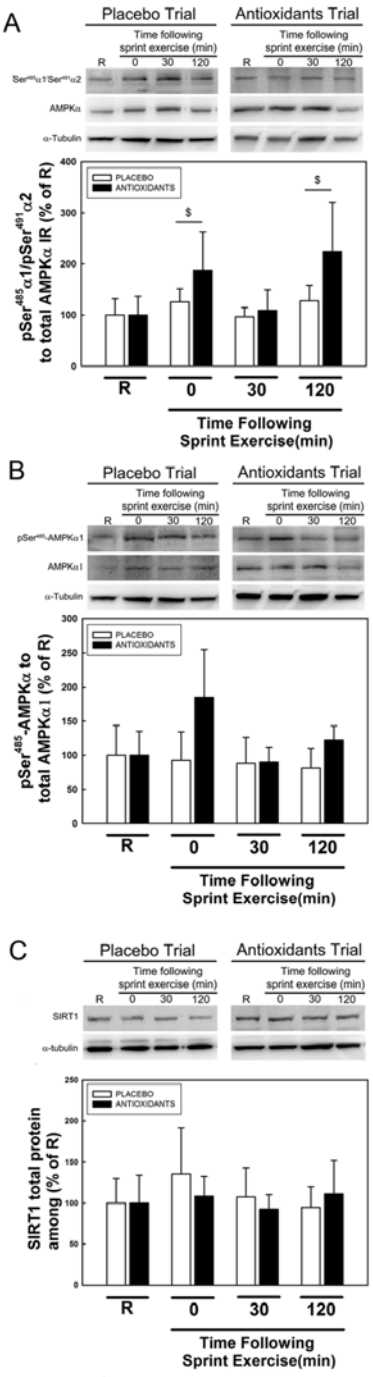
A
Placebo Trial
Antioxidants Trial
Time following sprint exercise (min)
R
0
30
120
AMPKα
α-Tubulin
PLACEBO
ANTIOXIDANTS
$
pSer485α1/pSer491α2 to total AMPKα IR (% of R)
Time Following Sprint Exercise(min)
B
pSer485-AMPKα1
AMPKα1
pSer485-AMPKα to total AMPKα1 (% of R)
C
SIRT1
α-tubulin
SIRT1 total protein among (% of R)

Figure 3.

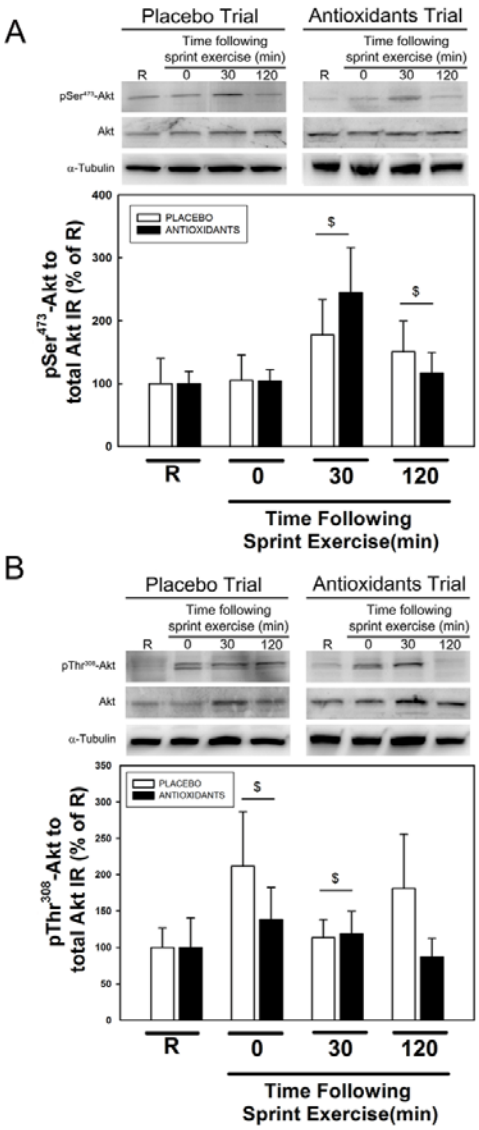
A
Placebo Trial
Antioxidants Trial
Time following sprint exercise (min)
R
0
30
120
pSer473-Akt
Akt
α-Tubulin
PLACEBO
ANTIOXIDANTS
pSer473-Akt to total Akt IR (% of R)
$
Time Following Sprint Exercise(min)
B
pThr308-Akt
pThr308-Akt to total Akt IR (% of R)

Figure 4.

Legends to figures

Figure 1. Levels of carbonylated proteins in plasma (A) and skeletal muscle (B) before and after a single Wingate test performed in placebo (open bars) or antioxidants (solid bars). Values in 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 and densitometry analysis (130 and 30 KDa) showing carbonylated proteins in plasma extracts. B) Western blot and densitometry analysis (45 and 35 KDa) showing carbonylated proteins in skeletal muscle extracts. Statistical analysis performed with logarithmically transformed data. N = 9 in both experimental conditions.

Figure 2. Levels of Thr^{172}-AMPKα (A), Ser^{221}-ACCβ (B), Thr^{286}-CaMKII (C), and $Thr^{184/187}$-TAK1 (D) before and after a single Wingate test performed in placebo (open bars) or antioxidants (solid bars). Values in 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*

panel: a representative western blot with antibodies against AMPKα, phospho-AMPKα and α-Tubulin. *Lower panel*: AMPKα phosphorylation densitometric values relative to total AMPKα. $ P<0.05 versus R. B) *Top panel*: a representative western blot with antibodies against ACCβ, phospho-ACCβ and α-Tubulin. *Lower panel*: ACCβ phosphorylation values relative to total ACCβ. $ P<0.05 versus R. Statistical analysis performed with logarithmically transformed data. C) *Top panel*: a representative western blot with antibodies against CaMKII, phospho-CaMKII and α-Tubulin. *Lower panel*: CaMKII phosphorylation values relative to total CaMKII. $ P<0.05 versus R. D) *Top panel*: a representative western blot with antibodies against TAK1, phospho-TAK1 and α-Tubulin. *Lower panel*: TAK1 phosphorylation values relative to total TAK1. Statistical analysis performed with logarithmically transformed data.

$ P<0.05 versus R. Statistical analysis performed with logarithmically transformed data. N = 9 in both experimental conditions.

Figure 3. Levels of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 (A), Ser^{485}-AMPKα1 (B) and SIRT1 (C) before and after a single Wingate test performed in placebo (open bars) or antioxidants (solid bars). Values in 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 panel*: a representative western blot with antibodies against AMPKα, phospho-Ser^{485}-α1/Ser^{491}-α2 and α-Tubulin. *Lower panel*: Ser^{485}-α1/Ser^{491}-α2 phosphorylation values relative to total AMPKα. $^{\$}$ $P<0.05$ versus R in normoxia. Statistical analysis performed with logarithmically transformed data. B) *Top panel*: a representative western blot with antibodies against AMPKα, phospho-Ser^{485}-α1 and α-Tubulin. *Lower panel*: Ser^{485}-AMPKα1 phosphorylation values relative to total AMPKα1. C) Top panel: a representative western blot with antibodies against SIRT1 and α-Tubulin. Lower panel: SIRT1 values. $^{\$}$ $P<0.05$ versus R. Statistical analysis performed with logarithmically transformed data. N = 9 in both experimental conditions.

