Ph.D. Thesis

MARCOS MARTÍN RINCÓN

PREVENTION OF MUSCLE MASS LOSS DURING SEVERE ENERGY DEFICIT BY EXERCISE AND PROTEIN INGESTION: MOLECULAR MECHANISMS

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PREVENCIÓN DE LA PÉRDIDA DE MASA MUSCULAR DURANTE EL DÉFICIT ENERGÉTICO SEVERO MEDIANTE EJERCICIO Y SOLUCIONES PROTEICAS: MECANISMOS MOLECULARES

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INFORMA:

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha 27 de julio de 2018 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada ***"Prevención de la pérdida de masa muscular durante el déficit energético severo mediante ejercicio y soluciones proteicas: mecanismos moleculares"*** presentada por el doctorando **D. Marcos Martín Rincón** y dirigida por los **Dres. José Antonio López Calbet y David Morales Álamo.**

Que la citada tesis doctoral reúne todos los requisitos exigidos por la normativa de este programa de doctorado y de esta universidad, para ser tramitada como tesis doctoral con mención internacional.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo el presente informe en Las Palmas de Gran Canaria, a veintisiete de julio de dos mil dieciocho

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Dirigida por el Dr. D. José Antonio López Calbet

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DEDICATION / DEDICATORIA

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1. LIST OF SCIENTIFIC PAPERS

The present thesis will be based on the three publications listed below, which will be referred to throughout the sections by their Roman numerals:

Article I (Martin-Rincon et al. 2018)

***Marcos Martin-Rincon**, Ismael Perez-Suarez, Alberto Pérez-López, Jesús Gustavo Ponce-Gonzále[1], David Morales-Alamo, Pedro de Pablos-Velasco, Hans-Christer Holmberg, Jose A.L. Calbet.*

Protein synthesis signalling in skeletal muscle is refractory to whey protein ingestion during a severe energy deficit evoked by prolonged exercise and caloric restriction. *Int J Obesity*, recently accepted on 25th June 2018, doi: 10.1038/s41366-018-0174-2.

Article II (Ström et al. 2018)

*Kristoffer Ström, David Morales-Álamo, Filip Ottosson, Anna Edlund, Line Hjort, Sine W. Jörgensen, Peter Almgren, Yuedan Zhou, **Marcos Martin-Rincon**, Carl Ekman, Alberto Pérez-Lopez, Ola Ekström, Ismael Pérez-Suárez, Markus Mattiasson, Pedro de Pablos-Velasco, Nikolay Oskolkov, Nils Wierup, Lena Eliasson, Allan Vaag, Leif Groop, Karin Stenkula, Céline Fernandez, Jose A. L. Calbet, Hans-Christer Holmberg, and Ola Hansson.*

N1-methylnicotinamide is a signalling molecule produced in skeletal muscle coordinating energy metabolism. *Scientific Reports* **8**, 3016, doi:10.1038/s41598-018-21099-1 (2018).

Article III (Martin-Rincon et al. 2017)

***Marcos Martin-Rincon**, David Morales-Alamo, Jose A.L. Calbet.*

Exercise-mediated modulation of autophagy in skeletal muscle. *Scand J Med Sci Sports*. 2018 Mar 28, 772-781, doi:10.1111/sms.12945 (2018). Epub 2017 Aug

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3. LIST OF SYMBOLS AND ABBREVIATIONS

Akt: Protein kinase B (PKB)

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

Atg: Autophagy-related

ATP: Adenosine triphosphate

BCAA: Branched-chain amino acid

BCL2: B-cell lymphoma 2

BNIP3: BCL-2/adenovirus E1B nineteen-kilodalton interacting protein 3

CTSD: cathepsin D

DNA: Deoxyribonucleic acid

DXA: Dual energy X-ray absorptiometry

EAA: Essential amino acid

eIF2α: Eukaryotic initiation factor 2 alpha

FAI: Free androgen index

FDR: False discovery rate

FFM: Fat-free mass

FM: Fat mass

FoxOs: Forkhead boxO family of transcription factors

GSK3β: Glycogen synthase kinase 3 beta

HPLC: Reversed-phase high-performance liquid chromatography

IPAQ: International physical activity questionnaire

kcal: Kilocalorie

kDa: Kilodalton

kJ: Kilojoule

LCD: Low calorie diet

LC3B: microtubule-associated protein 1A/1B-light chain 3

LM: Lean mass

MAFbx: muscle atrophy Fbox protein, also atrogin-1

min: minute/s

mRNA: messenger ribonucleic acid

mTOR: Mammalian target of rapamycin

MuRF1: Muscle RING finger-containing protein 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

PCR: Polymerase chain reaction

PI3K: Phosphatidylinositol 3-kinase

PSMD4: 26S proteasome non-ATPase regulatory subunit 4

p62: Sequestosome 1, also SQSTM1

p70S6K: p70 ribosomal S6 kinase, also S6K1

RDA: Recommended Dietary Allowance

RHEB: Ras homolog enriched in brain

SDS: Sodium dodecyl sulphate

Ser: Serine

SHBG: Sexual hormone-binding globulin

SH3GLB1: SH3 domain containing GRB2 like endophilin B1 gene, also Bif-1

Thr: Threonine

TP53INP2: Tumour protein p53 inducible nuclear protein 2

TRIM7: tripartite motif containing 7

TSC2: Tuberous sclerosis complex 2

Tyr: Tyrosine

ULK: unc-51-like kinase

VLCD: Very low calorie diet

µg: microgram

4E-BP1: eukaryotic translation initiation factor 4E-binding protein 1

SUMMARY

4. SUMMARY

Background: Overweight and obesity elevate the risk of many several diseases and poor health conditions, including cardiovascular disease, Type 2 diabetes and cancer, being considered as the epidemic of the twenty-first century. Nonetheless, obesity can be counteracted by reducing energy intake and/or increasing energy expenditure thereby achieving a negative energy balance. Combining exercise and diet further increases the effect of diet alone in weight loss ~0.3-1.1 kg, holding together or separately the capacity of reversing their associated metabolic alterations. Despite the existence of other strategies to generate a loss of weight, it has been shown that very low calorie diets (VLCD, <800 kcal/day) are more effective to induce a loss of fat and to maintain it over time. Intensified protocols can provoke fast reductions in body weight loss by a combination of long-lasting walking protocols and moderate energy restriction during short periods (3-10 days).

However, during weight loss regimes, concomitantly with fat mass (FM) there is marked reduction in fat-free mass (FFM) and particularly skeletal muscle, being ~25% of the total body weight loss achieved. The loss of FFM is higher with VLCDs than low calorie diets (LCDs, ~1000 kcal/day less than normal), representing a greater fraction of the total weight loss. Developing strategies to preserve muscle mass during interventions designed to reduce body weight holds crucial clinical relevance. Exercising while following LCD attenuates the loss of FFM, although data are limited on the effects of exercise to prevent or attenuate

muscle mass loss when the energy deficit is severe (i.e. combination of VLCD and high volume of exercise).

Endocrine signals play a crucial role on the changes in body weight and composition. An increase in testosterone stimulates muscle protein synthesis and muscle mass, while cortisol induces an increase in protein breakdown. Leptin can act as a sensor of energy availability while insulin induces an anabolic and anti-catabolic effect in muscle. During a severe energy deficit, cortisol increases while testosterone levels are reduced. Concomitantly, insulin levels are lowered, insulin resistance within the skeletal muscles is augmented and leptin levels may also be reduced at least with short-term energy restriction. These are the main reasons why a loss in muscle mass presents in a situation of severe energy restriction and therefore a ~10-40% of the total weight loss is FFM, primarily muscle mass.

During hypocaloric diets, increasing the ratio of protein to carbohydrate has been shown as an effective strategy to prevent the loss of FFM. Better preservation of lean mass and greater fat mass losses can be achieved by when the exercise is combined with a high intake of proteins (2-3-fold the recommended dietary allowance for protein (0.8 g/kg body mass)) than with lower amounts. Essential amino acids and particularly leucine are powerful activators of protein synthesis and exercise enhances their anabolic effect. It remains unknown whether a higher fraction or total protein content of the diet during a VLCD enables an attenuation of the muscle mass losses.

Protein turnover, the balance between protein synthesis and protein degradation, is affected by the degree of energy balance. During a situation of

energy deficit, there is a reduction in protein synthesis and increased in proteolysis, generating an overall negative protein balance and loss of skeletal muscle mass. Exercise and/or increasing the protein ratio in the diet attenuate the reduction in protein synthesis driven by negative energy balance, but this has only been investigated during moderate energy deficits and/or combined with resistance exercise. No studies have addressed protein synthesis and/or protein degradation responses during a severely deficient energy state and neither using low-intensity (aerobic) exercise in combination.

The signalling pathways coordinating protein synthesis and degradation interact in a highly complex and dynamic intracellular network with a constant crosstalk of signals that eventually regulate and control hypertrophic and atrophic events. Physical exercise and nutrient availability influence these signals as well as their interdependency. Protein synthesis at the molecular level is principally regulated by the Akt/mTor signalling pathway. The main degradation pathways in skeletal muscle are the autophagy-lysosomal and the ubiquitin-proteasome systems, which compose a complex framework of catabolic messages modulating one another and inseparably linked to energy availability.

In spite of the compelling number of studies demonstrating the protective effect of exercise and/or protein consumption in FFM loss during energy deficit, these effects have not been studied when the energy deficiency is high. Furthermore, the interaction between the transcriptional and molecular pathways driving protein synthesis and protein degradation during energy deficit are poorly

understood and no study has investigated these responses in a situation of severe energy deficiency.

The main objectives of this thesis are to study the influence of low-intensity exercise and/or protein ingestion on the regulation of muscle mass during severe energy deficit and to determine the underlying molecular mechanisms and key genes mediating this response in human skeletal muscle of overweight men.

Methods: Fifteen overweight, but otherwise healthy men underwent a pre-test (PRE); caloric restriction (3.2 kcals/kg body weight/day) + exercise (45 min one-arm cranking + 8h walking) for 4 days (CRE); followed by a control diet (CD) for 3 days, with a caloric content similar to pre-intervention while exercise was reduced to less than 10.000 steps per day. During CRE, participants ingested either whey protein (PRO, n=8) or sucrose (SU, n=7) (0.8 g/kg body weight/day). Muscle biopsies were obtained from the trained and untrained deltoid, and *vastus lateralis*. Blood samples were drawn after each experimental phase for hormonal and biochemical analysis. Analysis of levels of expression of individual proteins in muscle biopsies were obtained by immunoblotting (western blot). Changes in skeletal muscle gene expression were measured by microarray analysis.

Results: Following CRE and CD, serum concentrations of leptin, insulin, and testosterone were reduced, whereas cortisol and the catabolic index (cortisol/total testosterone) were increased. The Akt/mTor/p70S6K pathway and total eIF2α were unchanged, while total 4E-BP1 and $Thr^{37/46}$4E-BP1 were higher. After CRE, plasma BCAA and EAA were elevated, with a greater response in PRO group, and total GSK3β, $pSer^{9}$GSK3β, $pSer^{51}$eIF2α and $pSer^{51}$eIF2α/total eIF2α were reduced,

with a greater response of pSer^{9}GSK3β in the PRO group. The changes in signalling were associated with the changes in leptin, insulin, amino acids, cortisol, cortisol/total testosterone and lean mass. Muscle gene expression changed similarly with both diets. In the non-exercised and exercised arm, 39 and 44 genes were differentially expressed between PRE and CRE (FDR < 5%), respectively, without significant differences between them in either PRE, CRE or CD.

In the leg, 421 genes (FDR < 5%) were differentially expressed. A large proportion of the identified changes in gene expression induced by the four-day intervention was reversed by the three-day period with isoenergetic diet and limited exercise. The level of expression of a number of atrogenes involved in the ubiquitin-proteasome and the autophagy lysosomal pathways was upregulated after CRE, primarily in the legs. From PRE to CRE, six autophagy genes (e.g. *BNIP3, p62/SQSTM1, SH3GLB1*) were upregulated in the legs while one gene (*PARP1*) was downregulated. A single gene (*TP53INP2*) was downregulated in the non-exercised arm and none in the exercised arm (FDR < 5%). Six genes involved in the ubiquitin proteasome (*TRIM63/MuRF1* and *PSMD4*) were upregulated in the legs while one gene (*TRIM7*) was downregulated in the exercised arm from PRE to CRE. From CRE to CD, three ubiquitin-proteasome-related atrogenes were still upregulated (*TRIM63/MuRF1, RHEB and CTSD*) in the legs while the remaining upregulated genes were reversed in all extremities by the subsequent phase of isoenergetic diet and limited exercise (FDR < 5%).

Discussion: Protein synthesis through the axis Akt/mTor/p70S6K remained unchanged after the intervention, regardless of contractile activity. A partial

stimulation of protein synthesis was seen by elevated total 4E-BP1 and $Thr^{37}/^{46}$4E-BP1 and reductions in $pSer^{51}$eIF2α and the ratio $pSer^{51}$eIF2α/total eIF2α following CRE in all muscles. However, this was counteracted by the lowered levels of $pSer^{9}$GSK3β which might have increased the inhibitory action of GSK3β. This enhanced inhibitory action of GSK3β was higher in the protein group, which may explain why whey protein did not exert additional effects on protein synthesis and the preservation of muscle mass, which was similar in both groups. An inverse association between total 4E-BP1 and testosterone and free testosterone as well as between $pSer^{9}$GSK3β and total testosterone. The increase in $pSer^{51}$eIF2α from PRE to CRE was associated with the changes in cortisol. The elevated levels of cortisol following CRE may act simultaneously inhibiting protein synthesis via 4E-BP1 and its downstream p70S6K and increasing protein degradation through reducing the intake of amino acids by muscle cells and reducing phosphorylation of PI3K and Akt which in turn may activate FoxO family of transcription factors. A strong association was found between serum leptin concentration and the mean protein expression of total Akt, total GSK3β and $pSer^{9}$GSK3β in the three analysed muscles, which may suggest a role of leptin in easing a potential anabolic response of skeletal muscles upon re-feeding. These associations suggest a critical role of cortisol/testosterone in protein synthesis modulation.

Upregulation of expression levels of key genes encoding ubiquitin-ligases were found in the present study after the four-day intervention, as primarily shown by the augmented expression of *TRIM63/MuRF-1* and *PSMD4* in the legs. These genes have been shown to be upregulated during atrophic conditions and are associated with muscle catabolism. Low levels of insulin and elevated levels of

glucocorticoids trigger the activation of proteolysis and several models of muscle loss through activation of the ubiquitin-proteasome systems and therefore may have driven this upregulation. Similarly, we found changes in the expression levels of genes involved directly or indirectly in the autophagy-lysosomal system following the four days of severe energy restriction and prolonged low-intensity exercise. Primarily, the upregulation of *BNIP3* and *SH3GLB1*S support an increase in autophagy induction and autophagosome formation. On the contrary, the expression of *p62/SQSTM1* was upregulated. Accumulation of p62/SQSTM1 protein has been used as a marker of lysosomal degradation inhibition. Similarly, *TP53INP2* expression was downregulated. TP53INP2 protein has been shown to be necessary for complete autophagosome development, which together with the changes in *p62/SQSTM1* may suggest a blockade in autophagic flux. These results in combination with unpublished protein expression data fit well with studies showing that, after prolonged endurance exercise protocol (~>2 h), there is an increase in autophagy induction but the autophagic flux is blocked. We speculate with a plausible protein-sparing mechanism present in humans under high levels of cellular stress driven by energy deficiency and enormous levels of contractile activity.

Our findings showing anabolic resistance to whey protein ingestion during severe energy deficiency are in line with a recent article where protein synthesis was measured using stable isotopes during a 10-day low-calorie diet (40%-reduced energy intake). Protein synthesis was not altered by the administration of 3-fold the amount of protein given here.

Conclusions: This investigation demonstrates that exercise exerts a muscle mass sparing effect during a severe energy deficit in an exercise-dose dependent manner, where the reduced levels of plasma, testosterone, leptin and insulin blunt protein synthesis and translation initiation, primarily by a reduced phosphorylation of GSK3β at Ser^{9}, which constitutes a novel mechanism in human skeletal muscle under such conditions. An upregulation of the two principal proteolytic systems in skeletal muscle, the ubiquitin-proteasome and the autophagy-lysosomal systems, suggests a state of catabolism elicited primarily by the prolonged contractile activity executed by the legs. The ingestion of a diet consisting in solely whey protein or sucrose does not cause any additional muscle-sparing effect in skeletal muscle and does not affect the molecular regulation of protein synthesis nor protein degradation markers in human skeletal muscle under conditions of severe energy deficiency, despite of the volume of contractile activity performed.