Figure 4. Levels of Ser^{473}-Akt (A) and Thr^{308}-Akt (B), before and after a single Wingate test performed in placebo (open bars) or antioxidants (solid bars). Values in 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 panel*: a representative western blot with antibodies against Akt, phospho-Ser^{473}-Akt and α-Tubulin. *Lower panel*: Ser^{473}-Akt phosphorylation values relative to total Akt. $^{\$}P<0.05$ versus R. Statistical analysis performed with logarithmically transformed data. B) *Top panel*: a representative western blot with antibodies against Akt, phospho-Thr^{308}-Akt and α-Tubulin. *Lower panel*: Thr^{308}-Akt phosphorylation densitometric values relative to total Akt. $^{\$}P<0.05$ versus R. Statistical analysis performed with logarithmically transformed data. N = 9 in both experimental conditions.

ESTUDIO III

Skeletal muscle signaling during sprint exercise in severe acute hypoxia: role of free radicals

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Abstract

Compared to normoxia, sprint exercise in severe acute hypoxia elicits a much greater glycolytic rate, lower muscle pH at exhaustion and higher oxidative stress leading to a paradoxical blunt of the AMPK Thr^{172} phosphorylation. To determine if free radicals could play a role in blunting Thr^{172}-AMPKα phosphorylation nine voluntaries performed a single 30s sprint (Wingate test) in two occasions while breathing hypoxic gas (P_IO_2=75 mmHg): one after the ingestion of placebo (P) and another following the intake of antioxidants (A) (α-lipoic acid, vitamin C, and vitamin E), with a double blind design. Vastus lateralis muscle biopsies were obtained before and immediately after, 30 and 120 min post-sprint. Compared to the control condition, the ingestion of antioxidants resulted in lower plasma carbonylated proteins, lower elevation of AMP/ATP molar ratio, and reduced glycolytic rate ($P<0.05$) without significant effects on performance or VO_2. The ingestion of antioxidants did not alter the basal muscle signaling. Thr^{172}-AMPKα and $Thr^{184/187}$-TAK1 phosphorylation were not increased after the sprint regardless of the ingestion of antioxidants. Thr^{286}-CaMKII phosphorylation was increased after the sprint but,

this response was blunted by the antioxidants. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation increased immediately after the sprints coincident with increased Akt phosphorylation. In summary, this study shows that antioxidants attenuate the glycolytic response to sprint exercise in severe acute hypoxia and modify the muscle signaling response to exercise. Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, a known mechanism of Thr^{172}-AMPKα phosphorylation inhibition, is increased immediately after sprint exercise in hypoxia by a mechanism independent of free radicals.

Introduction

AMP-protein activated kinase (AMPK) is a well conserved metabolic master switch activated by Thr^{172} phosphorylation [1], which plays a critical role in the skeletal muscle response to exercise [2] and is required for mitochondrial biogenesis [3]. Thirty minutes after a single 30 s sprint Thr^{172}-AMPKα phosphorylation is markedly increased [4]. However, when the sprint is performed in severe acute hypoxia this Thr^{172}-AMPKα phosphorylation is blunted [5]. Compared to normoxia, sprint exercise in severe acute hypoxia elicits a much greater glycolytic rate, lower muscle pH at exhaustion and higher oxidative stress [5]. However, it remains unknown if the blunting effect of hypoxia on the Thr^{172}-AMPKα phosphorylation response to sprint exercise is due to increased free radicals-mediated signaling response and the molecular mechanisms involved have not been elucidated.

High intensity or fatiguing muscle contractions are strong inducers of AMPK Thr^{172} phosphorylation in human skeletal muscle [5-7]. AMPK Thr^{172} phosphorylation may be elicited by several AMPK kinases, but in skeletal muscle, the liver kinase B1 (LKB1), due to is sensitivity to the AMP/ATP ratio [8], has a

crucial role in exercise-induced AMPK Thr^{172} phosphorylation. Transforming growth factor-β-activated kinase 1 (TAK-1), which is activated by phosphorylation, may phosphorylate and activate LKB1 [9]. LKB1 can be also activated through deacetylation by the deacetylase sirtuin 1 (SIRT1), which is activated in response to the increase in $NAD^+/NADH.H^+$ ratio [10]. Although in cell cultures hypoxia produces Thr^{172}-AMPKα phosphorylation by a free radicals dependent mechanism [11], the sprint exercise expected Thr^{172}-AMPKα phosphorylation in skeletal muscle is prevented when the sprint is performed in hypoxia [5]. Since sprint exercise in hypoxia is associated to increased oxidative stress and protein carbonylation [5], we hypothesized that excessive free radical release during sprint exercise in hypoxia may contribute to blunt Thr^{172}-AMPKα phosphorylation by inhibiting LKB1 [5]. Compared to normoxia, sprint exercise in hypoxia is accompanied by a greater activation of glycolysis [5]. Since antioxidants reduce glycolytic activation in cell cultures, antioxidant administration prior to sprint exercise in hypoxia may also reduce the glycolytic rate, acidosis and free radicals production [12-14]. This may result in a higher $NAD^+/NADH^+$ ratio, which in turn may facilitate a greater activity

of LKB1 through SIRT1 deacetylation leading to higher Thr^{172}-AMPKα phosphorylation. Antioxidants may also help to maintain the intracellular levels of SIRT1 total protein (contributing to LKB1 activation), which has been reported to be reduced when sprint exercise is accompanied by high glycolytic rates and oxidative stress, as observed in severe acute hypoxia [5]. Alternatively, reactive oxygen species may activate CaMKII through modification of the Met-$^{281/282}$ pair within the regulatory domain [15]. Thus antioxidant ingestion prior to sprint exercise in hypoxia could facilitate Thr^{172}-AMPKα phosphorylation if the predominating mechanism for sprint exercise-induced Thr^{172}-AMPKα phosphorylation is, as thought, LKB1-dependend. However, if the main mechanism leading to Thr^{172}-AMPKα phosphorylation during sprint exercise depends on CaMKII activation by free radicals, then the ingestion of antioxidants should blunt the activating phosphorylation of CaMKII and, its downstream kinase AMPK. Finally, sprint exercise in hypoxia elicits Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation [5], an important mechanism of Thr^{172}-AMPKα phosphorylation inhibition [16-17], possibly mediated by Akt phosphorylation [18]. It remains unknown if free radicals could

play a role in eliciting Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation in skeletal muscle during sprint exercise.

Therefore, the main aim of this study was to determine if free radicals could play a role in blunting Thr^{172}-AMPKα phosphorylation in human skeletal muscle after sprint exercise in hypoxia and to dissect the potential signaling mechanism responsible for this response. By assessing Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 and Ser^{473}/Thr^{308}-Akt phosphorylation we expected to determine whether the Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 and it putative upstream kinase (Akt) are phosphorylated after sprint exercise by a free-radicals depending mechanism. A secondary aim was to determine if ingestion of antioxidants prior to sprint exercise in severe acute hypoxia reduces the glycolytic rate and to assess the effects of antioxidants on exercise performance and aerobic metabolism.

Materials and methods

Materials

The Complete protease inhibitor cocktail and the PhosSTOP phosphatase inhibitor were obtained from Roche Diagnostics

(Mannheim, Germany). All the primary antibodies used were from Cell Signaling Technology (Denvers, MA, USA) except the polyclonal anti-Phospho-AS160 (Thr^{642}, no. AT-7079; molecular weight (MW): 160 KDa) that was obtained from MBL International Corporation (Woburn, MA, USA), the anti-CaMKII antibody (no. sc-13082, MW: 50 KDa) that was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the monoclonal mouse anti-alpha-tubulin antibody (no. T-5168-ML, MW: 50 KDa) that was obtained from Biosigma (Sigma, St .Louis, MO, USA). The corresponding catalogue number of the antibodies from Cell Signaling were: anti-Phospho-AMPKα (Thr^{172}) no. 2531 (MW: 62 KDa), anti-AMPKα no. 2532 (MW: 62 KDa), anti-phospho-AMPKα1 (Ser^{485})/AMPKα2 (Ser^{491}) no. 4185 (MW: 62 KDa), anti-phospho-AMPKα1 (Ser^{485}) no. 4184 (MW: 62 KDa), anti-AMPKα1 no. 2795 (MW: 62 KDa), anti-Phospho-Acetyl-CoA Carboxylase (Ser^{221}) no. 3661 (MW: 280 KDa), anti-Acetyl CoA Carboxylase (ACC) no. 3662 (MW: 280 KDa), anti-SIRT1 no. 2310 (MW: 120 KDa), anti-Phospho-Akt (Ser^{473}) antibody no. 9271 (MW: 60 KDa), anti-Phospho-Akt (Thr^{308}) antibody no. 9275 (MW: 60 KDa), anti-Akt antibody no. 9272 (MW: 60 KDa), anti-Phospho-TAK1

(Thr$^{184/187}$) no. 4531 (MW: 82 KDa), anti- TAK1 no. 4505 (MW: 82 KDa), anti-Phospho-CaMKII (Thr^{286}) no. 3361 (MW: 50 KDa), and AS160 no. 2447 (MW: 160 KDa). The secondaries HRP-conjugated goat anti-rabbit (no. 111-035-144) and the HRP-conjugated donkey anti-mouse (no. 715-035-150) antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). The Immun-Blot™ PVDF Membranes, and the Inmmun-Star™ WesternC™ were from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK). The ChemiDoc XRS System and the image analysis software Quantity One© were obtained from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK).

Subjects

Nine healthy male physical education students (age = 25 ± 5 yr, height = 176.0 ± 5.1 cm, body mass = 79.4 ± 10.1 kg, body fat = 18.3 ± 6.7%) agreed to participate in this investigation (Table 1). 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. 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 (CEIH-2010-01).

General Procedures

The subjects' body composition was determined by DXA (Hologic QDR-1500, Hologic Corp., software version 7.10, Waltham, MA) as described elsewhere [19-20]. Subjects reported to the laboratory to complete different tests on separate days. First, their peak VO_2 (VO_2peak), HRmax and maximal power output (Wmax), in hypoxia (H, F_IO_2=0.104; barometric pressure 735-745 mmHg), 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, the subjects were familiarized with the experimental protocol (a single 30s-isokinetic Wingate tests at 100 RPM). On separate days they performed one 30s-isokinetic Wingate test at 100 RPM under hypoxia after the ingestion of either placebo (HP) or antioxidants (HA) with a double-blind design. Antioxidants were administered split into two doses with the first dose ingested 2 h before the sprint (at 07:00 a.m.) followed by a second dose 30 min later, i.e. 90 min prior to

the sprint. The first dose consisted of 300 mg of α-lipoic acid, 500 mg vitamin C, and 200 IU vitamin E, whereas the second included 300 mg α-lipoic acid, 500 mg vitamin C, and 400 IU vitamin E (water dispersible). Using electron paramagnetic resonance spectroscopy, this antioxidant cocktail has been shown to be effective in decreasing free radicals levels at rest and in response to exercise in humans [21]. Placebo microcrystalline cellulose capsules were of similar taste, colour, and appearance and were likewise consumed in two similarly timed doses [21]. On each trial day, the subjects reported to the laboratory at 8.00 a.m., after an overnight fast, and an antecubital vein was catheterized. After a 10 min resting supine period, a 20 ml blood sample was withdrawn and used to measure serum glucose and insulin. Right after, a muscle biopsy was obtained from the middle portion of the vastus lateralis muscle using the Bergstrom's technique with suction, as described elsewhere [22]. After the pre-exercise muscle biopsy, the subjects sat on the cycle ergometer for four minutes. During this period they breathed a hypoxic gas mixture from a Douglas bag containing 10.4% O_2 in N_2 (H). The Douglas bag was replenished with gas

from a cylinder specially prepared for the experiment (Carburos metálicos, gas mixture 206030, Las Palmas de Gran Canaria).

During both sprints, the subjects attempted to pedal as fast and hard as possible (i.e., all-out) from the start to the end of the exercise. Since the cycle Ergometer (Excalibur Sport, Lode, Groningen, NL) 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.

Peak power output was calculated as the highest work output performed during 1 s interval, and mean power output from the average work performed during the 30 s. Warm up was not allowed prior to 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 [23-25].

Within 10 seconds from the end of the sprint a second muscle biopsy was taken, and then another blood sample was obtained. During the following two hours the subjects were fasting, 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 minutes. For the last two biopsies a new incision was performed in the contralateral leg. To avoid injury-triggered activation of signaling cascades the muscle biopsies were obtained at least 3 cm apart, using the procedures described by Guerra et al. [26]. 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. The time needed to obtain and freeze the muscle biopsies was below 30 s.

Cycling economy tests.

Cycling economy was determined on two different days using 8-11 submaximal workloads at intensities between 50 to 90% of VO_2peak, at 100 RPM. Exercise intensities and pedaling rates were administered in random order, separated by rest periods of 6 min. To reduce thermal stress and minimize water losses due to sweating, subjects were fan cooled and ingested fresh water during the resting periods *ad libitum*. The duration of each submaximal bout was set at 10 min. The mean VO_2 registered during the last two

minutes was taken as representative of each submaximal exercise intensity. To relate VO_2 to power, linear regression equations were calculated by least square linear fit.

Oxygen uptake and hemoglobin oxygen saturation

Oxygen uptake was measured with a metabolic cart (Vmax N29; Sensormedics, California, USA), calibrated immediately before each test according to the manufacturer's instructions. Respiratory variables were analyzed breath-by-breath and averaged every 5 seconds during the Wingate test and every 20 seconds during the incremental and cycling economy tests. The highest 20-s averaged VO_2 recorded in hypoxia was taken as the VO_2peak. Hemoglobin oxygen saturation (SpO_2) was determined with a finger pulse oxymeter (Lode Excalibur Sport 925900, Groningen, The Netherlands).

Muscle metabolites

From each muscle biopsy, 30 mg of wet tissue were treated with 0.5 M $HClO_4$ and centrifuged at 15000 g at 4 °C for 15 min. The supernatant was neutralized with $KHCO_3$ 2.1M. ATP,

phosphocreatine (PCr), creatine (Cr), pyruvate (Pyr) and lactate (Lac) were enzymatically determined in neutralized extracts by fluorometric analysis [27-28]. Muscle metabolite concentrations were adjusted to the individual mean total creatine (PCr + Cr) because this mean should remain constant during the exercise [29]. The adjustment to the total creatine content accounts for the variability in solid non-muscle constituents, which may be present in the biopsies [30]. The glycolytic rate (GR) was calculated as GR= 0.5 x (ΔLac + ΔPyr) [31]. The free AMP/ATP molar ratio was estimated after calculating the adenosine diphosphate (ADP) concentration using the creatine kinase equilibrium apparent constant for resting conditions and exhaustion after a Wingate test [30], as described in Sahlin et al. [32]. Subsequently, adenosine monophosphate (AMP) concentration was calculated using the adenilate kinase apparent equilibrium constant for the same conditions [30]. The $[NAD^+]/[NADH.H^+]$ was calculated using the equilibrium constant for the lactate dehydrogenase [33-34].