RESUMEN GENERAL

(SUMMARY IN SPANISH)

5. RESUMEN GENERAL

Estado del tema: La obesidad y el sobrepeso elevan el riesgo de padecer un alto número de enfermedades crónicas, incluyendo enfermedades cardiovasculares, diabetes tipo 2 y cáncer y siendo considerada la epidemia del siglo XXI. Sin embargo, la obesidad puede ser contrarrestada a través de la reducción de la ingesta energética y/o el incremento del gasto energético, adquiriendo así un balance energético negativo. La combinación de ejercicio y dieta incrementa el efecto aislado de la dieta en la pérdida de peso en ~0.3-1.1 kg y ambos, de manera conjunta o separada son capaces de revertir las alteraciones metabólicas asociadas. A pesar de la existencia de otras estrategias para generar una pérdida de peso, se ha mostrado que las dietas muy bajas en calorías (<800 kcal/día) son más efectivas para inducir una pérdida de grasa y mantenerla en el tiempo. Los protocolos intensificados pueden provocar reducciones rápidas en el peso corporal a través de una combinación de caminatas de larga duración y un déficit energético moderado durante períodos cortos de tiempo (3-10 días).

No obstante, paralelamente a la pérdida de masa grasa, hay una marcada reducción de masa libre de grasa y particularmente músculo esquelético, el cual representa un ~25% del total de la pérdida de peso alcanzada. Durante dietas muy bajas en calorías, la pérdida de masa libre de grasa es superior a aquella obtenida con dietas bajas en calorías (~1000 kcal/día), en términos relativos a la pérdida total de peso. Desarrollar estrategias para preservar la masa muscular durante intervenciones diseñadas para perder peso dispone de una crucial importancia clínica. La realización de ejercicio físico durante dietas bajas en calorías atenúa la

pérdida de masa libre de grasa, sin embargo, la evidencia es muy limitada en sus efectos preservadores de masa magra cuando el déficit energético es severo, como, por ejemplo, durante la combinación de una dieta muy baja en calorías con un alto volumen de ejercicio.

Las señales endocrinas juegan un papel fundamental en los cambios en el peso y la composición corporal. El incremento de la testosterona estimula la síntesis de proteínas y la masa muscular, mientras que el cortisol induce un incremento en la degradación de proteínas. La leptina puede actuar como sensor de la disponibilidad de energía mientras que la insulina induce un efecto anabólico y anticatabólico en el músculo esquelético. En una situación de déficit energético severo, el cortisol aumenta mientras que los niveles de testosterona se reducen. Simultáneamente, los niveles de insulina bajan y la resistencia a la insulina es aumentada mientras los niveles de leptina son también disminuidos, al menos durante la restricción calórica de corta duración. Estas son las razones principales por las cuales hay una pérdida de masa libre de grasa (de un 10 a un 40% de la pérdida de peso) durante una situación de déficit energético severo.

Durante una dieta hipocalórica, incrementar la ratio de proteínas respecto a carbohidratos se ha mostrado como una estrategia efectiva para prevenir la pérdida de masa libre de grasa. Una mayor preservación de masa magra y una mayor pérdida de grasa pueden lograrse combinando ejercicio con una alta ingesta de proteínas (2-3 veces la cantidad diaria recomendada para proteína (0.8 g/kg peso corporal), comparado con ingestas más bajas en proteínas. Los aminoácidos esenciales y en particular la leucina son potentes activadores de la síntesis de

proteínas, y el ejercicio mejora este efecto anabólico. Se desconoce si una mayor fracción de proteínas en el contenido total de calorías durante una dieta muy baja en calorías permite una disminución de la pérdida de masa muscular.

La tasa de recambio de proteínas o balance entre la síntesis y la degradación de proteínas se ve afectada por el grado en el que se encuentre el balance energético. En un déficit energético severo, hay una reducción de la síntesis de proteínas y un incremento de la degradación o proteólisis, generando un balance proteico negativo global y la pérdida de masa de músculo esquelético. El ejercicio y/o el incremento de la ratio de proteínas en la dieta atenúan la reducción en la síntesis de proteínas desencadenada por un balance energético negativo, pero esto sólo ha sido investigado en déficits energéticos moderados y/o en combinación con ejercicio de fuerza. No existen estudios que hayan abordado la respuesta en la síntesis y/o la degradación de proteínas durante un estado de déficit energético severo ni tampoco cuando este es combinado con ejercicio de baja intensidad (aeróbico).

Las vías de señalización que coordinan la síntesis y la degradación de proteínas interaccionan en una red intracelular altamente compleja y dinámica con constante comunicación cruzada entre señales que regulan y controlan los eventos hipertróficos y atróficos en el músculo. El ejercicio físico y la disponibilidad de nutrientes influyen en estas señales, así como en su interdependencia. A nivel molecular, la síntesis de proteínas está principalmente regulada por la vía de señalización de Akt/mTor. Las principales vías de degradación de proteínas en el músculo esquelético son las vías de la ubiquitina-proteosoma y la autofagia-lisosoma, que componen un complejo marco de mensajes catabólicos que se

modulan entre ellos y están unidos inseparablemente al estado de disponibilidad energética.

A pesar del convincente número de estudios demostrando el efecto protector del ejercicio y/o el consumo de proteínas para preservar la masa libre de grasa durante un déficit energético, estos efectos no han sido estudiados durante un estado de déficit energético severo. Además, la interacción entre las vías transcripcionales y moleculares que modulan la síntesis y la degradación de proteínas en un déficit energético son escasamente comprendidos y no hay estudios que hayan investigado estas respuestas cuando este déficit es severo.

Los objetivos de esta tesis son el estudio de la influencia del ejercicio de baja intensidad y/o la ingestión de proteínas en la regulación de la masa muscular durante el déficit energético severo y determinar los mecanismos moleculares subyacentes y principales genes dirigiendo esta respuesta en músculo esquelético de varones con sobrepeso.

Métodos: Quince hombres con sobrepeso, pero sin ningún otro problema de salud, llevaron a cabo una fase pre-test (PRE); restricción calórica (3.2 kcal/kg peso corporal/día) + ejercicio (45 min de pedaleo uni-braquial + 8h de caminata) durante cuatro días (CRE); seguido de una fase de dieta control (CD) durante 3 días, con un contenido calórico similar al de la medición pre-intervención mientras el ejercicio físico fue limitado a 10000 pasos al día como máximo. En la fase CRE, los participantes ingirieron o bien una solución de proteína de suero de leche (PRO, n=8) o sacarosa (SU, n=7) (0.8 g/kg peso corporal/día). Se extrajeron biopsias musculares del deltoides entrenado y del deltoides control (no entrenado) y del

vasto lateral de una pierna. Se extrajeron muestras de sangre al final de cada fase para determinaciones analíticas hormonales y bioquímicas. Las expresiones de proteínas individuales en las biopsias musculares fueron medidas por inmunoblot (Western blot). Los cambios en la expresión génica en las biopsias musculares fueron medidas a través de chips de ADN.

Resultados: Después de las fases CRE y CD, la concentración sérica de leptina, insulina y testosterona fueron reducidos, mientras que el cortisol y el índice catabólico (cortisol/testosterona total) aumentaron. La vía Akt/mTor/p70S6K y la expresión de eIF2α total permanecieron sin cambios, mientras que 4E-BP1 total y $Tyr^{37/46}$4E-BP1 aumentaron. Después de CRE, los BCAA y EAA en plasma se elevaron, con una respuesta mayor en grupo PRO, y GSK3β total, $pSer^{9}$GSK3β, $pSer^{51}$eIF2α and $pSer^{51}$eIF2α/total eIF2α se redujeron, con una mayor respuesta de $pSer^{9}$GSK3β en el grupo PRO. Los cambios en la señalización se asociaron con los cambios en leptina, insulina, aminoácidos, cortisol, cortisol/testosterona total y masa magra.

No hubo efectos significativos en la expresión génica debida a la dieta (proteína de suero de leche o sacarosa). En el brazo control y el brazo entrenado, la expresión de 39 y 44 genes se vio alterada entre PRE y CRE (FDR < 5%), respectivamente, sin cambios significativos entre ellos después de PRE, CRE o CD.

En la pierna, 421 genes (FDR < 5%) tuvieron una expresión diferencial tras la intervención. Una larga proporción de los cambios identificados en la expresión génica inducida por los cuatro días de intervención fueron revertidos por los tres días de dieta isoenergética y ejercicio limitado. Los niveles de expresión de varios

atrogenes pertenecientes a las vías de la ubiquitina-protesoma y la autofagia-lisosoma fueron aumentados después de la fase CRE, principalmente en las piernas. De PRE a CRE, seis genes autofágicos (p.ej. *BNIP3*, *p62/SQSTM1*, *SH3GLB1*) aumentaron su expresión en las piernas mientras que sólo un gen disminuyó su expresión (*PARP1*). Un solo gen (*TP53INP2*), redujo su expresión en el brazo control y ninguno en el brazo ejercitado (FDR < 5%). Seis genes de la vía de la ubiquitina-proteosoma (p.ej. *TRIM63/MuRF1* and *PSMD4*) incrementaron su expresión in las piernas, mientras que un gen (*TRIM7*) redujo su expresión en el brazo entrenado de PRE a CRE. De CRE a CD, la expresión de tres genes de la vía ubiquitina-proteosoma se mantuvieron todavía aumentados (*TRIM63/MuRF1*, *RHEB* and *CTSD*) en las piernas mientras que el resto de genes con expresión alterada en CRE fueron revertidos in todos los miembros con la sucesiva fase de dieta control y ejercicio limitado (FDR < 5%).

Discusión: La síntesis de proteínas a través del eje Akt/mTor/p70S6K permaneció sin cambios tras la intervención, independiente del nivel de actividad contráctil muscular. Una estimulación parcial de la síntesis de proteínas fue vista con el aumento de 4E-BP1 total y $Tyr^{37/46}$4E-BP1 y la reducción en $pSer^{51}$eIF2α y la ratio $pSer^{51}$eIF2α/total eIF2α después de CRE en todos los músculos estudiados. No obstante, este efecto fue contrarrestado por la disminución en los niveles de $pSer^{9}$GSK3β, el cual induce un aumento en la acción inhibitoria de GSK3β. Esta incrementada acción inhibitoria de GSK3β fue superior en el grupo que ingirió proteínas, lo cual podría explicar por qué la proteína de suero no produjo un efecto adicional en la síntesis de proteínas ni en la preservación de masa muscular, los cuales fueron similares en los dos grupos. Hubo una correlación inversa

significativa entre los niveles de expresión de 4E- BP1 total, testosterona y testosterona libre, así como entre pSer^{9}GSK3β y testosterona total. El incremento de expresión de pSer^{51}eIF2α de PRE a CRE se correlacionó con los cambios en cortisol. La elevación en los niveles de cortisol después de CRE podría actuar simultáneamente inhibiendo la síntesis de proteínas a través de 4E-BP1 y su objetivo descendente p70S6K e incrementando la degradación de proteínas a través de la reducción de la captación de aminoácidos por las células musculares y reduciendo la fosforilación de PI3K y Akt, los cuales a su vez podrían facilitar la expresión de la familia FoxO de factores de transcripción. Una alta correlación fue encontrada entre los niveles de leptina y la expresión proteica media de Akt, GSK3β total y pSer^{9}GSK3β en los tres músculos analizados, lo que podría sugerir un rol de la leptina en facilitar una potencial respuesta anabólica del músculo esquelético al producirse el cese de la restricción calórica. Estas asociaciones sugieren un rol crítico del eje cortisol/testosterona en la modulación de la síntesis proteica.

En este estudio, se encontró un aumento de la expresión de genes clave que codifican ligasas de la ubiquitina tras la intervención de cuatro días, como es demostrado principalmente por el incremento de la expresión de *TRIM63/MuRF-1* y *PSMD4* en las piernas. Se ha demostrado previamente se produce un aumento en la expresión de estos genes en situaciones de atrofia asociadas al catabolismo muscular. Niveles bajos de insulina y niveles elevados de glucocorticoides desencadenan la activación de la proteólisis y varios modelos de pérdida de masa muscular a través de la activación de la vía de la ubiquitina-proteosoma y que por lo tanto podrían haber generado este aumento en la vía. De modo similar,

encontramos cambios en la expresión de genes envueltos directa o indirectamente en la vía autofágica-lisosómica tras los 4 días de intervención combinando ejercicio prolongado y restricción calórica severa. Principalmente, el incremento en la expresión de *BNIP3* y *SH3GLB1S* apoya un incremento en la inducción de la autofagia y en la formación de autofagosomas. Por el contrario, la expresión de *p62/SQSTM1* fue aumentada. La acumulación de la proteína p62/SQSTM1 se ha usado como marcador de inhibición de la degradación lisosomal. En la misma línea, la expresión de *TP53INP2* mostró una reducción. La proteína TP53INP2 se ha mostrado necesaria para el completo desarrollo autofagosómico, lo que junto con los cambios en expresión de *p62/SQSTM1* podría sugerir un bloqueo en el flujo autofágico. Estos resultados en combinación con datos proteicos no publicados recientemente obtenidos concuerdan con estudios recientes que han mostrado que, tras la realización de ejercicio de resistencia de larga duración (~>2 h), se produce una inducción de la autofagia acompañado de un bloqueo en el flujo autofágico. Nosotros especulamos con un posible mecanismo de ahorro/reservorio proteico presente en humanos al ser sometidos a niveles altos de estrés celular generados por una combinación de una restricción calórica severa y un enorme volumen de actividad contráctil.

Nuestros hallazgos mostrando resistencia anabólica a la ingestión de proteína de suero durante un déficit energético severo van en línea con un reciente artículo que mide la síntesis de proteínas de manera directa mediante isótopos radiactivos durante 10 días de dieta baja en calorías (reducción de un 40% de la dieta habitual). La síntesis proteica no se vio alterada por cantidades de proteína

tres veces superiores a las presentes en la presente investigación, lo cual respalda nuestros hallazgos, aunque durante una restricción calórica moderada.

Conclusiones: Esta investigación demuestra que el ejercicio ejerce un efecto preservador de la masa muscular durante el déficit energético severo de manera dosis-dependiente, donde la reducción en los niveles de plasma, testosterona, leptina e insulina mitigan la síntesis de proteínas y la iniciación de la traducción, primariamente a través de la reducción de la fosforilación de GSK3β en Ser^{9}, lo cual constituye un nuevo mecanismo en músculo esquelético humano bajo estas condiciones. La activación génica de las dos principales vías proteolíticas en el músculo esquelético, la vía de la ubiquitina-proteosoma y de la autofagia-lisosoma, sugieren un estado de catabolismo principalmente provocado por el excepcional volumen de actividad contráctil presente en las piernas. Los resultados muestran no efecto adicional de la ingesta aislada de proteína de suero ni de carbohidratos en la activación de la síntesis de proteínas ni en la activación de las vías proteolíticas en músculo esquelético humano bajo condiciones de déficit energético severo, pese a la inclusión de diferentes volúmenes de actividad contráctil.

INTRODUCTION

6. INTRODUCTION

6.1. THE ROLE OF SEVERE ENERGY DEFICIENCY IN OBESITY - EFFECTS OF EXERCISE

The number of individuals who are overweight or obese has greatly risen over the last two decades and continues increasing worldwide (Sassi, 2010). Overweight and obesity elevates the risk of many chronic diseases and poor health conditions, including cardiovascular disease, Type 2 diabetes and cancer, being described as the epidemic of the twenty-first century (WHO, 2003; James *et al.*, 2004; Prentice, 2006). The total estimated cost of diabetes diagnosed in the U.S. in 2017 was $327 billion, which is an increase by more than 26% in 5 years (Association, 2018).

In Spain, over half of the population aged 25-60 years is overweight (Body Mass Index, BMI $\geq$ 25 kg/m^2) of which ~15% are obese (BMI $\geq$ 30 kg/m^2) which locates it among the higher rates compared to related countries (Aranceta-Bartrina *et al.*, 2005; OECD, 2017). This trend continues growing, despite some mild encouraging signs of slowdown present in adult men, where obesity has stabilised and overweight slightly reduced by ~1.5% between 2011 and 2014 (INE, 2014).

Notwithstanding, obesity can be counteracted by reducing energy intake and/or increasing energy expenditure thereby achieving a negative energy balance (Bray, 1987). Several strategies can be utilised to produce body weight losses.

Dieting alone has been shown to produce on average a moderate weight loss ranging ~0.5-11 kg. Nonetheless, meta-analysis data have estimated that the

combination of diet and exercise further increases this effect by ~0.3-1.1 kg (Garrow & Summerbell, 1995; Miller *et al.*, 1997; Shaw *et al.*, 2009) (Figure 1).

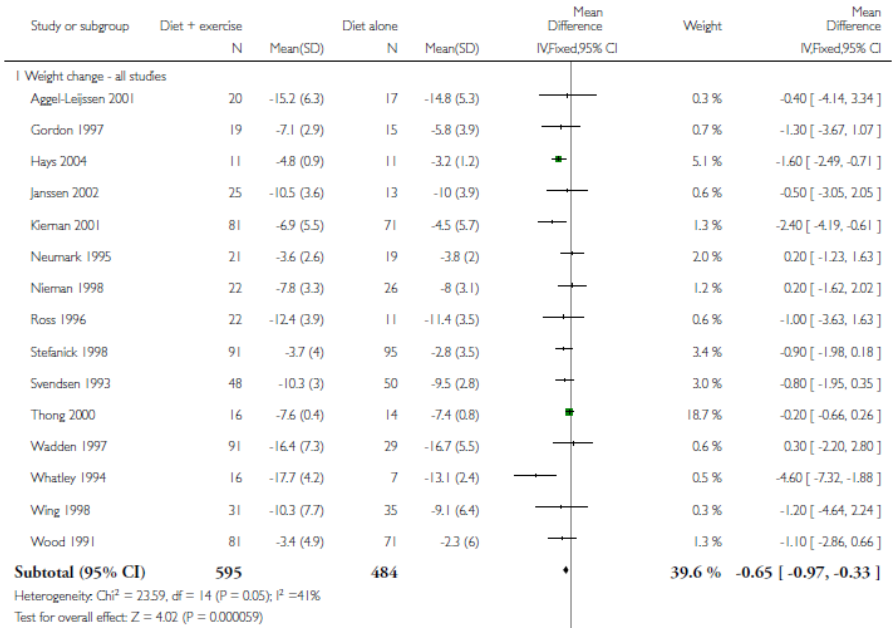


Figure 1. Meta-analysis of studies comparing the effects of exercise plus diet versus diet alone in weight loss. n=1049. Graph obtained from Shaw *et al.* (2009).

Moreover, exercise and/or dieting can totally or partially revert the obesity-related metabolic alterations (Steinberg *et al.*, 2004; Solomon *et al.*, 2008; Goodpaster *et al.*, 2010; Kelly *et al.*, 2011). Other methods such as surgical removal of subcutaneous fat tissue (large-volume liposuction or dermolipectomy) or bariatric surgery (also named weight-loss surgery) have expanded in the last decade. Weight losses achieved by means of lipectomy have presented very limited prevalence over time (Seretis *et al.*, 2015). In the case of bariatric surgery, which aims at reducing the absorptive and digestive capacity of the gastrointestinal tract, its application has become increasingly safe and effective (Jensen *et al.*, 2014),

achieving weight losses of ~15-20% and reported as highly durable in the long-term (Sjostrom, 2013; Shah *et al.*, 2018). However, the costs are elevated, ranging from 6000 to 12000 euros, depending on the surgical procedure applied and this implies that this procedures are frequently cost-prohibitive (Igel *et al.*, 2018).By contrast, it has been shown that very low calorie diets (VLCD, <800 kcal/day) are more effective to produce fat loss and to maintain it over time (Vogels & Westerterp-Plantenga, 2007; Dulloo *et al.*, 2016).

A fast reduction in total body weight can be also achieved with intensified protocols involving a combination of long-lasting walking protocols and moderate energy restriction during short periods (3-10 days) (Carlson & Fröberg, 1967; Marniemi *et al.*, 1984; Shpilberg *et al.*, 1990).

6.2. LEAN-MASS SPARING EFFECT OF EXERCISE DURING SEVERE ENERGY DEFICIENCY – ENDOCRINE REGULATION

During weight loss elicited by a situation of energy deficiency, concomitantly with fat mass (FM) there is marked reduction in fat-free mass (FFM) induced by catabolism of muscle and particularly skeletal muscle, which accounts for ~25% or a higher proportion of the total body weight loss achieved (Weinheimer *et al.*, 2010). Differences exist between normal-weight and overweight and obese subjects in this regard. Mean FFM losses for normal-weight subjects are usually ~35% or higher (Johnson *et al.*, 1994; Bosy-Westphal *et al.*, 2009), while in overweight or obese subjects this losses range ~20-30% (Ross *et al.*, 2000; Weiss *et al.*, 2007; Santanasto *et al.*, 2011; Bosy-Westphal & Muller, 2014; Magkos *et al.*, 2016). This implies a more favourable FFM to FM ratio in

obese people during weight loss induced by diet. In VLCDs, the loss of FFM is superior to that attained with low calorie diets (LCDs, ~1000 kcal/day less than normal), highlighting the relevance of the extent of the energy deficit in the degree of FFM loss (Chaston *et al.*, 2007).

An excessive loss of fat FFM may be detrimental, since lean tissues and, in particular skeletal muscle, account for most resting metabolism (Johnstone *et al.*, 2005), is essential for the preservation of bone mass (Aloia *et al.*, 1995; Vicente-Rodriguez *et al.*, 2005) and to maintain the capacity to perform physical work (Marks & Rippe, 1996). Furthermore, a lowered loss of FFM during VLCD has the capacity of enhancing the long-term maintenance of weight (Vogels & Westerterp-Plantenga, 2007; Dulloo *et al.*, 2016). For these reasons, there is considerable interest in minimizing the loss of FFM during interventions designed to reduce body weight.

Randomized control trials have shown that exercising while following LCDs attenuates loss of FFM in middle-age and older obese individuals of both sexes (Janssen & Ross, 1999; Rice *et al.*, 1999; Janssen *et al.*, 2002; Andreou *et al.*, 2011; Villareal *et al.*, 2011). However, a small number of studies (Chomentowski *et al.*, 2009; Ryan & Harduarsingh-Permaul, 2014) have reported some conflicting results, particularly regarding endurance-based modalities of exercise.

Nevertheless, data are limited on the effects of exercise to prevent or attenuate muscle mass loss when the energy deficit is severe, that is when a VLCD is combined with a high volume of exercise, similar to that observed in Ironman

triathlon competitions (Kimber *et al.*, 2002), without the confounding effects of other macronutrients in the diet.

In addition, it remains unknown whether this effect is limited to the group of muscles directly and/or primarily involved in the sportive task (i.e. trained muscles) (Zorzano *et al.*, 2009) or provides a more generalized impact in the skeletal muscle system , as indicated by certain reports using different exercise modalities (Ballor *et al.*, 1988; Janssen & Ross, 1999; Janssen *et al.*, 2002; Chomentowski *et al.*, 2009).

6.2.1. Endocrine regulation driven by severe energy deficit

Alteration of endocrine pathways play a pivotal role in the changes in weight, body composition and particularly the level of muscle mass gain/loss during fed/fasting states. Multiple factors regulate the endocrine response, such as age, sex, genetics, physical activity, body composition, health status or diet. Testosterone has a role in stimulating skeletal muscle protein synthesis and muscle mass (Rooyackers & Nair, 1997) and has been associated with changes in fat mass, suggesting a plausible role in regulating adiposity (De Maddalena *et al.*, 2012). On the contrary, cortisol is a glucocorticoid-class hormone that induces an increase in protein breakdown (Gore *et al.*, 1993) and is appointed to downregulate leptin levels (Zakrzewska *et al.*, 1997). Leptin possesses an important role in the regulation of glucose and fatty acid metabolism, acting as a sensor of energy availability (Dyck *et al.*, 2006). Importantly, insulin holds an anabolic and anti-catabolic effect in skeletal muscle (Chow *et al.*, 2006). Other hormones such as thyroid hormones (regulation of metabolic rate (Kim, 2008)) and ghrelin (appetite sensing and food

intake (De Vriese & Delporte, 2007)) are relevant in the regulation of protein metabolism

During energy deficit, the activation of muscle proteolysis is indispensable to maintain an appropriate amino acid provision to the liver and to maintain a sufficient level of hepatic glucose production when carbohydrate (CHO) provision is insufficient (Cahill, 2006). Furthermore, during a severe energy deficit, as induced by VLCD, there is an activation of the hypothalamus-pituitary-adrenal axis which induces an increase in the circulating levels of cortisol while there is an inhibition of the hypothalamus-pituitary-gonadal axis which in men produces a reduction in plasma testosterone levels (Chan *et al.*, 2003). Concomitantly, insulin levels are lowered and insulin resistance within the skeletal muscles is augmented (Chan *et al.*, 2003; McMurray & Hackney, 2005; Afolabi *et al.*, 2007), and thus a severe energy deficit occurs with a marked increase in the cortisol/testosterone catabolic index and with resistance to the anabolic effect of insulin. Circulating leptin levels have been shown to be reduced with short-term energy restriction. Furthermore, obesity is associated with and resistance to insulin and leptin (Bates & Myers, 2003; Steinberg *et al.*, 2006; Anubhuti & Arora, 2008; Myers *et al.*, 2008) which have been linked to activation of the pro-inflammatory pathways JNK (c-Jun N-terminal kinase) e IKK (inhibitor of nuclear factor kappa-B kinase complex)–NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Hirosumi *et al.*, 2002; Bandyopadhyay *et al.*, 2006; Sriwijitkamol *et al.*, 2006), increased protein tyrosine phosphatase 1B (PTP1B) (Zabolotny *et al.*, 2008) and suppressor of cytokine signalling 3 (SOCS3) (Bjørbæk *et al.*, 2000), and downregulation of the number of leptin receptors (OBRs) (Fuentes *et al.*, 2010).

Figure 2 illustrates the dramatic changes in cortisol and testosterone to a period of 5-7 days of military operations where infantry cadets were subjected to extreme levels of physical stress, energy deficiency (energy expenditures ranging 8000-11000 kcal/day, weight losses 8-12 kg) and sleep deprivation (1-3 h/day) (Opstad, 2001).

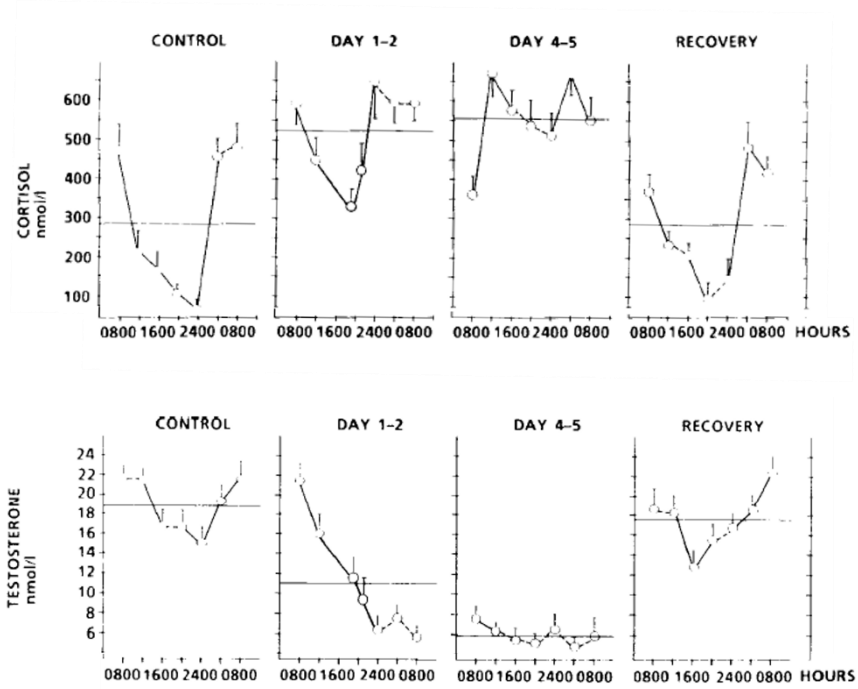


Figure 2. Circadian fluctuations in plasma levels of cortisol and testosterone before (control), throughout and after (recovery 4-5 days after) a 5-day military training course in cadets with heavy physical exercise in combination with energy and sleep deprivation. Data are means ± standard deviation. Statistically significant differences between time points (P<0.01) are shown with thick lines and non-significant with dotted lines. Horizontal lines represent 24-h means. Adapted from Opstad (2001).

These are the main reasons why a loss in muscle mass presents in a situation of severe energy restriction and therefore a ~10-40% of the total weight loss is FFM, primarily muscle mass (Chaston *et al.*, 2007).

6.3. EFFECTS OF DIET ON LEAN MASS DURING SEVERE ENERGY DEFICIENCY

Protein consumption below the Recommended Dietary Allowance (RDA) of 0.8 g/kg/day (Trumbo *et al.*, 2002) results in loss of FFM during weight maintenance (energy balance) or weight gain (positive energy balance) (Bray *et al.*, 2012; Campbell *et al.*, 2015) by ~0.2-0.5%/week (Cava *et al.*, 2017). Higher than the RDA for protein does not benefit FFM during positive or neutral energy balance (Baer *et al.*, 2011; Bray *et al.*, 2012)

During hypocaloric diets, increasing the ratio of protein to carbohydrate has been shown as an effective strategy to prevent the loss of FFM (Piatti *et al.*, 1994; Farnsworth *et al.*, 2003; Layman *et al.*, 2003; Krieger *et al.*, 2006; Leidy *et al.*, 2007; Meckling & Sherfey, 2007; Arciero *et al.*, 2016). Consequently, during periods of pronounced energy deficit primarily caused by demanding physical exercise, as frequently occurs in athletes and military personnel, it is currently recommended that protein doses higher than RDA (i.e. ~1.2-2.0 g/kg/day) are consumed to optimise skeletal muscle mass maintenance (Phillips, 2006; Pasiakos *et al.*, 2013). Studies have also supported that overweight and obese subjects can be benefited from two times the RDA during sustained weight loss interventions (Mettler *et al.*, 2010; Wycherley *et al.*, 2012a; Wycherley *et al.*, 2012b; Pasiakos *et al.*, 2013). Nonetheless, this muscle mass sparing effect of protein has only been clearly demonstrated in connection with long-lasting interventions (i.e. > 6 weeks) involving moderate energy deficits (Johnston *et al.*, 2004), and appears to depend on the total daily intake of protein (Pasiakos *et al.*, 2013) and the distribution of

protein throughout the day (Cava *et al.*, 2017). Mettler *et al.* (2010) clearly showed in young resistance-trained athletes that, during two weeks of hypocaloric diet (while maintaining their weight-training regime) consisting of 60% of habitual energy intake, a diet containing ~2.3 g/kg was superior to 1.0 g/kg for lean body mass preservation as illustrated in Figure 3. These findings agree with other studies (Longland *et al.*, 2016), evidencing that shorter protocols lasting less than 5 weeks using LCD combined with resistance training or high-intensity interval training with a higher protein intake are more efficient in preserving lean mass and promoting fat mass loss that a control diet with lower daily protein intake.

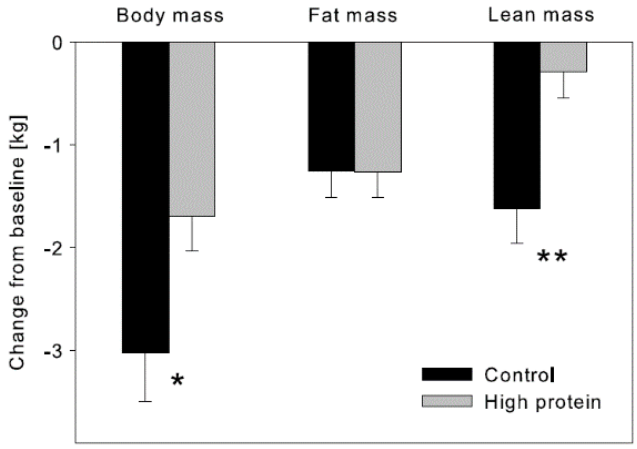


Figure 3. On the right, lean mass losses following either a high (2.3 g/kg) or control (1.0 g/kg) protein diet during a 2-week weight loss protocol (n=10 for each group). *Significant difference between groups. **Significant difference between groups (P=0.006). *Significant difference between groups (P=0.036). Figure obtained from Mettler *et al.* (2010).