Total protein extraction, electrophoresis, and Western blot analysis

Muscle protein extracts were prepared as described previously [35] and total protein content was quantified using the bicinchoninic acid assay [36]. Briefly, proteins were solubilized in sample buffer containing 0.0625 M Tris -HCl, pH 6.8, 2.3% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 5% (vol/vol) beta-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. Equal amounts (50 μg) of each sample were electrophoresed on 7.5–10% SDS-PAGE using the system of Laemmli [37] and transferred to Hybond-P membranes. To determine Thr^{172}-AMPKα, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2, Ser^{485}-AMPKα1, Ser^{221}-ACCβ, Ser^{473}-Akt, Thr^{308}-Akt, $Thr^{184/187}$-TAK1, Thr^{286}-CaMKII and Thr^{642}-AS160 phosphorylation levels, antibodies directed against the phosphorylated and total form of these kinases were diluted in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T) (BSA-blocking buffer). SIRT1 was assessed in membranes incubated with a SIRT1 antibody (diluted in BSA-blocking buffer). To control for differences in loading and transfer efficiency across membranes, membranes were incubated with a monoclonal mouse

antialpha-tubulin antibody diluted in TBS-T with 5% blotting grade blocker non-fat dry milk (blotto-blocking buffer). No significant changes were observed in alpha-tubulin protein levels during the experiments (data not shown). Antibody-specific labeling was revealed by incubation with an HRP-conjugated goat anti-rabbit antibody (1:20,000) or an HRP-conjugated donkey anti-mouse (1:10,000) antibody both diluted in 5% blotto blocking buffer and visualized with the Immun-Star™ WesternC™ kit (Bio-Rad Laboratories, Hemel Hempstead Hertfordshire, UK). The densitometry analyses were carried out immediately before saturation of the immunosignals. Specific bands were visualized with the Immun-Star™ WesternC™ kit, using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with the image analysis program Quantity one© (Bio-Rad laboratories, Hercules, CA, USA). Muscle-signaling data were represented as a percentage of immunostaining values obtained for the phosphorylated form of each kinase relative the respective total form. In all gels a human muscle sample obtained from a healthy young man was used as an internal control, to reduce inter-gel variability.

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 µIU/ml.

Serum Glucose and blood lactate

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 minutes into the recovery period, using a Lactate Pro analyzer (Arkay, JA) [38].

Protein carbonylation

Protein carbonylation in skeletal muscle and plasma was assessed by immunobloting detection of protein carbonyl groups using "OxyBlot" protein oxidation kit (Intergen Company, Purchase,NY) as previously described [39]. Protein carbonylation data were represented as a percentage of immunostaining values.

Statistics

Variables were checked for normal distribution by using the Shapiro-Wilks test. When necessary, the analysis was carried out on logarithmically transformed data. First, the pre-exercise values were

compared between the two conditions using a Student's t-test. Since not significant differences between conditions were observed prior to the start of the sprint, the individual responses were normalized to the band densities or level of phosphorylation observed just before the start of the Wingate test. A repeated-measures ANOVA over time and oxidative stress condition with two levels (hypoxia placebo vs. hypoxia antioxidants) was used to compare the responses with the value prior to the start of the Wingate test. When there was a significant condition effect or condition x time interaction (placebo vs. antioxidants), pairwise comparisons at specific time points, were adjusted for multiple comparisons with the Holm–Bonferroni method. The relationship between variables was determined using linear regression analysis. The areas under the curve (AUC) were determined using the trapezoidal rule and compared between conditions with paired Student t-tests. 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

Performance and ergospirometric variables

The ergoespirometric and performance-related variables are reported in Table 1. Neither exercise performance nor any of the ergoespirometric variables assessed were affected by the ingestion of antioxidants.

Serum lactate, glucose, insulin, and carbonylated plasma proteins

Peak blood lactate concentration during the recovery period was similar in both conditions (Table 1). Basal serum glucose and insulin concentrations were not altered by the ingestion of antioxidants. Compared to resting, serum glucose concentration was increased by 10% immediately after the sprints, and remained at this level 30 min later (time effect, $P<0.05$) (Table 2). Compared to pre-exercise values, serum insulin concentration was elevated by 2.3-folds 30 minutes after the sprint (time effect, $P<0.05$) (Table 2) and then decreased, with a similar response in both conditions.

Basal levels of plasma carbonylated proteins were not altered by the ingestion of antioxidants. However, compared to the

placebo condition, antioxidant ingestion resulted in 23% lower plasma carbonylated proteins after the sprints ($P<0.05$) (Fig. 1a).

Muscle metabolites

The changes observed in muscle metabolites are shown in Table 3. Basal muscle metabolites were not altered by the ingestion of antioxidants. The ingestion of antioxidants resulted in a lower elevation of AMP/ATP molar ratio immediately after the sprint ($P<0.05$). Lower muscle pyruvate and lactate concentrations were observed after the ingestion of antioxidants ($P<0.05$), due to a 34% lower glycolytic rate ($P<0.05$). After the sprints, the Lac/Pyr ratio was increased by 15 and 6.5-fold, respectively in HP and HA ($P<0.05$; ANOVA antioxidants x time interaction $P<0.05$). Immediately after the sprints, the $NAD^+/NADH.H^+$ ratio was reduced to a similar level, however the relative reduction was lower after the ingestion of antioxidants (88%) than after the ingestion of placebo (95%) (ANOVA condition x time interaction $P<0.05$).

Skeletal muscle carbonylated proteins

Antioxidants did not alter the basal levels of muscle carbonylated proteins which remained unchanged after the sprints (Fig. 1b).

Muscle signaling

The ingestion of antioxidants did not alter the basal levels of Thr^{172}-AMPKα phosphorylation, ACCβ phosphorylation, Thr^{286}-CaMKII phosphorylation, $Thr^{184/187}$-TAK1 phosphorylation, Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, Ser^{485}-AMPKα1 phosphorylation, Ser^{473}-Akt phosphorylation, and Thr^{308}-Akt phosphorylation, nor total SIRT1 protein content. Thr^{172}-AMPKα phosphorylation remained at similar levels after both sprints (Fig. 2a). However, ACCβ phosphorylation was increased by 2.4 and 2.2-fold immediately after and 30 min into the recovery period, respectively (both, $P<0.05$). Compared to pre-exercise values, ACCβ phosphorylation was decreased by 25% two hours after the sprints, with a similar response in both conditions (Fig. 2b).

Antioxidant ingestion resulted in a 36% lower AUC during all the recovery period for the Thr^{286}-CaMKII phosphorylation compared to the placebo condition ($P<0.05$) (Fig. 2c). No

statistically significant changes were observed in $Thr^{184/187}$-TAK1 phosphorylation after both sprints (Fig. 2d).

Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation level was increased by 81% immediately after the sprints (time effect, $P<0.05$) (Fig. 3a) with a similar response in both conditions. No significant changes in Ser^{485}-AMPKα1 phosphorylation were observed after both sprints (Fig. 3b). Not significant changes were observed on SIRT1 total protein levels (Fig. 3c).