Interestingly, in conflict with these results, preservation of lean body mass was not enhanced in infantry cadets consuming 0.5 or 0.9 g/kg/day dietary protein during eight days of energy restriction, arduous work and sleep deprivation causing a daily energy deficit of ~2.300 kcal/day (Alemany *et al.*, 2008).

Essential amino acids (EAAs) and particularly leucine are powerful activators of protein synthesis (Blomstrand *et al.*, 2006; Dreyer *et al.*, 2008) and exercise specifically enhances the anabolic effect of amino acid ingestion (Lundholm & Schersten, 1977; Blomstrand *et al.*, 2006; Dreyer *et al.*, 2008; Kakigi *et al.*, 2014). It remains unknown whether a higher fraction or total protein content of the diet during a VLCD enables an attenuation of the muscle mass loss.

6.4. PROTEIN TURNOVER AND REGULATION OF MUSCLE MASS

Skeletal muscle mass contains ~40% of total body protein stores and is responsible for ~30-50% of whole body metabolism. Its regulation depends upon the dynamic and constant process of protein turnover, which is the balance between protein synthesis and protein degradation (Calloway, 1975; Burd *et al.*, 2009). Protein turnover is affected by the degree of energy balance. During a situation of energy balance, muscle protein synthesis and protein breakdown simultaneously acting over a period are equal, and therefore net protein balance is neutral and skeletal muscle mass remains stable (Burd *et al.*, 2009). On the contrary, a negative energy balance yields a reduction in muscle protein synthesis in fasted and fed states (Pasiakos *et al.*, 2013; Areta *et al.*, 2014; Hector *et al.*, 2015) which partly explains the reduction in FFM under these conditions. During an energy deficit caused by energy restriction and/or exercise, there is also an overall increase in whole-body proteolysis, amino acid oxidation and nitrogen excretion (Tsalikian *et al.*, 1984; Nair *et al.*, 1987; Hoffer & Forse, 1990; Knapik *et al.*, 1991; Farnsworth *et al.*, 2003; Pikosky *et al.*, 2008), which in turn generates an overall marked negative protein balance.

Exercise and/or nutritional interventions aiming for a higher protein diet attenuate the reduction in muscle protein synthesis driven by an energy-deficient state, and this may explain the muscle mass sparing effect (Areta *et al.*, 2014). However, these studies have focused on the effects of resistance exercise and the interaction of endurance exercise and energy restriction in muscle protein synthesis remains widely unknown, with no study particularly addressing a severely deficient energy state in combination with exercise.

Compared to muscle protein synthesis, little attention has been given to the regulation of muscle proteolytic responses during energy deficit and its interaction with exercise (Carbone *et al.*, 2014; Hector *et al.*, 2018) and no study to date has particularly addressed the regulation of protein breakdown during a combination of a severe energy deficit and aerobic exercise.

Studies are lacking addressing the molecular markers, such as gene and protein expression driving the responses in muscle protein synthesis and degradation during a severe energy deficit combined with prolonged exercise.

6.5. MOLECULAR PATHWAYS REGULATING SKELETAL MUSCLE MASS

The signalling pathways coordinating protein synthesis and degradation interact in a highly complex and dynamic intracellular network with a constant crosstalk of signals that eventually regulate and control hypertrophic and atrophic events. Physiological stressors such as physical exercise or nutrient availability influence these signals as well as their interdependency (Figure 4).

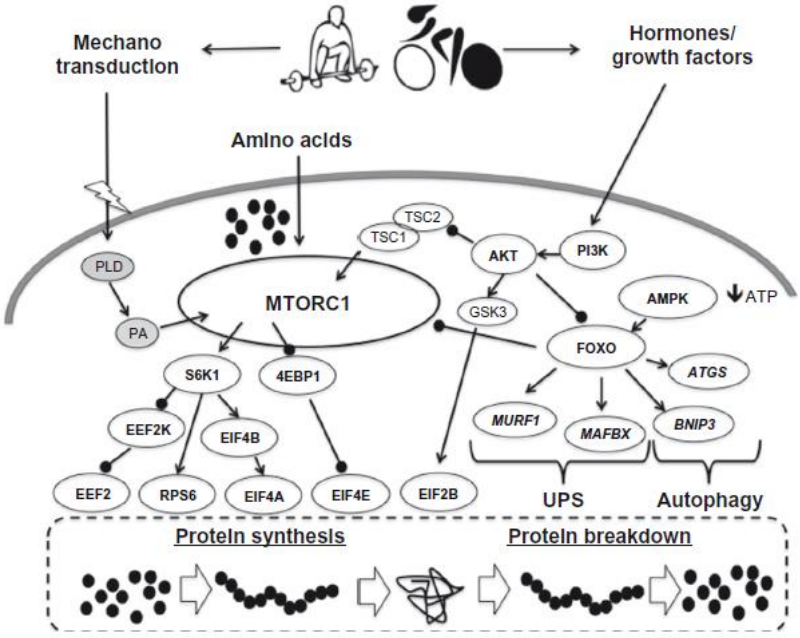


Figure 4. *Simplification of the interplay between the main molecular signalling pathways regulating skeletal muscle mass*. Physiological stresses such as exercise and energy deprivation trigger a complex cascade of molecular events initially driven by FoxO3, AMPK and mTOR, depending upon the severity of the energy deficiency and the type of exercise (modality, intensity and duration). AMPK induces autophagy activation by directly phosphorylating ULK1. Anabolic stimuli activate insulin signalling leading to mTOR activation, which acts as an autophagy inhibitor via downregulation of the ULK1complex. AMPK blocks mTOR directly or by activating TSC2 via phosphorylation. FoxO3 coordinates a transcriptional program that promotes several autophagy genes. FoxO3 is phosphorylated and inhibited by Akt. Phosphorylated FoxO3 by Akt leads to exclusion from the nucleus. FoxO3 is upregulated by AMPK during prolonged energy deficit. Of note is the interdependency between pathways. Scheme extracted from Atherton *et al.* (2015).

Protein synthesis at the molecular level is principally regulated by the Akt (Protein kinase B or PKB)/mTor (mammalian target of rapamycin) signalling pathway. The main degradation pathways in skeletal muscle are the autophagy-lysosomal and the ubiquitin-proteasome systems, which compose a complex framework of catabolic messages modulating one another and inseparably linked to energy availability (Figure 4).

6.5.1. Protein synthesis molecular pathway

The increased expression of a determined protein requires the transcription of a specific sequence of amino acid encoded into DNA to mRNA. Through reactions of translation initiation, the mRNA sequence is then used to assemble the chain of amino acids forming a functional protein for its subsequent elongation and termination of the peptide chain.

Protein synthesis in muscle is stimulated by the pathway involving the mTor protein kinase, which is considered the main regulator of translation initiation and elongation in muscle. mTor is activated through phosphorylation by Akt (Figure 4). Activated mTor forms two distinct complexes, TORC1 and TORC2. TORC1 is sensitive to inhibition by rapamycin and propagates downstream signalling by phosphorylating p70 ribosomal S6 kinase (p70S6K, also referred to as S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (also referred to as PHAS-1), resulting in their activation and inhibition, respectively (Egerman & Glass, 2014). Phosphorylation of 4E-BP1 and p70S6K by mTor are essential for protein synthesis and hypertrophy in muscle (Egerman & Glass, 2014). Akt can promote protein synthesis by inhibiting glycogen synthase kinase 3 beta (GSK3β),

impeding its inhibitory action on protein synthesis (Frame & Cohen, 2001). Protein synthesis requires sufficient and appropriate amino acid supply, and amino acid deprivation induces the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), thereby impeding translation initiation (Baird & Wek, 2012).

6.5.2. Protein degradation molecular pathway: ubiquitin- proteasome and autophagy-lysosomal systems

Protein degradation in skeletal muscle primarily depends upon the activation of the two major proteolytic pathways: the ubiquitin-proteasome and the autophagy-lysosome systems (Schakman *et al.*, 2013). Both are involved in the loss of muscle (proteins, organelles and cytoplasm) and are transcriptionally regulated, with a group of genes (named atrophy-related genes or "atrogenes") whose up- or down-regulation has been associated with catabolic conditions and therefore skeletal muscle atrophy (Bodine *et al.*, 2001; Lecker *et al.*, 2004; Sandri, 2013).

The ubiquitin-proteasome system removes targeted proteins through the 26S proteasome by covalently attaching a chain of ubiquitin molecules (Mitch & Goldberg, 1996). It primarily depends on the activity of the E3 Ubiquitin Ligases, enzymes that transfer the ubiquitin onto the substrate for recognition by the proteasome. Subsequently, deubiquitinating enzymes remove the ubiquitin chain and allow the recycle and reuse of ubiquitin in new conjugation reactions (Sandri, 2013). There are only a few muscle-specific E3 Ubiquitin ligases demonstrated to be up-regulated during muscle loss. Particularly important are muscle RING finger-containing protein 1 (*MuRF1*) and muscle atrophy Fbox protein (*MAFbx* or *atrogin-1*). Using knockout mice models in either of these genes, animals

evidenced partial resistance against the atrophying stimulus elicited by denervation (Bodine *et al.*, 2001; Gomes *et al.*, 2001). Importantly, inhibition of *MAFbx* expression prevents from muscle atrophy during fasting (Cong *et al.*, 2011).

The expression of *MuRF1* and *atrogin-1* is regulated by the forkhead boxO family of transcription factors (FoxOs) (Eijkelenboom & Burgering, 2013).

Autophagy is a process where dysfunctional cell components are identified, degraded and its constituents recycled as building blocks for the synthesis of new components of improved quality through the lysosomal machinery. Autophagy is also required for a physiological adaptation to regular exercise (angiogenesis, mitochondrial biogenesis, insulin sensitivity, and muscle hypertrophy) (He *et al.*, 2012a; Lira *et al.*, 2013; Sin *et al.*, 2016). Until now, three main autophagy pathways have been identified. The best-understood mechanism, macroautophagy, (hereafter referred to as autophagy) consists of a dynamic process in which a double-membrane cytoplasmic vesicle called autophagosome selectively engulfs dysfunctional or damaged proteins, organelles, and fractions of cytoplasm. Subsequently, the completed autophagosome fuses with the lysosome (forming the autolysosome) for an eventual degradation of the sequestered components via lysosomal hydrolases. Autophagy pathway ends with lysosome digestion and release of the molecular components of engulfed materials, mostly amino acids, and presumably lipids and sugars, that may follow complete degradation or be recycled by the cell for the de novo synthesis of molecules and cellular structures.

Up- or down-regulation of autophagy is one of the main mechanisms of cellular adaptation to stress. When cellular stress is increased, as for example

limited nutrient availability (Mizushima *et al.*, 2004) or exercise (He *et al.*, 2012b), autophagy flux (defined as a measure of autophagic degradation activity) (Loos *et al.*, 2014)) is elevated to provide energy substrates and to adapt cellular structures to the newly elevated demands.

The discovery of the so-called "autophagy-related (Atg) genes" early in the 90s in experiments conducted in yeast, meant a broad expansion in the understanding of the autophagy-lysosomal pathway (Wang & Klionsky, 2003). Several mammalian homologues have been identified to date. Among them, the unc-51-like kinases (ULK) and Beclin1 (homologues of Atg1 and Atg6, respectively) modulate the signalling pathways leading to autophagy activation (Nazarko & Zhong, 2013). Autophagosome formation and targeting of damaged substrates requires the microtubule-associated protein 1A/1B-light chain 3 (LC3B, homologue of Atg8) and its lipidated form after conjugation with phosphatidylehanolamine (PE), namely LC3B-II (Weidberg *et al.*, 2010). Up-regulation of BCL-2/adenovirus E1B nineteen-kilodalton interacting protein 3 gene (*BNIP3*) has been shown to induce autophagy and support mitochondrial integrity (Hamacher-Brady *et al.*, 2006; Glick *et al.*, 2012). Recruitment of the sequestosome 1 (p62/SQSTM1) to autophagosome is present during autophagy activation, being required for formation and degradation of polyubiquitin substrates (Pankiv *et al.*, 2007).

Skeletal muscle is one of the tissues with highest basal autophagy flux and greater capacity to increase autophagy flux (Mizushima *et al.*, 2004). Muscle-specific knockout models in mice have demonstrated that inactivation of genes

coding for autophagy-lysosomal proteins results in muscle loss (Mammucari *et al.*, 2007; Mammucari *et al.*, 2008; Masiero *et al.*, 2009).

In human skeletal muscle, given the unfeasibility of most *in vivo* assessments (Loos *et al.*, 2014), frequently autophagic flux is indirectly assessed by determining the changes in molecular markers of the different steps involved in autophagy, mainly adenosine monophosphate-activated kinase (AMPK), ULK, the FoxOs, LC3, and p62/SQSTM1. AMP-activated protein kinase. For the purpose of this research, only the principal markers which are also linked directly to ubiquitin-proteosome and/or protein synthesis pathways will be mentioned in this section. For a more detailed insight, these markers have recently been reviewed in Martin-Rincon *et al.* (2018a).

AMPK is considered a crucial activator of autophagy (Klionsky *et al.*, 2016). AMPK activation (as occurs in humans by exercise-elicited AMPKα Thr^{172} phosphorylation) is necessary for exercise to induce autophagy (Egan *et al.*, 2011; Smiles *et al.*, 2017). AMPK promotes autophagy by directly phosphorylating and activating ULK. ULK1 is considered the predominant isoform involved in autophagy (Klionsky *et al.*, 2016). Particularly, phosphorylation of ULK1 at the 757-serine residue by mTORC1 prevents ULK1 activation (Kim *et al.*, 2011). AMPK inhibits mTORC1 complex by direct phosphorylation (Inoki *et al.*, 2003; Gwinn *et al.*, 2008) and also by phosphorylating and activating tuberous sclerosis complex 2 (TSC2) (Zoncu *et al.*, 2011). Thus, AMPK acts simultaneously on two processes that synergize to activate autophagy: directly, by activating ULK1, and indirectly by impeding mTOR-dependent inhibition of ULK1.

The important role played by ULK1 in autophagy has been confirmed using loss-of-function genetic models showing that lack of ULK1 impedes the conversion of LC3-I to LC3-II in mice (Kim *et al.*, 2011).

ULK1 effects may be reinforced by FoxOs (FoxO1, FoxO3 and FoxO4), among which FoxO3 up-regulates autophagy-genes expression in skeletal muscle (Mammucari *et al.*, 2007). FoxO3 activation is facilitated in catabolic conditions such as ultraendurance exercise (Zhao *et al.*, 2007; Jamart *et al.*, 2012b) and by nutrient deprivation and lack of growth factors (IGF-1 (insulin-like growth factor 1), insulin, etc.), resulting in reduced intracellular Akt activity (Eijkelenboom & Burgering, 2013). However, during sprint (Guerra *et al.*, 2010), endurance (Camera *et al.*, 2010), and resistance exercise (Camera *et al.*, 2010) Akt is phosphorylated and activated, which may negatively regulate autophagy by phosphorylating threonine residues of FoxO3 (Mammucari *et al.*, 2007; Zhao *et al.*, 2007). PhosphoThr^{32}-FoxO3 remains in the cytosol (Imae *et al.*, 2003) and only translocates to the nucleus to stimulate the autophagy-gene program when dephosphorylated (Sandri *et al.*, 2004; Milan *et al.*, 2015). Akt can also inhibit autophagy by blunting Thr^{172}-AMPK phosphorylation via Ser^{485}-AMPKα1/ Ser^{491}-AMPKα2 phosphorylation (Guerra *et al.*, 2010; Dagon *et al.*, 2012; Hardie & Ashford, 2014; Hawley *et al.*, 2014). The exercise-induced activation of Akt is more prominent when the exercise is performed in the fed than the fasted state, likely due to the additive effect of the postprandial increase of insulin and exercise on Akt activation (Guerra *et al.*, 2010). Moreover, AMPK phosphorylates FoxO3a in Ser^{588} increasing its nuclear localisation and activity (Greer *et al.*, 2007; Sanchez *et al.*, 2012).