Ser^{473}-Akt phosphorylation was similarly increased after both sprints (32% and 2.3-fold immediately and 30 min after the sprints, respectively, $P<0.05$) (Fig. 4a). Thr^{308}-Akt phosphorylation was increased by 2.7-fold and 80% immediately and 30 min after the sprints, respectively ($P<0.05$). Compared to the placebo condition, the ingestion of antioxidants attenuated Thr^{308}-Akt phosphorylation by 47% ($P<0.05$) (Fig. 4b).

Thirty minutes after both sprints, Thr^{642}-AS160 phosphorylation was similarly increased by 50% (Fig. 4c).

Discussion

This study was designed to determine if increased free radical-mediated signaling could account for the blunting effect of severe hypoxia on the Thr^{172}-AMPKα phosphorylation response to sprint exercise in human skeletal muscle. To attenuate free radical-mediated signaling subjects ingested and antioxidant cocktail containing α-lipoic acid, vitamin C and E which have been shown to decrease circulating free radicals at rest (~98%) and during exercise (~85%) in humans skeletal muscle as assessed in vivo by electron parametric resonance (EPR) spectroscopy, a highly sensitive technique [21]. In fact our study reveals lower levels of plasma carbonylated proteins after the sprints performed following the ingestion of the antioxidant cocktail, implying that this antioxidant cocktail efficiently counteracted oxidative stress in our experimental setting. We have shown for the first time in humans that the administration of antioxidants prior to sprint exercise modifies the energy metabolism during the sprint, reducing the glycolytic rate without a negative effect on performance or aerobic metabolism. Consequently, the muscle signaling response to sprint exercise is modified by the ingestion of antioxidants. However, in

contrast with our hypothesis, the Thr^{172}-AMPKα phosphorylation response to sprint exercise was not restored, implying that increased oxidative stress is not the main mechanism blunting the Thr^{172}-AMPKα phosphorylation during sprint exercise in hypoxia. Moreover, this investigation has shown that free radicals play a role in exercise-induced Thr^{286}-CaMKII phosphorylation in skeletal muscle, which is completely abrogated by the ingestion of antioxidants. The fact that the lack of increase in Thr^{286}-CaMKII phosphorylation was not followed by lower Thr^{172}-AMPKα phosphorylation after the ingestion of antioxidants could indicate that Thr^{286}-CaMKII phosphorylation is not required for maintaining Thr^{172}-AMPKα phosphorylation at resting levels. Regardless of antioxidant ingestion, $Thr^{184/187}$-TAK1 phosphorylation was not altered in response to sprint exercise. This coincides with our previous study in which no response of $Thr^{184/187}$-TAK1 phosphorylation to sprint exercise was observed in normoxia [5]. Thus changes on $Thr^{184/187}$-TAK1 phosphorylation do not account for the blunting effect of hypoxia on sprint exercise-induced Thr^{172}-AMPKα phosphorylation.

We have also examined the influence of antioxidant ingestion on the exercise-induced Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, which inhibits Thr^{172}-AMPKα phosphorylation [16, 18, 40]. Sprint exercise in hypoxia induced an immediate Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, which was not altered by the ingestion of antioxidants. Thus, during sprint exercise in hypoxia the main mechanism that could account for the blunting effect on Thr^{172}-AMPKα phosphorylation is the immediate Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation, which was not prevented by antioxidants. A similar inhibitory mechanism of Thr^{172}-AMPKα phosphorylation has been also observed when sprint exercise is performed one hour after the ingestion of 75 g of glucose [6].

Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation is elicited by Akt, which was activated by Thr^{308} and Ser^{473} phosphorylation immediately after the sprint, regardless of the antioxidant status. This increase in Akt activity is supported by the subsequent phosphorylation of its downstream target AS160. Although antioxidants attenuated the Thr^{308}-Akt phosphorylation response to sprint, this effect was more marked 30 min after the sprint and had

no influence on the post-exercise increase of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation. Thirty minutes after the sprint, Thr^{308}-Akt phosphorylation, but not Ser^{473}-Akt phosphorylation, had returned to resting levels. Since Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation had also returned to resting pre-exercise levels at 30 min into the recovery process, this is compatible with Thr^{308}-Akt phosphorylation as necessary for Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation in response to sprint exercise, confirming our previous results [5].

Compared to normoxia, sprint exercise in severe acute hypoxia is accompanied by reduced SIRT1 protein levels, which combined with a lower NAD^+/$NADH.H^+$ ratio at the end of the sprint could led to lower LKB1 activation by SIRT1 [10] and, hence, reduced Thr^{172}-AMPKα phosphorylation [5]. In cell cultures H_2O_2 induces JNK1 phosphorylation causing Ser^{47}-SIRT1 phosphorylation [41]. SIRT1 phosphorylation facilitates its translocation to the nucleus, increases its deacetylase activity and commits SIRT1 to degradation at proteasome [41]. Thus, to determine if free radicals produced during sprint exercise in hypoxia may induce SIRT1 protein degradation in human skeletal

we assessed SIRT1 protein levels after the sprint performed with or without antioxidants. Although the area under curve during the recovery period for the SIRT1 protein levels was almost twice as high that after the control sprint, this difference did not reach statistical significance (P=0.22); but we cannot rule out a type II error due to the high variability of these blots. Antioxidants attenuated the reduction of NAD^+/$NADH.H^+$ caused by sprint exercise in hypoxia and this should have facilitated a greater LKB1 activity, however, it did not restore the normal Thr^{172}-AMPKα phosphorylation response to sprint exercise. This may be explained by the fact that the AMP/ATP ratio, which is considered the main mechanism eliciting LKB1 activation and Thr^{172}-AMPKα phosphorylation, was less increased after the ingestion of antioxidants. Thus, the combined effect of a lesser reduction of NAD^+/$NADH.H^+$ and lower increase of the AMP/ATP ratio may have cancelled each other resulting in unchanged LKB1 activity levels after both sprints.

Antioxidants attenuate the skeletal muscle glycolytic response to sprint exercise in humans.

In cell cultures glycolysis is upregulated by ROS [42-43] and this response is blunted by α-lipoic acid and other antioxidants [42]. In the present investigation we have shown that an antioxidant cocktail containing α-lipoic acid attenuates the glycolytic response during sprint exercise in hypoxia. The latter might have been mediated by the lower increase of the AMP/ATP ratio after the ingestion of antioxidants. An alternative mechanism that could explain a lower glycolytic rate after the ingestion of antioxidants is a reduced production of fructose 2,6-bisphosphate (the most potent activator of phosphofructokinase (PFK) 1, which is the main regulatory enzyme determining the glycolytic rate) by phosphofructokinase 2/ fructose biphosphatase 2 (PFK2/FBPase2") [44]. The liver isoform of PFK2/FBPase2, which is also expressed in skeletal muscle, is a bifunctional enzyme that when phosphorylated at its Ser-^{32} changes its activity from kinase to phosphatase lowering fructose 2,6-bisphosphate concentration [31, 45]. If antioxidants favor PFK2/FBPase2 phosphorylation in human skeletal muscle remains unknown.

Although the lactate ion is antioxidant [46], lactate production during exercise is always associated with acidosis, which is a potent oxidative condition [13-14]. The present investigations show that the antioxidant cocktail administered prior to a single bout of sprint exercise reduced muscle lactate accumulation, attenuating the reduction of muscle pH. Thus antioxidant effects of the cocktail administered may have result not only from the free radicals scavenging properties of these antioxidants [21], but also from the reduction of the glycolytic rate and subsequent acidosis [13-14].

Marked Ser^{221}-ACCβ phosphorylation despite lack of Thr^{172}-AMPKα phosphorylation in response to sprint exercise.

Although Ser^{221}-ACCβ phosphorylation is a downstream kinase for AMPK [47], the present investigation confirms previous studies showing a dissociation between Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylations immediately after sprint exercise [4-6]. Thus alternative Ser^{221}-ACCβ phosphorylation mechanism must be recruited during sprint exercise [48].As a novelty, this study indicates that Ser^{221}-ACCβ phosphorylation does not depends on

free radicals, which if anything could attenuate ACCβ phosphorylation.

In summary, we have shown that the expected Thr^{172}-AMPKα phosphorylation response to sprint exercise is blunted when the sprint is performed in severe acute hypoxia by a mechanism independent of oxidative stress likely involving Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation by Akt. Sprint exercise-elicited Thr^{286}-CaMKII phosphorylation is in part mediated by a free radicals depending mechanism. However, neither CaMKII nor TAK1 appear to explain the abrogation of Thr^{172}-AMPKα phosphorylation during sprint exercise in hypoxia. We have also shown that antioxidants reduce the glycolytic rate elicited by sprint exercise in human skeletal muscle, attenuating the increases of the AMP/ATP and NAD^+/$NADH.H^+$ ratios, which are critical signaling triggers in skeletal muscle. Interestingly, the reduction in the glycolytic rate after the ingestion of antioxidants was without any negative effect on performance. New experiments will be needed to determine whether the antioxidants improve the energy efficiency during sprint exercise.

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Disclosures

The authors have no conflict of interest to disclose.

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Table 1. Physical characteristics and ergoespirometric variables during sprint exercise in hypoxia placebo and hypoxia antioxidants (mean±SD).

	Hypoxia Placebo	Hypoxia Antioxidants
Age (years)	25.2 ± 4.7	
Height (cm)	176.0 ± 5.1	
Weight (Kg)	79.4 ± 10.1	
% body fat	18.3 ± 6.6	
Two-Legs lean mass	19.73 ± 2.37	
HRmax (Beats/min)	172.0 ± 9.7	
VO_2peak (L/min)	2.586 ± 0.208	
Wmax (W)	285.2 ± 32.1	
Wpeak (W)	944.3 ± 131.3	999.1 ± 129.0
Wpeak/Kg LLM	48.5 ± 3.7	51.6 ± 6.2
Wmean (W)	539.8 ± 69.0	546.5 ± 64.2
Wmean/Kg LLM	27.8 ± 1.9	28.1 ± 1.7
O_2 Demand (L/min)	7.830 ± 0.765	7.922 ± 0.762
Accumulated VO_2 (L)	0.847 ± 0.123	0.830 ± 0.175
O_2 deficit (L)	3.068 ± 0.399	3.131 ± 0.430
O_2 deficit/Wmean	5.70 ± 0.45	6.09 ± 0.82
La peak (mmol/L)	11.9 ± 2.0	11.7 ± 1.5
Wingate SpO_2	80.9 ± 7.0	78.1 ± 6.1
Wingate $P_{ET}O_2$	48.8 ± 3.3	49.4 ± 4.7

Wmax: maximal intensity during the incremental exercise test to exhaustion; Wpeak: peak power output during the Wingate test; LLM: lean mass of the lower extremities; Wmean: mean power output during the Wingate test; Accumulated VO_2: amount of O_2 consumed during the 30s Wingate test; SpO_2: Hemoglobin saturation (pulsoximetry); $P_{ET}O_2$: end tidal O_2 pressure.

Table 2. Glucose and Insulin concentrations prior to and during the recovery period after the sprint exercise in hypoxia placebo and hypoxia antioxidants (mean ± SD).

		R	0 min	30 min	120 min	
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Interaction
Glucose (mg/dL)	Hyp. Placebo	88.3 ± 4.5	100.4±11.9 $	102.8 ± 13.0 $	87.9 ± 7.2	P=0.38
	Hyp. Antioxidants	91.5 ± 6.9	99.0±7.3 $	99.2 ± 10.7 $	87.02 ± 7.2	
Insulin (µIU/mL)	Hyp. Placebo	5.4 ± 3.3	5.6 ± 3.0	11.5 ± 6.1 $	6.1 ± 4.2	P= 0.07
	Hyp. Antioxidants	6.2 ± 5.0	8.7 ± 5.3$	14.8 ± 11.5 $	6.2 ± 3.9	

0 min corresponds to immediately after the Wingate test. $ P< 0.05 versus resting (R) values.

Table 3. Muscle metabolites before and immediately after a 30-second sprint in hypoxia placebo and hypoxia antioxidants (mean ± SD).

	Hypoxia Placebo				Hypoxia Antioxidants			
	Resting		Post-sprint		Resting		Post-sprint	
	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
ATP (mmol/Kg)	4.84 ± 1.00		3.2 ± 1.63 $		5.17 ± 1.17		3.11 ± 0.81 $	
AMP/ATP (mmol/mol) bc	5.5 ± 2.3		184.2 ± 255.4 $		3.7 ± 2.5		25.0 ± 16.6 e$	
PCr (mmol/Kg)	16.50 ± 1.52		5.99 ± 3.32 $		18.28 ± 2.28		8.26 ± 2.76 $	
Cr (mmol/Kg)	11.92 ± 1.52		22.42 ± 3.32 $		10.20 ± 2.28		21.61 ± 3.13 $	
Pyruvate (mmol/Kg) b	0.14 ± 0.05		0.23 ± 0.07 $		0.09 ± 0.04		0.17 ± 0.07 $a	
Lactate (mmol/Kg) b	2.1 ± 1.3		55.4 ± 20.1 $		3.1 ± 2.0		38.3 ± 10.6 $ a	
Lac/Pyr b d	17.1 ± 14.3		258.4 ± 120.4 $		38.6 ± 24.8		250.5 ± 110.1$	
[NAD+]/([NADH.H+]) x10^{7} bd	897 ± 667		42 ± 21 $		339 ± 262		42 ± 16 $a	

$ Post-sprint vs. resting (same condition); a P<0.05 Post-sprint in hypoxia placebo versus hypoxia antioxidants.

b P<0.05 for Antioxidants x time interaction; c AMP/ATP ratio calculated from the CK and AK apparent equilibrium constants for free AMP and ADP; d Statistical analysis performed with normalized values, taking the resting values as 100%; e Relative increase between

Legends to figures

Figure 1. Levels of carbonylated proteins in plasma (A) and skeletal muscle (B) before and after a single Wingate test performed in hypoxia placebo (open bars) or hypoxia antioxidants (solid bars). Values in 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 and densitometry analysis (130 and 30 KDa) showing carbonylated proteins in plasma extracts. Statistical analysis performed with logarithmically transformed data. * $P<0.05$ Hypoxia placebo versus hypoxia antioxidants .B) Western blot and densitometry analysis (45 and 35 KDa) showing carbonylated proteins in skeletal muscle extracts. Statistical analysis performed with logarithmically transformed data. * $P<0.05$ Hypoxia placebo versus hypoxia antioxidants. N = 9 in both experimental conditions.