Prolonged endurance exercise is likely needed to promote FoxO3 signalling (Jamart *et al.*, 2012a; Jamart *et al.*, 2012b), although few hours after a single bout of bicycling for 120 min at 60% of VO2max, FoxO3 mRNA and protein levels were already increased (Stefanetti *et al.*, 2015). In contrast to endurance exercise, FoxO3 protein levels remained unchanged after a single bout of resistance exercise (Stefanetti *et al.*, 2015). The increase in FoxO3 protein after endurance exercise is also observed in the trained state (Stefanetti *et al.*, 2015). The importance of FoxO3 for autophagy is further supported by suppression of autophagic flux in FoxO3 knockout mice (Milan *et al.*, 2015).

Studies on how nutrition interacts with exercise in the regulation of autophagy are scarce and controversial, and none has focused on the skeletal muscle autophagic responses to a severe energy deficit in humans.

To summarize, in spite of the compelling number of studies demonstrating the protective effect of exercise and/or protein consumption in FFM loss during energy deficit, these effects have not been studied when the energy deficiency is high. Furthermore, the interaction between the transcriptional and molecular pathways driving protein synthesis and protein degradation during energy deficit are poorly understood and no study has investigated these responses in a situation of severe energy deficiency.

Given the high degree of crosstalk between protein synthesis and degradation via several molecular markers such as Akt, FoxOs and AMPK, the integration of the signalling pathways controlling protein turnover and muscle mass

is necessary for a profound understanding of the mechanisms regulating muscle mass during these conditions.

AIMS

7. AIMS

The overall aim of this thesis is to characterize the potential influence of low-intensity exercise and/or protein ingestion on the regulation of muscle mass during severe energy deficit and to study the underlying molecular mechanisms and key genes mediating this response in human skeletal muscle of overweight men. More specifically, we aimed at:

1. To ascertain whether the potential muscle-mass sparing effect of exercise during a severe energy deficit is dependent on the amount of exercise performed and therefore more pronounced in the active muscles, or holds a systemic effect in the skeletal muscle system.

2. To examine whether whey protein ingestion and the subsequent increase in circulating amino acids would potentiate the anabolic stimulus driven by exercise during a severe energy deficit.

3. To determine the acute effects of a severe energy deficit and its derived neuroendocrine response on the principal molecular markers regulating the protein synthesis signalling cascade and its interaction with different amounts of low-intensity exercise.

4. To examine changes in gene expression of key genes involved in protein degradation, primarily the autophagy-lysosomal and the ubiquitin-proteasome proteolytic systems.

HYPOTHESES

8. HYPOTHESES

The thesis has been undertaken considering the following hypothesis:

1. Low-intensity exercise during severe energy deficit will attenuate the loss of skeletal muscle mass.

2. A diet consisting solely of whey protein will lessen the loss of muscle mass during severe energy deficit and this effect will be enhanced locally by exercise in a dose-dependent manner in overweight men.

3. The potential preservation of muscle mass will be modulated primarily by the changes in plasma levels of cortisol, testosterone, leptin and amino acids.

4. The muscle mass-sparing effect of exercise will be mediated by an activation of the Akt/mTor/p70S6K/4E-BP1 pathway induced by whey protein ingestion and potentiated by elevated levels of circulating leucine and essential amino acids.

5. The expression of key genes involved in the ubiquitin-proteasome and autophagy-lysosomal proteolytic systems will be increased after caloric restriction in combination with low-intensity exercise but to a lesser extent in the group ingesting a whey protein diet.

MATERIALS AND METHODS

9. MATERIALS AND METHODS

9.1. SUBJECT CHARACTERISTICS

A detailed description of our study population has been reported previously (Calbet *et al.*, 2015; Calbet *et al.*, 2017). Table 1 summarizes the background characteristics of our 15 overweight men assigned randomly to ingest a diet consisting solely of sucrose (SU; n=7) or whey protein (Whey PRO; n=8) during caloric restriction phase (CRE). The sample size was calculated to reveal any significant difference ≥1.5-fold larger than the coefficient of variation (which was <10% in most cases) between the mean values for any individual variable, with a significance level of $P < 0.05$ and statistical power of 0.8. After being informed of potential risks and benefits, all subjects provided their written consent to participate in the study. Ethical approval was obtained from the Regional Ethical Review Board of Umeå University (Umeå, Sweden), as well as the ethical committee of the University of Las Palmas de Gran Canaria (Canary Islands, Spain).

Table 1. The baseline characteristics of our subjects.

	Diet	
	Sucrose (n=7)	Whey protein (n=8)
Age (years)	38.7 ± 8.2	43.0 ± 8.0
Height (cm)	181 ± 5.5	180 ± 4.2
Weight (Kg)	98 ± 12.0	100 ± 14.9
BMI (Kg/m^2)	29.9 ± 3.1	30.9 ± 4.2
Lean mass (Kg)	63.1 ± 3.1	65.4 ± 6.0
Fat mass (Kg)	31.5 ± 9.1	31.4 ± 9.2
Body Fat (%)	31.6 ± 5.3	30.9 ± 4.1
VO_2max (L/min)	3.8 ± 0.3	3.9 ± 0.3
Physical activity (IPAQ) (kJ/day)	2161 ± 1318	2515 ± 1209

IPAQ: International Physical Activity Questionnaire (previously published in Calbet et al. 2015 and Calbet et al. 2017); BMI: body mass index. The values presented are means ± standard deviation.

9.2. INTERVENTION PROTOCOL

The protocol consisted of a pre-test phase (PRE), a phase of caloric restriction combined with exercise for 4 days (CRE), followed by a control diet with reduced exercise for 3 days (CD) (Fig. 5). During the PRE, and at the end of the CRE and CD phases, body composition was assessed (Lunar iDXA, GE Healthcare, Madison, WI, USA) (Calbet *et al.*, 2001); 20 mL blood samples were drawn with the subjects in the supine position; and thereafter muscle biopsies were taken. All of these procedures were performed in the morning following a 12-h overnight fast.

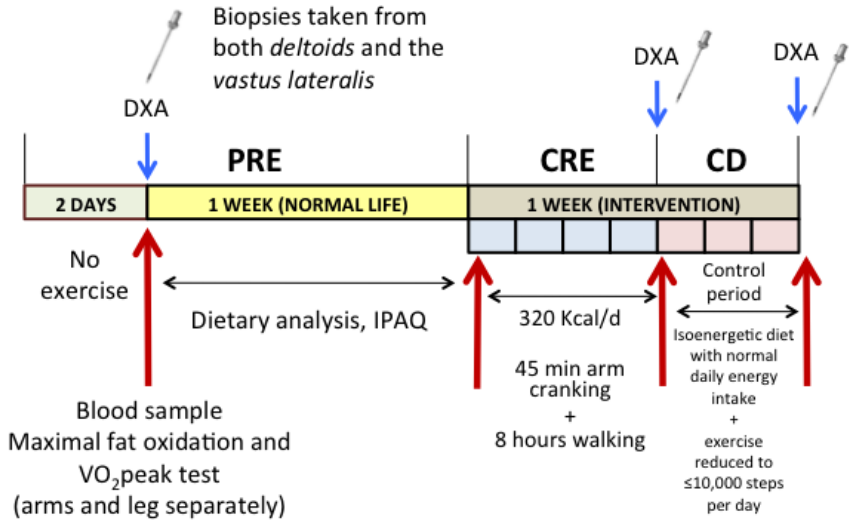


Figure 5. Experimental protocol of the study.

During CRE, participants ingested a diet consisting solely in either whey protein (PRO, n=8) or sucrose (SU, n=7) (0.8 g/kg body weight/day) in a double-blind fashion. The solution of whey protein also contained Na^+ (308 mg/L) and K^+ (370 mg/L), as did the sucrose solution (160 mg of Na^+ and 100 mg of K^+ per L).

The subjects drank 0.5 L of this solution in the morning (immediately prior to the arm-cranking session) and again at midday and at 20.00 h. In addition, both groups were allowed to drink a hypotonic rehydrating solution containing Na^+ (160 mg/L), Cl^- (200 mg/L), K^+ (100 mg/L), citrate (700 mg/L), and sucrose (3g/L) *ad libitum*.

Every day during the CD phase, the participants ate three standardized meals containing their normal daily intake of energy (as assessed by weighing all food ingested during the 7-day pre-test period) and were not allowed to walk more than 10,000 steps. This phase was designed to allow replenishment of body water and stabilization of body weight.

9.3. ASSESSMENT OF PHYSICAL ACTIVITY, NUTRITION AND BODY COMPOSITION

The short version of the International Physical Activity Questionnaire was employed to assess daily energy expenditure due to physical activity (Craig *et al.*, 2003). During the 7-day PRE phase, the participants kept a dietary record and their food intake was analysed (Dietist XP, Kost & Näringsdata, Bromma, Sweden). During the CD phase, all participants were provided a diet with the same energy content as that recorded during PRE, and the food ingested weighed. Energy intake was also calculated employing the Dietist XP program. During this phase, the sucrose and whey protein groups ingested 2256 ± 513 and 2086 ± 450 kcal/day (means ± SD), respectively.

In the morning following a 12-h overnight fast, body composition was determined by bioimpedance (Gibson *et al.*, 2008) and dual-energy x-ray absorptiometry as reported elsewhere (Calbet *et al.*, 2001; Perez-Gomez *et al.*,

2008). To perform the bioimpedance measurements, the participants were requested to stand upright and to grasp the handles of the InBody 720 to make contact with a total of 8 electrodes (2 for each foot and hand). Lean mass (LM) as assessed with the iDXA was defined as LM = total tissue mass - fat mass - bone mineral content

9.4. HORMONAL AND BIOCHEMICAL ANALYSIS

After a 12-h overnight fast, 30 mL blood samples were drawn from an antecubital vein directly into Vacutainer Tubes (REF: 368499; 368498). Some samples were collected in tubes containing EDTA and centrifuged for 5 min at 4000 revolutions per minute (rpm) and 4 °C, to obtain plasma; while others were centrifuged for 10 min at 4000 rpm and 4 °C to prepare serum. All of these samples were aliquoted on tubes precooled on ice water and rapidly stored at -80 º C until further analysis.

9.4.1. Glucose, leptin, cortisol, total and free testosterone and SHBG

The concentration of glucose in serum was measured with the hexokinase procedure, utilizing kits from ABX Pentra (Horiba Medical, Montpellier, France). Serum insulin was quantified by an electrochemiluminescence immunoassay (ECLIA) performed with reagent kits and a Modular Analytics Analyzer E170 (Roche Diagnostics SL, Barcelona, Spain), at a sensitivity of 0.2 µU/mL and with corresponding intra- and inter-assay coefficients of variation of 2.0 and 2.6%, respectively. Serum leptin was determined by the enzyme-linked immunosorbent assay (ELx800 Universal Microplate Reader, Bio-Tek Instruments, Inc, Winooski, Vermont, USA), employing reagent kits from Linco Research (#EZHL-80SK, St

Charles, Missouri, USA). In this case, the sensitivity was 0.05 ng/mL and the intra and inter-assay coefficients of variation were 3.8% and 4.4%, respectively 1. Cortisol and total testosterone were measured with chemiluminescence enzyme immunoassays (Immulite 2000 Cortisol, Ref. L2KCO2, Immulite 2000 Total Testosterone, Ref. L2KTW2; Siemens) exhibiting sensitivities of 5.5 and 0.5 nmol/L and intra- and inter-assay coefficients of variation of 6.2 and 7.3%, and 8.2 and 9.1%, respectively. Free testosterone was determined by a radioimmunoassay (Coat-A-Count Free Testosterone, Ref. TKTF1; Siemens) with a sensitivity of 0.5 pmol/L and intra- and inter-assay coefficients of variation of less than 8%. Sexual hormone-binding globulin (SHBG) was assessed with a chemiluminescence enzyme immunoassay (Immulite SHBG, Ref. L2KSH2; Siemens) characterized by a sensitivity of 0.02 nmol/L and intra- and inter-assay coefficients of variation of 2.7 and 5.2%, respectively. The free androgen index (FAI) was calculated as [TT (nmol/L) / SHBG (nmol/L)] x 100.

9.4.2. Amino acids

Following automated precolumn derivatization of plasma amino acids with o-phthalaldehyde, the resulting derivatives were separated by reversed-phase high-performance liquid chromatography (HPLC) (on a 5-µm Resolve C18 column; Waters), and quantified by fluorescence detection. The derivatization reagent was prepared by dissolving 50 mg o-phthalaldehyde in 1 mL of methanol and then adding 9 mL potassium borate buffer (0.4 mol/L, pH 10) and a drop of 2-mercaptoethanol. Solvent A consisted of phosphate buffer (0.1 mol/L, pH 7.0)/methanol/tetrahydrofuran (92:2:2) and Solvent B methanol/water (65:35). The

HPLC system contained two Model 510 pumps, a PCM Pump Control Module, a WISP 710 autosampler and a Model 470 fluorescence detector, all from Waters (Barcelona, Spain). Measurements were performed with an excitation wavelength of 338 nm and emission wavelength of 425 nm and the data collected and processed by a Model 860 Waters Networking Computer System.

9.5. MATERIALS

The Complete protease inhibitor cocktail and PhosSTOP phosphatases inhibitor cocktail were obtained from Roche Diagnostics (Mannheim, Germany; #04693116001 and #04906845001, respectively). All primary antibodies used were from Cell Signalling Technology (Danvers, MA, USA). The horseradish peroxide (HRP)-conjugated secondary anti-rabbit antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA; catalogue sc-2030). The Immun-Blot Polyvinylidene difluoride (PVDF) membranes, the Inmmun-StarTM WesternCTM and the Protein Plus Precision All Blue Standards 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. The corresponding catalogue numbers of the primary antibodies from Cell Signalling were as follows: anti-phospho-Akt (Ser^{473}), no. 9271; anti-phospho-eIF2α (Ser^{51}), no. 9721; anti-phospho-GSK-3β (Ser^{9}), no. 5558, anti-phospho-mTor (Ser^{2448}), no. 2971; anti-phospho-p70s6k (Thr^{389}), no. 9205; anti- phospho-4E-BP1 ($Thr^{37/46}$), no. 9459; anti-Akt, no.9272; anti-eIF2α, no. 9722; anti-GSK-3β, no. 9325, anti-mTor, no. 2972; anti-p70S6k, no. 9202; and the anti-4E-BP1, no.

9452. The monoclonal mouse anti-α-tubulin antibody (no. T-5168-ML) was obtained from Biosigma (Sigma, St. Louis, MO).

9.6. MUSCLE ANALYSIS

9.6.1. Biopsy sampling

Three muscle biopsies were taken from each deltoid muscle (middle portion) and from the middle portion of the *vastus lateralis* using Bergstrom´s technique without suction, as describe elsewhere. After disinfection of the skin, 1-2 ml local anaesthetic (Lidocaine 2%) was injected into the skin and subcutaneous tissue, taking care not to penetrate below the superficial fascia. Thereafter, a 6-7 mm incision was made and the biopsy Bergstrom-type needle inserted. The muscle sample (~100 mg) was dissected free of any debris and fat tissue present, and immediately frozen in liquid nitrogen at -80°C until further analysis.

9.6.2. Extraction of total protein

Extracts of muscle protein were prepared as previously described (Morales-Alamo *et al.*, 2013) and total protein content quantified using the bicinchoninic acid assay (Smith *et al.*, 1985). In brief, 30 mg of muscle were homogenized in urea lysis buffer (6 M urea, 1% Sodium dodecyl sulphate (SDS) and 1X Complete Protease Inhibitor and phosphatases PhosphoStop 1X) and the lysate then centrifuged for 15 min at 20,000 rpm at 4° C. The resulting supernatant containing the protein fraction, was diluted with electrophoresis loading buffer (62.50 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, bromophenol blue).

9.6.3. Western Blotting

The optimal antibody concentration and the total protein amount to be loaded was first determined by loading a gradient of protein extracts at concentrations between 20 and 45 μg. The linear relation between total protein concentration loaded and quantitative intensity of the bands was calculated. After confirming linearity in this range, equal amounts for the same protein determination (30-35 μg) of each sample were electrophoresed with 10% SDS-PAGE using the system of Laemmli (Laemmli, 1970) and transferred to Immun-Blot PVDF Membrane for Protein Blotting (Bio-Rad Laboratories). To compensate for variability between gels, the 9 samples from each subject and 4 control samples (human muscle) were equally loaded onto the same gel. When the coefficient of variation for the control samples was >20% the blot was repeated. The sample protein bands were normalized to the mean value of the band densities of the 4 controls.