Figure 2. Levels of Thr^{172}-AMPKα (A), Ser^{221}-ACCβ (B), Thr^{286}-CaMKII (C), and $Thr^{184/187}$-TAK1 (D) before and after a single Wingate test performed in hypoxia placebo (open bars) or hypoxia antioxidants (solid bars). Values in 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 panel*: a representative western blot with antibodies against AMPKα, pThr^{172}-AMPKα and α-Tubulin. *Lower panel*: pThr^{172}-AMPKα densitometric values relative to total AMPKα. Statistical analysis performed with logarithmically transformed data. B) *Top panel*: a representative western blot with antibodies against ACCβ, phospho-ACCβ and α-Tubulin. *Lower panel*: ACCβ phosphorylation values relative to total ACCβ. $^{\$}$ P<0.05 versus R. Statistical analysis performed with logarithmically transformed data. C) *Top panel*: a representative western blot with antibodies against CaMKII, phospho-CaMKII and α-Tubulin. *Lower panel*: CaMKII phosphorylation values relative to total CaMKII. * P<0.05 Hypoxia placebo versus hypoxia antioxidants. Statistical analysis performed with logarithmically transformed data. D) *Top panel*: a representative western blot with antibodies against TAK1, phospho-TAK1 and α-Tubulin. *Lower panel*: TAK1 phosphorylation values relative to total TAK1. N = 9 in both experimental conditions.

Figure 3. Levels of Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 (A), Ser^{485}-AMPKα1 (B), and SIRT1 (C) before and after a single Wingate test performed in hypoxia placebo (open bars) or hypoxia antioxidants (solid bars). Values in 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 panel*: a representative western blot with antibodies against AMPKα, phospho-Ser^{485}-α1/Ser^{491}-α2 and α-Tubulin. *Lower panel*: Ser^{485}-α1/Ser^{491}-α2 phosphorylation values relative to total AMPKα. $^{\$}P<0.05$ versus R .Statistical analysis performed with logarithmically transformed data. B) *Top panel*: a representative western blot with antibodies against AMPKα, phospho-Ser^{485}-α1 and α-Tubulin. *Lower panel*: Ser^{485}-AMPKα1 phosphorylation values relative to total AMPKα1. Statistical analysis performed with logarithmically transformed data. C) *Top panel*: a representative western blot with antibodies against SIRT1 and α-Tubulin. Statistical analysis performed with logarithmically transformed data. *Lower panel*: SIRT1 values. N = 9 in both experimental conditions.

Figure 4. Levels of Ser^{473}-Akt (A), Thr^{308}-Akt (B), and Thr^{642}-AS160 (C) before and after a single Wingate test performed in hypoxia placebo (open bars) or hypoxia antioxidants (solid bars).Values in 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 panel*: a representative western blot with antibodies against Akt, phospho-Ser^{473}-Akt and α-Tubulin. *Lower panel*: Ser^{473}-Akt phosphorylation values relative to total Akt. $^{\$}P<0.05$ versus R. Statistical analysis performed with logarithmically transformed data. B) *Top panel*: a representative western blot with antibodies against Akt, phospho-Thr^{308}-Akt and α-Tubulin. *Lower panel*: Thr^{308}-Akt phosphorylation densitometric values relative to total Akt. $^{\$}$ $P<0.05$ versus R.* $P<0.05$ Hypoxia placebo versus hypoxia antioxidants. Statistical analysis performed with logarithmically transformed data. (C) *Top panel*: a representative western blot with antibodies against AS160, phospho-Thr^{642}-AS160 and α-Tubulin. *Lower panel*: Thr^{642}-AS160 phosphorylation values relative to total AS160. $^{\$}P<0.05$ versus R . N = 9 in both experimental conditions.

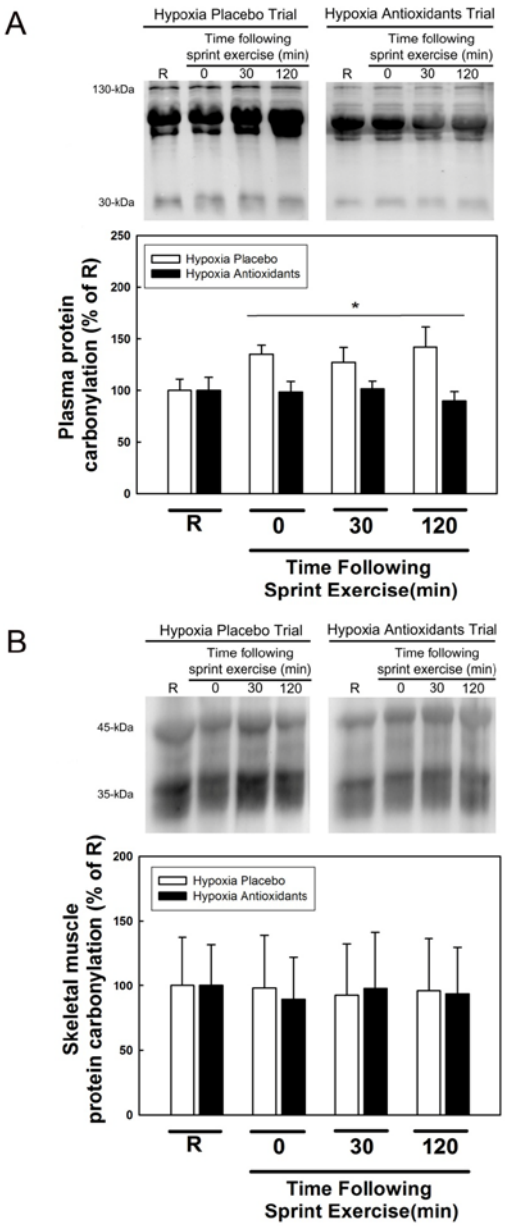
A
Hypoxia Placebo Trial
Hypoxia Antioxidants Trial
Time following sprint exercise (min)
R
0
30
120
130-kDa
30-kDa
Hypoxia Placebo
Hypoxia Antioxidants
Plasma protein carbonylation (% of R)
*
Time Following Sprint Exercise(min)
B
45-kDa
35-kDa
Skeletal muscle protein carbonylation (% of R)

Figure 1.

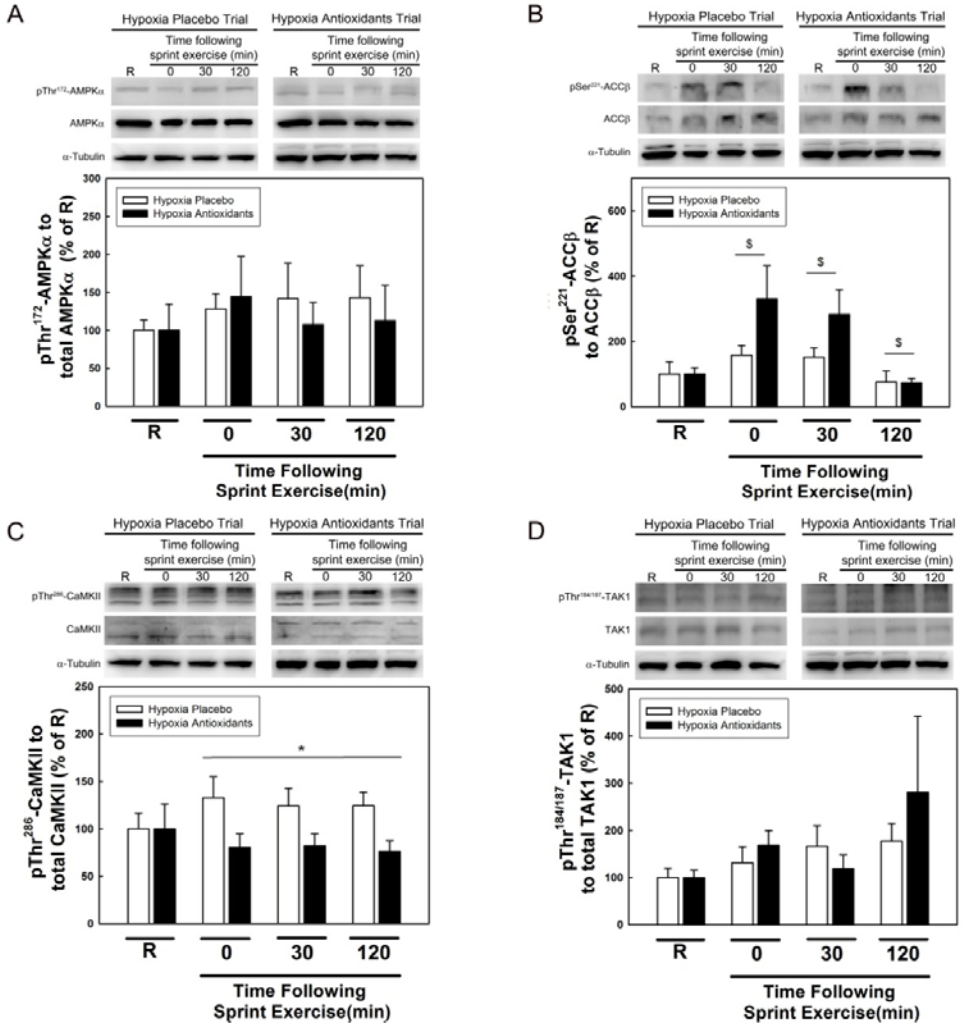
A
Hypoxia Placebo Trial
Hypoxia Antioxidants Trial
Time following sprint exercise (min)
R 0 30 120
pThr172-AMPKα
AMPKα
α-Tubulin
Hypoxia Placebo
Hypoxia Antioxidants
pThr172-AMPKα to total AMPKα (% of R)
Time Following Sprint Exercise(min)
B
pSer221-ACCβ
ACCβ
pSer221-ACCβ to ACCβ (% of R)
$
C
pThr286-CaMKII
CaMKII
pThr286-CaMKII to total CaMKII (% of R)
*
D
pThr184/187-TAK1
TAK1
pThr184/187-TAK1 to total TAK1 (% of R)

Figure 2.

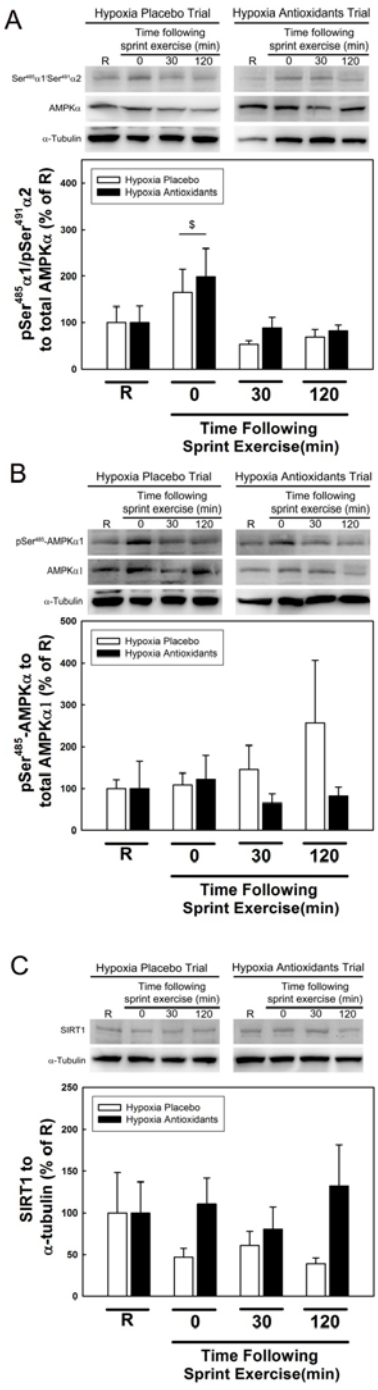
A
Hypoxia Placebo Trial
Hypoxia Antioxidants Trial
Time following sprint exercise (min)
R 0 30 120
Ser485α1/Ser491α2
AMPKα
α-Tubulin
Hypoxia Placebo
Hypoxia Antioxidants
$
pSer485α1/pSer491α2 to total AMPKα (% of R)
Time Following Sprint Exercise(min)
B
pSer485-AMPKα1
AMPKα1
pSer485-AMPKα to total AMPKα1 (% of R)
C
SIRT1
SIRT1 to α-tubulin (% of R)

Figure 3.

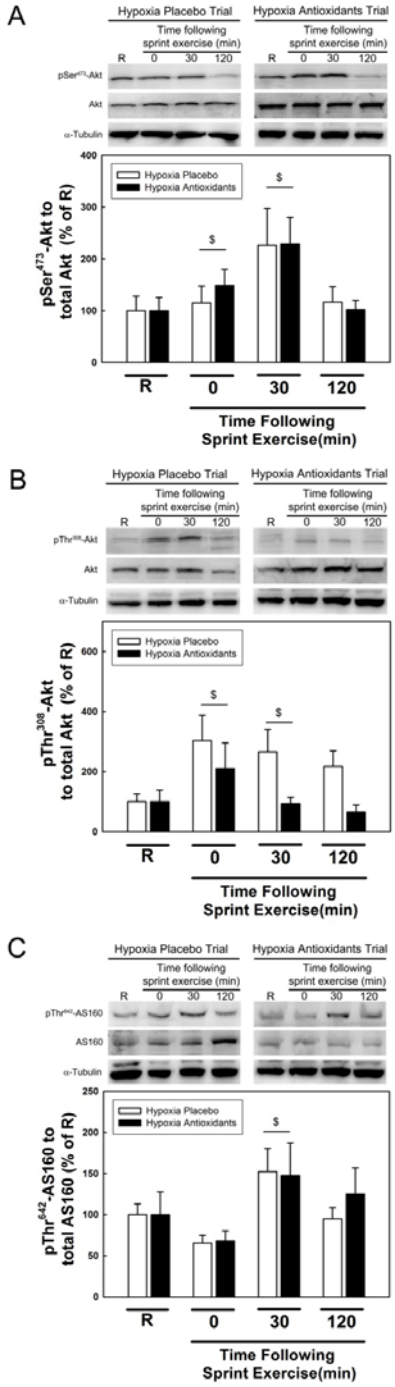
A
Hypoxia Placebo Trial
Hypoxia Antioxidants Trial
Time following sprint exercise (min)
R 0 30 120
pSer473-Akt
Akt
α-Tubulin
Hypoxia Placebo
Hypoxia Antioxidants
pSer473-Akt to total Akt (% of R)
$
R 0 30 120
Time Following Sprint Exercise(min)
B
Hypoxia Placebo Trial
Hypoxia Antioxidants Trial
Time following sprint exercise (min)
R 0 30 120
pThr308-Akt
Akt
α-Tubulin
Hypoxia Placebo
Hypoxia Antioxidants
pThr308-Akt to total Akt (% of R)
$
R 0 30 120
Time Following Sprint Exercise(min)
C
Hypoxia Placebo Trial
Hypoxia Antioxidants Trial
Time following sprint exercise (min)
R 0 30 120
pThr642-AS160
AS160
α-Tubulin
Hypoxia Placebo
Hypoxia Antioxidants
pThr642-AS160 to total AS160 (% of R)
$
R 0 30 120
Time Following Sprint Exercise(min)

Figure 4.