Membranes were blocked for 1 h in 4% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (BSA-blocking buffer) and incubated overnight with primary antibodies. For all targeted proteins, the membranes were initially incubated with the phospho-specific antibodies and, after detection, the antibodies were stripped off using a buffer containing (RestoreTM PLUS Western Blot Stripping Buffer from ThermoFisher Scientific, Surfact Anionic), and subsequently re-blocked and re-incubated with antibodies against total protein. All antibodies were diluted in 4% BSA-blocking buffer with the exception of phospho-eIF2α (Ser^{51}) which was diluted in 5% Blotto blocking

buffer. After incubation with primary antibodies, the membranes were incubated with an HRP-conjugated anti-rabbit antibody (diluted 1:5000 in Blotto blocking buffer) and subsequent visualization with Immmuno Western CTM-Star™ (Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, UK) using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) until saturation of the signal. Finally, the bands in the exposition immediately prior to saturation of the signal were quantified with the Quantity One © image analyzer (Bio-Rad Laboratories, Hercules, CA, USA). To control for differences in loading and transfer efficiency, the membranes were subsequently stained with reactive brown and these parameters quantified. Likewise, membranes were incubated with a monoclonal mouse anti-α-tubulin antibody (Blotto-blocking buffer). No significant changes were observed in α-tubulin protein levels nor in Reactive Brown total protein amount (data not shown).

9.7. MICRORRAY ANALYSIS

RNA was extracted from 10-20 mg of muscle biopsies using a TissueLyser II (Qiagen) and the RNeasy Fibrous Tissue mini kit (Qiagen). RNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (A260 / A280 > 1.8 and A260 / A230 > 1.0 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was evaluated using the 2100 Bioanalyzer Instrument (Agilent Technologies). Input RNA, 100ng for the leg and 200ng for the arm samples, was used for subsequent biotinylation and hybridization. TotalPrep™-96 RNA Amplification Kit (Thermo Fisher Scientific) was used to generate biotinylated, amplified cRNA for subsequent hybridization of 750ng (150 ng / µl) on the Human

HT-12 v4 Expression BeadChip (Illumina) and scanning on the iScan system (Illumina). The raw data was read and quantified using Illumina Genome Studio version 1.1.1. Summary level expression data was read using the Bioconductor beadarray package. Autophagy-related genes were extracted from The Human Autophagy Database (HADb, http://www.autophagy.lu/index.html). Ubiquitin proteasome-related genes obtained from mUbiSida for mammalian Ubiquitination Site Database (http://reprod.njmu.edu.cn/mUbiSiDa).

9.8. STATISTICAL ANALYSIS

All values were examined for normal distribution using the Shapiro-Wilks test and, when necessary, transformed logarithmically before analysis. A repeated-measures analysis of variance (ANOVA) with time and the two different diets (sucrose versus whey protein) was applied for analysis of the mean responses. When a significant effect of diet or interaction between diet and time was indicated, pairwise comparisons at specific time-points were adjusted for multiple comparisons with the Holm–Bonferroni procedure. The relationship between variables was examined by simple linear regression. The changes in the levels of phosphorylated kinases from PRE to CRE ((CRE-PRE) x 100/PRE) (average of all three muscles) were analysed for potential correlations with the plasma concentrations of hormones and amino acids. Unless otherwise stated, the values reported are means ± standard deviations. Statistical significance was set at a P-value ≤ 0.05 and all statistical analyses were performed using SPSS v.21 (SPSS Inc., Chicago, IL, USA).

Differential gene expression analysis was performed using the Bioconductor limma package. P-values were corrected using the False Discovery Rate method (FDR), with a false discovery rate acceptance of < 0.05.

RESULTS

10. RESULTS

10.1. BODY COMPOSITION AND LEAN MASS

The changes in body composition, performance, blood lipids and neuroendocrine status have been previously reported in detail (Calbet *et al.*, 2015; Calbet *et al.*, 2017; Perez-Suarez *et al.*, 2017). After CRE, lean mass was reduced by 6 and 4% in the arms and the legs, respectively (both, $P<0.001$; $P<0.05$ for time x extremity interaction), with no significant differences between the two diets ($P=0.34$) (Figure 6). In spite of a mild negative energy balance during CD, there was a recovery of ~50% of the muscle mass lost during CRE from the four limbs after replenishment of body water stores during CD (Calbet *et al.*, 2015). Accordingly, after the three days on the control diet, the relative lean mass losses from the legs and trained arm were 57% and 29% less than the losses from the control arm ($P<0.05$) (Calbet *et al.*, 2017).

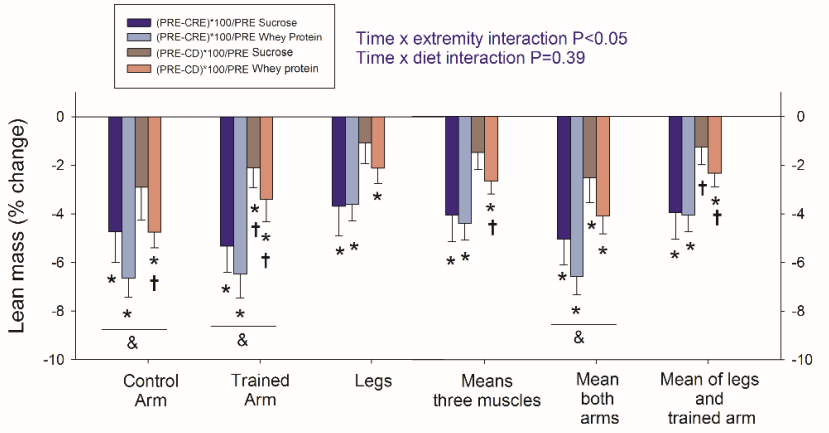


Figure 6. Changes in lean mass (fat-free mass - bone mass) by dual-energy x-ray absorptiometry. PRE: baseline tests; CRE: caloric restriction (13.4 kJ/day) and exercise (45 min arm cranking and 8 hours walking per day), for 4 days; CD: three days on a diet, isoenergetic with that observed during the PRE phase + reduced exercise. The vertical bars represent the mean values, and the error bars the standard error of the mean. Sucrose in dark colors (n=7) and whey protein in light colors (n=8). * $P < 0.05$ compared to PRE; † $P < 0.05$ compared to CRE; & $P < 0.05$ arms compared to legs. Extracted from Calbet *et al.* (2017).

10.2. PROTEIN CONCENTRATION IN MUSCLE BIOPSIES

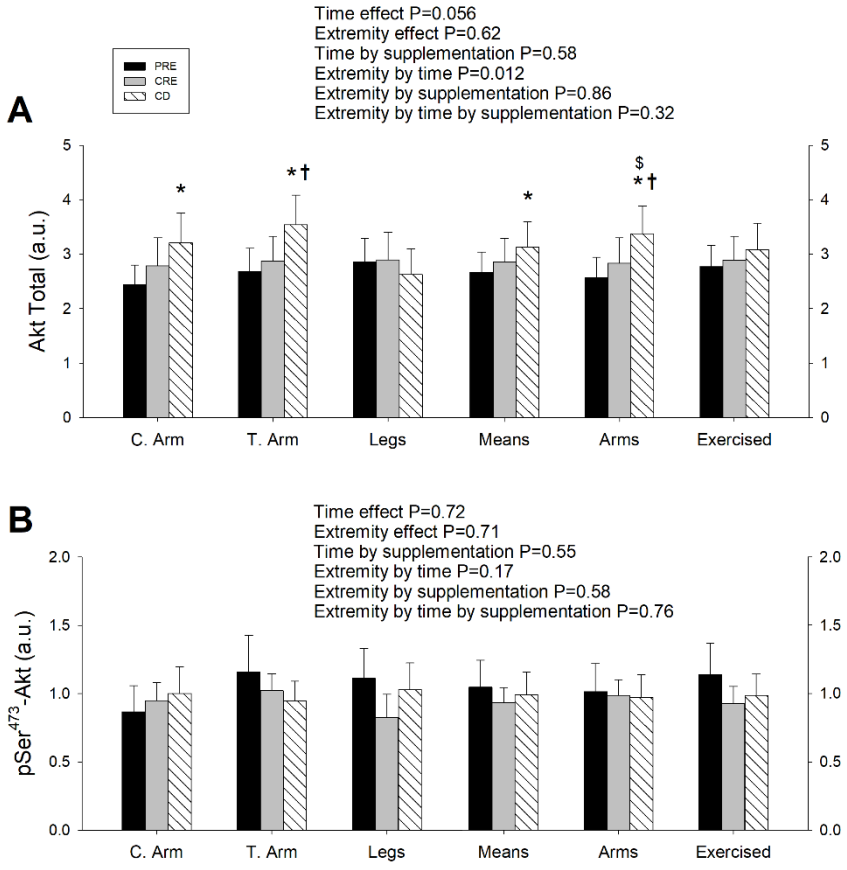
At baseline, the arms and leg muscles had a similar protein concentration (181.7±15.4 and 188.8±12.9 g/kg wet muscle, mean of both arms and the *vastus lateralis*, respectively, P=0.19). After CRE the protein concentration in the muscle biopsies (mean of the three muscles) was increased by 4.9% (P=0.010), remaining non-significantly elevated (2.2%, P=0.34 compared to baseline values) after the three days on a control diet (P=0.84 for extremity x time interaction).

10.3. HORMONAL AND METABOLIC CHANGES

The changes in plasma concentrations of amino acids and hormones have also been previously documented (Calbet *et al.*, 2017). In brief, plasma concentrations of asparagine, the three branched-chain amino acids (BCAAs) and EAAs were elevated after CRE, with more pronounced increases in the whey protein group (Calbet *et al.*, 2017). Serum concentrations of insulin and free testosterone, as well as the FAI were reduced similarly in both groups, whereas cortisol and the catabolic index (cortisol/total testosterone and cortisol/free testosterone) rose after CRE and CD (Calbet *et al.*, 2017), without significant differences between groups (see Table 4 in Calbet *et al.* (2017)).

10.4. PROTEIN SYNTHESIS SIGNALLING IN SKELETAL MUSCLE

Representative immunoblots are presented in Figure 12. In comparison to the PRE period, the level of total Akt (Figure 7A) was increased to a similar extent in both groups following CD, but this effect was limited to the arm muscles ($P<0.05$ for extremity x time interaction). However, neither pSer^{473}Akt (Figure 7B) nor the ratio pSer^{473}Akt /total Akt (Figure 7C) were altered significantly during the intervention (Figure 5).



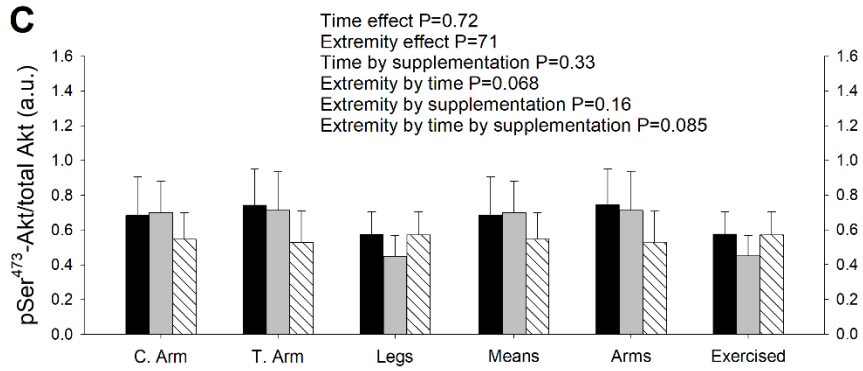
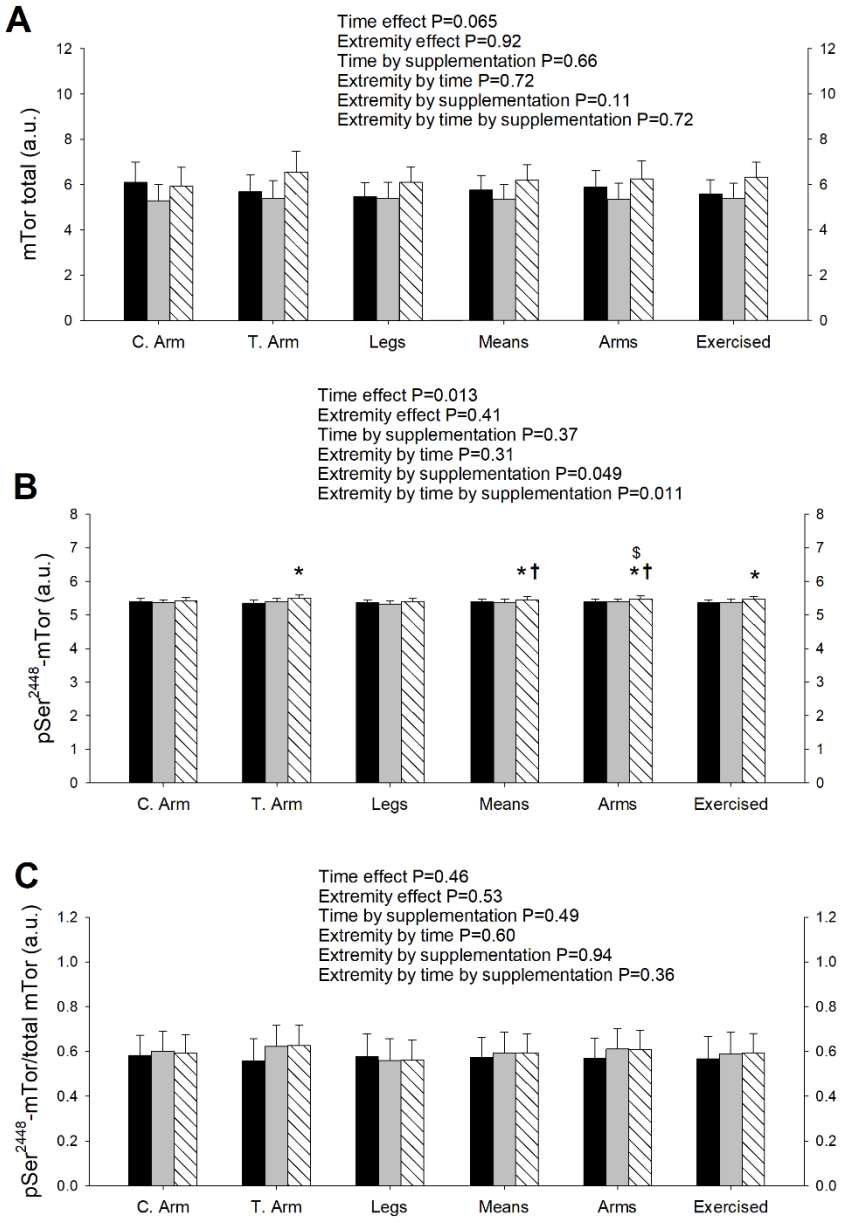


Figure 7. Protein expression levels of total and phosphorylated Akt (A-C) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * P < 0.05 compared to PRE; † P < 0.05 compared to CRE; $ P < 0.05 compared to the legs; § P < 0.05 compared to the legs.

The changes observed in the total level of mTor were not statistically significant (Figure 8A), while pSer^{2448}mTor (Figure 8B) was elevated following CD compared with PRE and CRE (P<0.05 for time effect), primarily due to the increase in the trained arm (P<0.05). This response was more marked in the sucrose than in the protein-supplemented group (P<0.05 for extremity x supplementation). pSer^{2448}mTor did not change significantly in the legs. Concomitantly, pSer^{2448}mTor/total mTor (Figure 8C) and total p70S6K (Figure 8D) were unchanged, while pThr^{389}p70S6K was undetectable.


A
Time effect P=0.065
Extremity effect P=0.92
Time by supplementation P=0.66
Extremity by time P=0.72
Extremity by supplementation P=0.11
Extremity by time by supplementation P=0.72
mTor total (a.u.)
C. Arm
T. Arm
Legs
Means
Arms
Exercised
B
Time effect P=0.013
Extremity effect P=0.41
Time by supplementation P=0.37
Extremity by time P=0.31
Extremity by supplementation P=0.049
Extremity by time by supplementation P=0.011
pSer2448-mTor (a.u.)
C
Time effect P=0.46
Extremity effect P=0.53
Time by supplementation P=0.49
Extremity by time P=0.60
Extremity by supplementation P=0.94
Extremity by time by supplementation P=0.36
pSer2448-mTor/total mTor (a.u.)

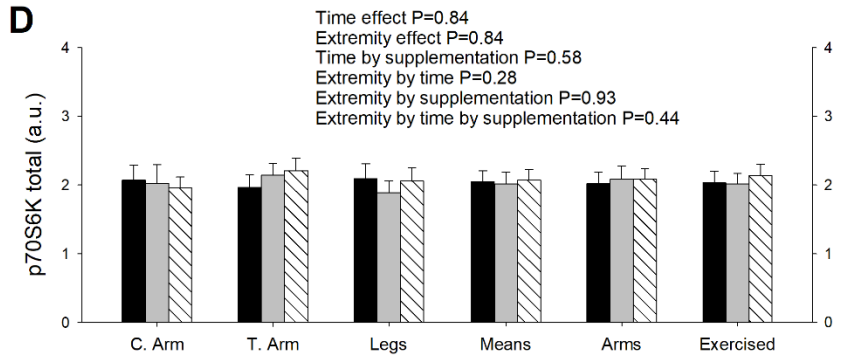
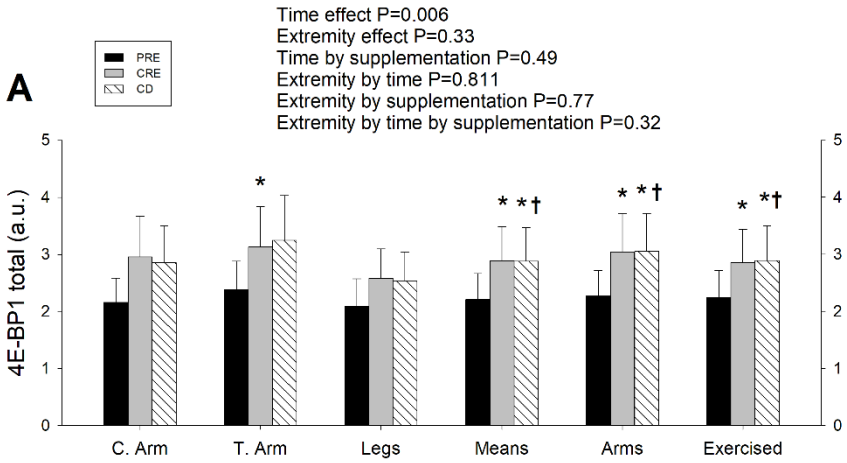


Figure 8. Protein expression levels of total and phosphorylated mTOR (A-C) as well as total p70S6K (D) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * P < 0.05 compared to PRE; † P < 0.05 compared to CRE; $ P < 0.05 compared to the legs; § P < 0.05 compared to the legs.

Regardless of the type of supplementation, the mean levels of total 4E-BP1 (Figure 9A) and Thr$^{37/46}$4E-BP1 (Figure 9B), a downstream target of mTor, were elevated after CRE and remained so after CD, although Thr$^{37/46}$4E-BP1/total 4E-BP1 did not change significantly (Figure 9C).



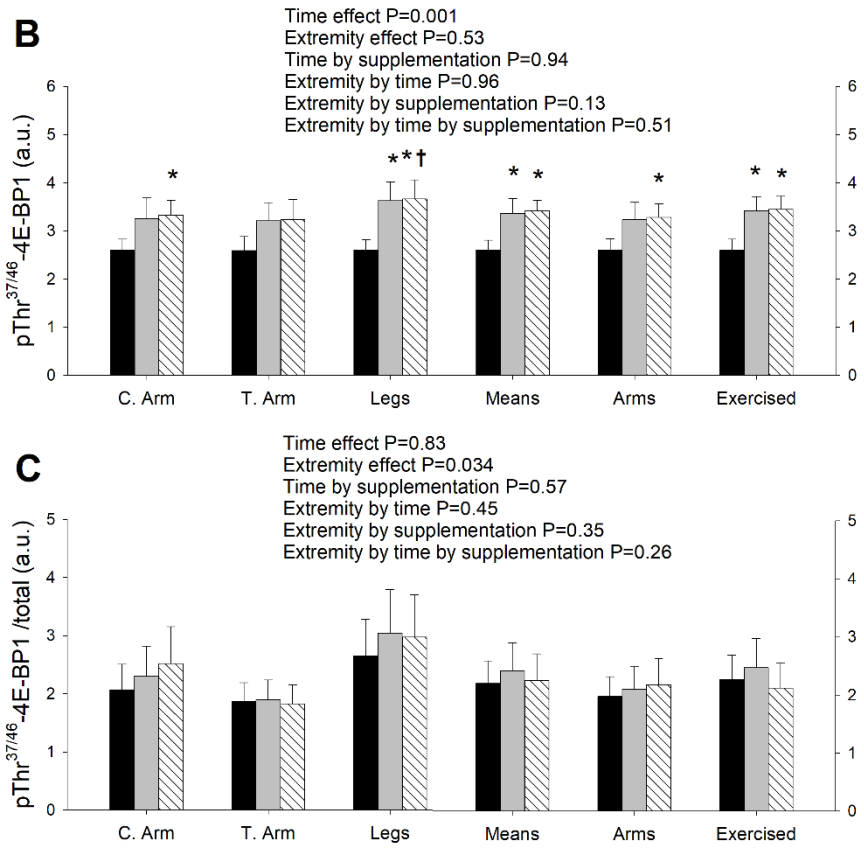


Figure 9. Protein expression levels of total and phosphorylated 4E-BP1 (A-C) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * P < 0.05 compared to PRE; † P < 0.05 compared to CRE; $ P < 0.05 compared to the legs; § P < 0.05 compared to the legs.

The total GSK3β (Figure 10A) was reduced in all muscles after CRE and remained so after CD. Ser^{9}GSK3β phosphorylation (Figure 10B) was also lowered after CRE, but returned to PRE levels after CD, resulting in higher pSer^{9}GSK3β/total GSK3β ratio (Figure 10C). These changes in pSer^{9}GSK3β were more pronounced in the protein than the sucrose group.

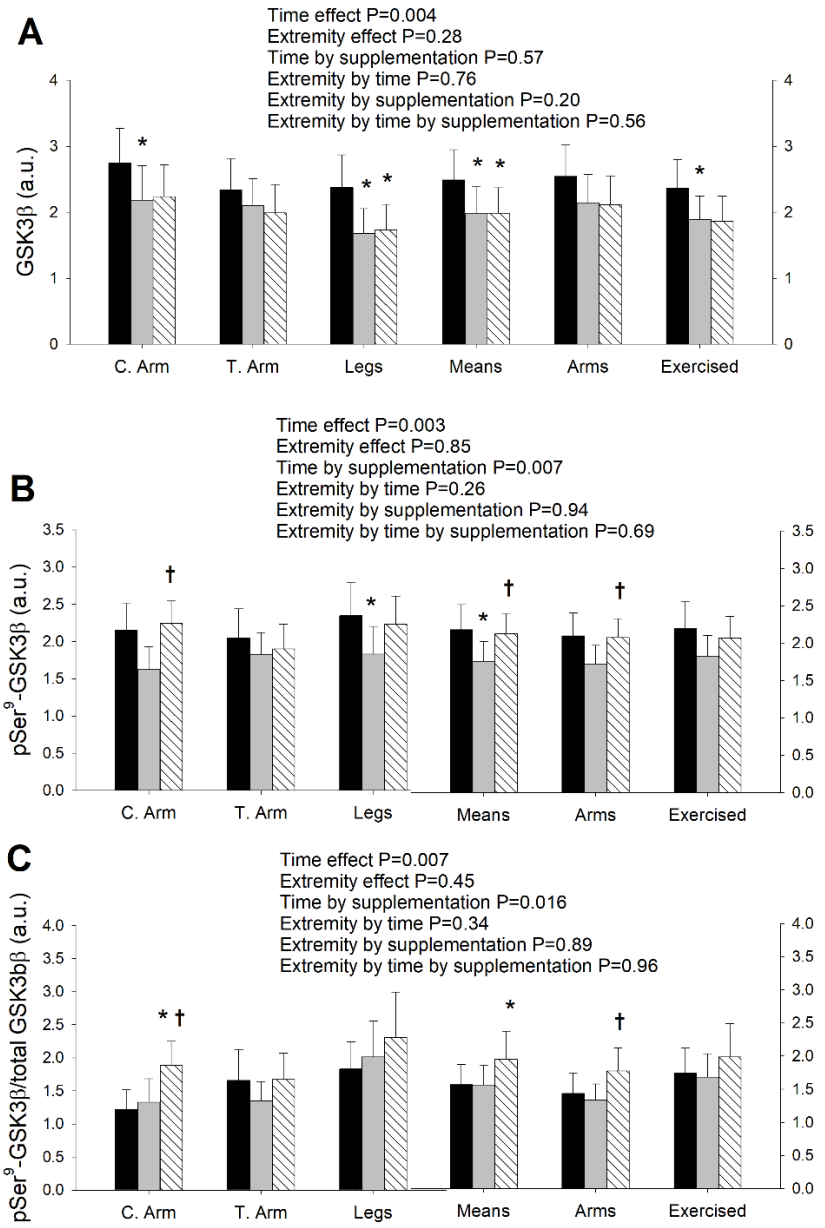


Figure 10. Protein expression levels of total and phosphorylated GSK3β (A-C) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * P < 0.05 compared to PRE; † P < 0.05 compared to CRE; $ P < 0.05 compared to the legs; § P < 0.05 compared to the legs.

Total eIF2α (Figure 11A) did not change significantly, while pSer^{51}eIF2α (Figure 11B) and the pSer^{51}eIF2α/total eIF2α ratio (Figure 11C) were reduced in all muscles after CRE, and remained so after CD.

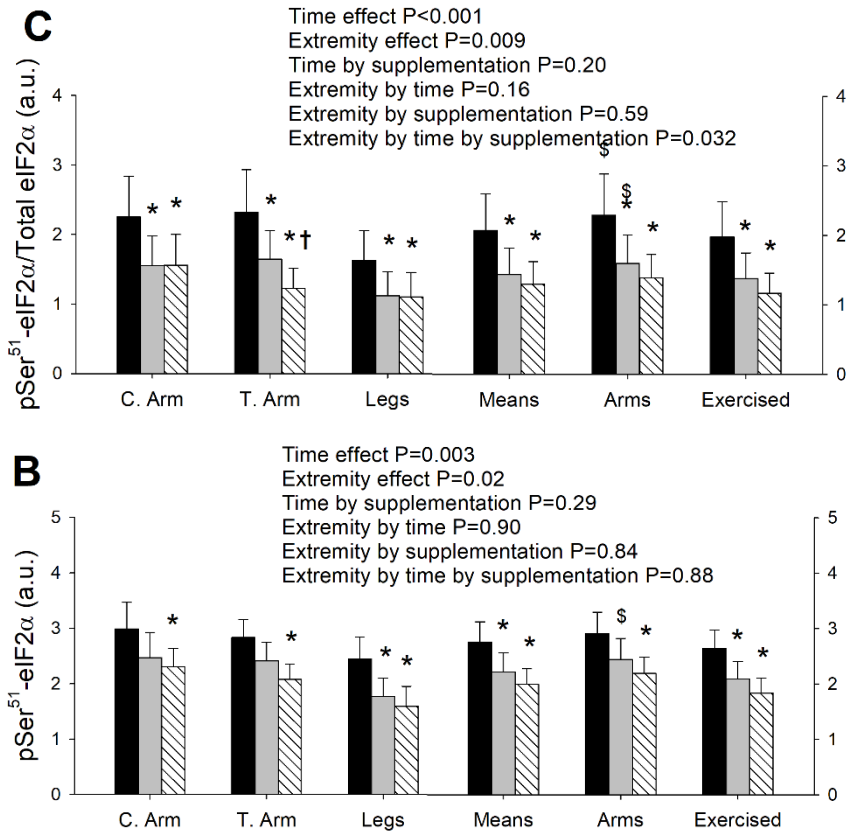


Figure 11. Protein expression levels of total and phosphorylated eIF2α (A-C) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * P < 0.05 compared to PRE; † P < 0.05 compared to CRE; $ P < 0.05 compared to the legs; § P < 0.05 compared to the legs.

10.5. ASSOCIATIONS BETWEEN CHANGES IN PLASMA AMINO ACIDS, HORMONES, LEAN MASS AND MUSCLE SIGNALLING

Akt. There was a linear relationship between Akt protein expression (mean of the three muscles) and the serum concentrations of leptin (r=0.41, P=0.005; n=45), insulin (r=0.33, P=0.026; n=45), Homeostasis Model Assessment (HOMA) (r=0.33, P=0.028; n=45), cortisol/total testosterone (r=0.32, P=0.033; n=45), cortisol/free testosterone (r=0.36, P=0.015; n=45), total testosterone (r=-0.40, P=0.007; n=45), free testosterone (r=-0.42, P=0.004; n=45). The increase in Akt protein expression from PRE to CD (mean of the three muscles) was inversely associated with the corresponding change in insulin (r=-0.72, P=0.003; n=15) and leucine (r=-0.58, P=0.024; n=15). Likewise there was a negative association between the increase in Akt protein expression from PRE to CRE and from PRE to CD with the corresponding changes in cortisol (r=-0.39, P=0.032; n=30), cortisol/total testosterone (r=-0.43, P=0.018; n=30), and cortisol/free testosterone (r=-0.40, P=0.028; n=30). pSer^{473}Akt level (mean of the three muscles) was inversely associated with the serum concentration of EAA (r=-0.36, P=0.015; n=45) and the cortisol /total testosterone (r=-0.31, P=0.038; n=45).

The expression level of mTor (mean of the three muscles) was inversely associated with the serum concentration of EAAs (r=-0.32, P=0.033; n=45). There was a negative association between the increase in mTor protein expression from PRE to CRE and from PRE to CD with the corresponding changes in cortisol (r=-0.40, P=0.028; n=30), cortisol/total testosterone (r=-0.38, P=0.041; n=30). A positive association was observed between the changes from PRE to CRE in pSer^{2448}mTor

in the trained arm and the changes in BCAA (r=0.62, P=0.014; n=15), leucine (r=0.73, P=0.002; n=15) and betahydroxybutyrate (r=0.75, P=0.001; n=15). The corresponding correlation between the changes (mean of the three muscles) of pSer^{2448}mTor with BCAA (r=0.55, P=0.035; n=15) and leucine (r=0.65, P=0.008; n=15) was also statistically significant.

The expression level of total 4E-BP1 was associated with total testosterone (r=-0.29, P=0.049; n=45) and free testosterone (r=-0.42, P=0.005; n=45). The changes from PRE to CRE in Total 4E-BP1 protein expression levels (mean of the three muscles) were associated with the changes in insulin (r=-0.56, P=0.031; n=15), and also when the changes from PRE to CD were included (r=-0.38, P=0.041; n=30), as well as with the changes from PRE to CD in cortisol (r=-0.55,

P=0.035; n=15) and cortisol/total testosterone (r=-0.53, P=0.041; n=15). The changes in 4E-BP1 expression from PRE to CRE and from PRE to CD were associated with the changes in cortisol (r=-0.48, P=0.007; n=30). The increase from PRE to CD in Thr$^{37/46}$4E-BP1 (mean of the three muscles) was associated to the corresponding changes in the serum concentrations of BCAA (r=-0.56, P=0.030; n=15). Likewise, the changes in Thr$^{37/46}$4E-BP1/total 4E-BP1 from PRE to CRE and from PRE to CD were associated with the corresponding changes in cortisol (r=0.37, P=0.043; n=30).

Total GSK3β (mean of the three muscles) was associated with the serum concentration of leptin (r=0.43, P=0.003; n=45), insulin (r=0.46, P=0.001; n=45), and EAA (r=-0.33, P=0.026; n=45). pSer^{9}GSK3β was associated with the serum concentration of leptin (r=0.39, P=0.008; n=45) and insulin (r=0.48, P=0.001;

n=45). The increase of pSer^{9}GSK3β (mean of the three muscles) from PRE to CRE and from PRE to CD was associated with the corresponding increase of leptin (r=0.65, P<0.001; n=30), insulin (r=0.38, P=0.040; n=30) and total testosterone (r=0.40, P=0.027; n=30). Likewise, the changes of pSer^{9}GSK3β/total GSK3β (mean of the three muscles) from PRE to CRE and from PRE to CD were associated with the changes in leptin (r=0.52, P=0.003; n=30), insulin (r=0.37, P=0.044; n=30), BCAA (r=-0.39, P=0.032; n=30), and total testosterone (r=0.52, P=0.003; n=30).

Total eIF2α (mean of the three muscles) was associated with the serum concentration of leptin (r=0.29, P=0.053; n=45), insulin (r=0.34, P=0.024; n=45), and EAA (r=-0.33, P=0.027; n=45). The change in total eIF2α from PRE to CRE

(mean of the three muscles) was associated to the change in BCAA (r=0.56, P=0.029; n=15) and leucine (r=0.58, P=0.023; n=15). pSer^{51}eIF2α (mean of the three muscles) was also associated with the serum concentration of leptin (r=0.34, P=0.021; n=45). The change in pSer^{51}eIF2α from PRE to CRE (mean of the three muscles) was associated with the change in leptin (r=0.58, P=0.023; n=15; for the trained arm only r=0.86, P<0.001; n=15) and cortisol (r=0.65, P=0.009; n=15), while the change from PRE to CD was associated to the change in insulin (r=0.50 P=0.055; n=15). The expression levels of pSer^{51}eIF2α/Total eIF2α were associated to the serum concentration of free testosterone (r=0.30 P=0.042; n=45). The change from PRE to CRE in pSer^{51}eIF2α/Total eIF2α (mean of the three muscles) was associated to the change in leptin (r=0.51, P=0.050; n=15), while the change from PRE to CD was associated with the change in insulin (r=0.72, P=0.003; n=15).

10.6. ASSOCIATIONS BETWEEN CHANGES IN MUSCLE SIGNALLING RESPONSES AND LEAN MASS

There was a positive association between the mean lean mass and the mean $Thr^{37/46}$4E-BP1/total 4E-BP1 (r=0.85, P=0.004, n=9) and the mean $pSer^{9}$GSK3β/total GSK3β (r=0.75, P=0.021, n=9, each point is the mean of the 15 subjects). In the trained arm, there was an association between the changes in lean mass from PRE to CRE and the changes in Akt protein expression (r=0.59, P=0.022, n=15), and from PRE to CD with the changes in $pSer^{51}$eIF2α (r=0.51, P=0.051, n=15) and $pSer^{51}$eIF2α/total eIF2α (r=-0.58, P=0.024, n=15). An association was also observed between the changes from PRE to CD in lean mass (all muscles) and the corresponding changes in $pSer^{473}$Akt (r=-0.33, P=0.028, n=45) and the $pSer^{473}$Akt/total Akt (r=-0.34, P=0.023, n=45). In the control arm, the change in lean mass from PRE to CRE was associated with the change in $pSer^{9}$GSK3β/total GSK3β (r=0.69, P=0.004, n=15).

In the legs, there was a negative association between the changes from PRE to CRE in lean mass and the changes in p70S6k (r=-0.57, P=0.027, n=15). Likewise, there was an association between the changes from PRE to CD in lean mass and the corresponding changes in total mTor (r=-0.56, P=0.03, n=15) and the ratio $pSer^{2448}$mTor/total mTor (r=0.52, P=0.050, n=15), $Thr^{37/46}$4E-BP1 (r=-0.70, P=0.004, n=15).

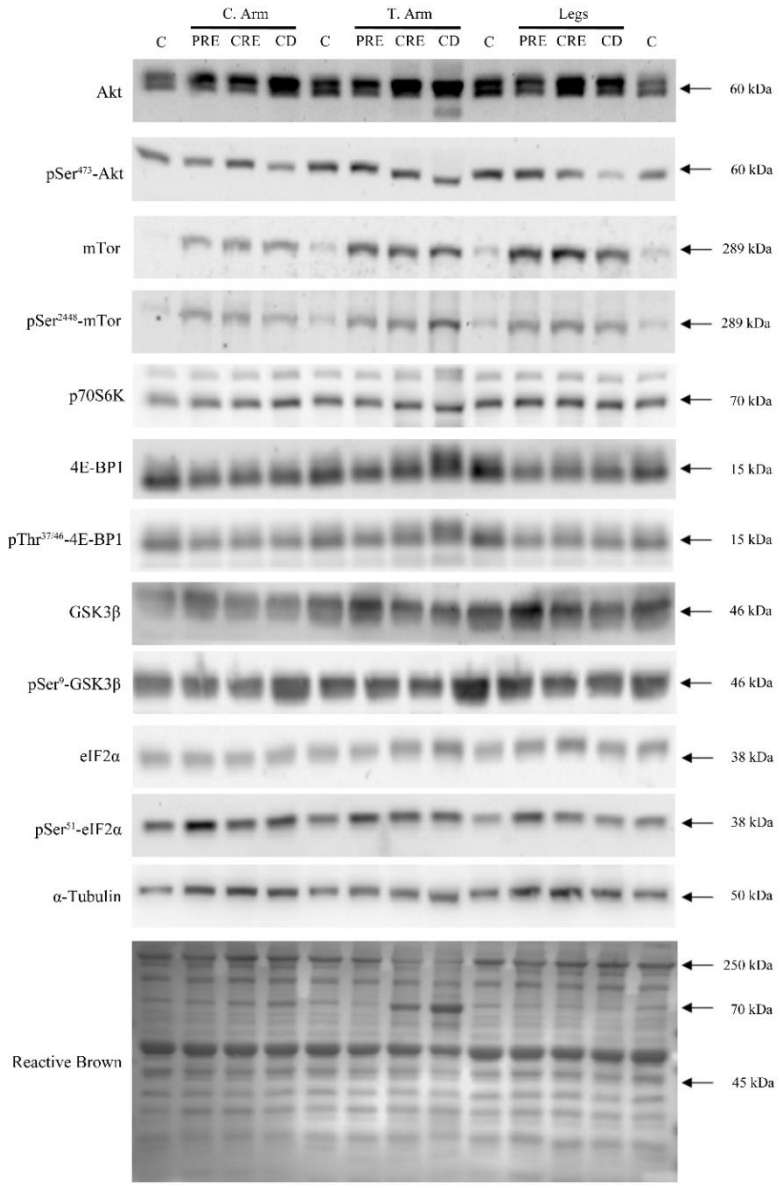


Figure 12. Representative images of protein expression levels (Western Blot) for each protein of interest, its regulatory phosphorylations and total protein amount loaded (Reactive Brown staining) for a single subject from the sucrose group. From top to bottom: Akt, pSer^{473}Akt, mTor, pSer^{2448}mTor, p706SK, 4E-BP1, pThr^{37}/^{46}4E-BP1, GSK3β, pSer^{9}-GSK3β, eIF2α, pSer^{51}eIF2α, and α-tubulin. A non-intervention human muscle sample (C) was included in quadruplicate as a loading control. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs. Based on the applied molecular standards, approximated molecular weights are indicated by arrows on the right.

10.7. mRNA EXPRESSION IN SKELETAL MUSCLE UNDER SEVERE ENERGY DEFICIT: REGULATION OF AUTOPHAGY-LYSOSOMAL AND UBIQUITIN-PROTEASOME SYSTEMS

Muscle gene expression remained unchanged due to diet (whey protein or sucrose) and therefore the two diet groups were merged for all subsequent analyses (n = 15). In the non-exercised arm, 39 genes were differentially expressed between PRE and CRE (FDR < 5%), 14 showed higher and 25 lower expression at CRE. In the exercised arm, of the 44 genes differentially expressed (FDR < 5%), 22 genes showed higher and 22 lower expression (CRE vs PRE, FDR < 5%) (see *Article II*, Ström *et al.* (2018) electronic Supplementary table S2) No significant differences between exercised and control arm in either PRE, CRE or CD.

In the leg, 421 genes (FDR < 5%) were differentially expressed, 206 increased and 215 decreased (CRE vs. PRE) (see *Article II*, Ström *et al.* (2018) electronic Supplementary table S2). This larger number of differentially expressed genes in the leg could reflect the higher amount of exercise performed by this muscle group during the intervention. The 10 most differentially expressed genes at CRE compared with PRE in the exercised, non-exercised arm and leg muscles are reported in *Article II* section (see appendix, *Article II*, Table 1). Genes differentially expressed in more than one tissue are displayed in supplementary figure 1 (see appendix, *Article II*), *e.g.* 19 genes were found in all three muscle tissues. The only gene found to be differentially expressed between CRE and CD (FDR < 5%) in the non-exercised arm was carnitine palmitoyltransferase 2 (*CPT2*). In the exercised arm, three genes, including *CPT2*, were differentially expressed (CRE vs. CD, FDR

< 5%). Of them, *CPT2* and high-mobility group protein B2 (*HMGB2*) were regulated in the opposite direction compared to CRE versus PRE. In the leg, 147 genes were found differentially expressed between CRE and CD (FDR < 5%) and out of these 121 (82%) were regulated in the opposite direction compared to CRE versus CD, *i.e.* a large proportion of the identified changes in gene expression induced by the 4-day intervention was reversed by the 3-day period with isoenergetic diet and limited exercise. All differentially expressed genes (FDR < 5%) in PRE vs CRE and CRE vs. CD can be found in *Article II*, Ström *et al.* (2018), electronic Supplementary Tables S2 and S3, respectively.

The level of expression of a number of atrogenes involved in the ubiquitin-proteasome and the autophagy lysosomal pathways was upregulated primarily in the legs (Table 2).

From PRE to CRE, six autophagy genes were upregulated in the legs (e.g. *BNIP3, p62/SQSTM1, SH3GLB1* (SH3 domain containing GRB2 like endophilin B1)) while one gene was downregulated. A single gene was downregulated in the non-exercised arm (i.e. *TP53INP2* (tumour protein p53 inducible nuclear protein 2) and none in the exercised arm (FDR < 5%). Six genes involved in the ubiquitin proteasome (e.g. *TRIM63/MuRF1* and *PSMD4* **(**26S proteasome non-ATPase regulatory subunit 4)) were upregulated in the legs while one gene (*TRIM7,* tripartite motif containing 7) was downregulated in the exercised arm from PRE to CRE. From CRE to CD, three atrogenes were still upregulated (*TRIM63/MuRF1*, *RHEB* (Ras homolog enriched in brain) and *CTSD (*cathepsin D)) in the legs while the remaining upregulated genes were reversed in all extremities by the subsequent

phase of isoenergetic diet and limited exercise (FDR < 5%). Altogether, these results indicate an upregulation of the principal catabolic pathways following CRE which is exacerbated in the legs, the muscle group submitted to a higher amount of exercise.

NON-EXERCISED ARM				EXERCISED ARM				LEG			
TargetID	**Entrez GeneID**	**Fold change**	**FDR adj. P-value**	**TargetID**	**Entrez GeneID**	**Fold change**	**FDR adj. P-value**	**TargetID**	**Entrez GeneID**	**Fold change**	**FDR adj. P-value**
**TP53INP2*	58476	0.55	0.00623	$TRIM7*	81786	0.51	0.02776	$CDKN1A*	1026	3.01	0.01522
								$MYC*	4609	2.21	0.00023
								**BNIP3*	664	1.79	0.00068
								**CTSD*	1509	1.75	0.00000
								**SQSTM1*	8878	1.67	0.00061
								$TRIM63/ MURF1*	84676	1.58	0.01417
								**SH3GLB1*	51100	1.48	0.00233
								$RHEB*	6009	1.46	0.00031
								**ITGA3*	3675	1.40	0.03242
								$PSMD4*	5710	1.36	0.01523
								$**PARP1*	142	0.80	0.01355

Table 2. Ubiquitin-proteasome (marked by $ symbol) and autophagy-lysosomal-related (marked by *) genes ranked on fold change (up or down) in skeletal muscle from non-exercised arm (control deltoid), exercised arm and leg (*vastus lateralis*) following the 4-day intervention (PRE vs. CRE).

DISCUSSION

11. DISCUSSION

The findings from the present investigation have demonstrated that during a severe energy deficit there is an impeded activation of the protein signalling cascade primarily mediated by a reduced Ser^{9}GSK3β which blunts translation initiation. Moreover, we have shown that the skeletal muscles become refractory to the anabolic effects of whey protein ingestion during a severe energy deficit and this effect cannot be counteracted by moderate or large amounts of contractile activity. Furthermore, the changes observed in gene expression indicate that a severe energy deficit despite the presence of low-intensity exercise does not favour muscle anabolism and, on the contrary, protein catabolism is upregulated by an increased expression of genes involved in the ubiquitin-proteasome and autophagy-lysosomal systems, with these effects being reversed by three days of isoenergetic diet and restricted exercise.

Although protein synthesis in skeletal muscle is known to be stimulated by amino acids to a greater extent when the muscle has been exercised, the current study shows that when the energy deficit is severe or extreme, protein synthesis becomes resistant to the anabolic effect of amino acids and moderate-to-large amounts of low-intensity exercise do not ameliorate this anabolic resistance. The present study also shows for the first time that during a severe energy deficit the serum concentration of the anabolic hormones insulin and testosterone are reduced, blunting the translation initiation due to insufficient inactivation of GSK3β through phosphorylation at $serine^{9}$. *In vitro* and *in vivo* experiments have shown that phosphorylation of Akt, in skeletal muscle is stimulated by insulin, testosterone

and leptin, and Ser^{9}GSK3β phosphorylation is catalysed by Akt (Kohn *et al.*, 1995; White *et al.*, 2013; Garcia-Carrizo *et al.*, 2016) (Figure 13). The associations observed here between the reductions in serum insulin, leptin and pSer^{9}GSK3β support a mechanistic link between the drop in these hormones and the lack of activation of protein synthesis, despite the fact that the circulating concentrations of amino acids were increased in both groups. As a consequence, circulating amino acids cannot be incorporated into muscle proteins during a severe energy deficit and are left available for gluconeogenesis, which is essential to maintain the glycaemia under these conditions (Felig *et al.*, 1969). Cell experiments give support to this interpretation as it has been shown that insulin, via Akt activation, phosphorylates GSK3β (Proud, 2006), while leptin can signal in skeletal muscle through extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), which is also able to elicit Ser^{9}GSK3β phosphorylation (Ding *et al.*, 2005). Moreover, we found that pSer^{9}GSK3β was also associated with total testosterone, which via Akt activation elicits GSK3β phosphorylation (White *et al.*, 2013).

Protein degradation is increased during energy deficiency (Carbone *et al.*, 2014) as well as after endurance- or resistance-type exercise performed in the fasted state (Kumar *et al.*, 2009) as a mechanisms aiming to remove damaged structures and allow for muscle remodelling and adaptation to exercise. Genetic knockout models have clearly shown an upregulation of several E3 Ubiquitin ligases in various atrophy models, being the most widely studied MAFbx and MuRF1 (Bodine *et al.*, 2001; Gomes *et al.*, 2001). We have shown for the first time in human skeletal muscle an upregulated expression of atrogenes and autophagy-related genes following prolonged exercise combined with a very low calorie diet,

which are primarily present locally in the skeletal muscles subjected to an exceptional level of exercise. In contrast with previous findings, protein ingestion did not altered the overexpression of ubiquitin proteasome-related genes (Carbone *et al.*, 2014) nor the expression of genes involved in autophagy (Smiles *et al.*, 2015).

11.1. THE ACUTE EFFECTS OF SEVERE ENERGY DEFICIT ON THE SIGNALLING CASCADES THAT REGULATE PROTEIN SYNTHESIS

The signalling activity of the Akt/mTor/p70S6K axis regulating protein translation did not manifest any significant changes after four days of a severe energy deficit close to 5500 kcal/day, regardless of the level of contractile activity or protein supplementation. A partial and transient activation of the Akt/mTor/p70S6K axis during the first hours after the ingestion of supplements is plausible as there was an activation of two downstream targets of mTor. However, given that the biopsies were performed in the fasted state following a 12-h rest period, these effects on protein translation may have escaped detection. These was suggested by the elevation of the levels of total 4E-BP1 and $Thr^{37/46}$4E-BP1, a downstream target of mTor, in all muscles after CRE. This conclusion is also supported by the reductions of $pSer^{51}$eIF2α and the ratio $pSer^{51}$eIF2α/total eIF2α observed in all muscles after CRE, which should have facilitated translation initiation (Baird & Wek, 2012).

However, this stimulating effect elicited by the elevated $Thr^{37/46}$4E-BP1 and the reduced $pSer^{51}$eIF2 α and $pSer^{51}$eIF2α/total eIF2α ratio was counteracted by the decrease of $pSer^{9}$GSK3ß after CRE. GSK3β intervenes in the regulation of protein

synthesis, cell proliferation and survival (Huo *et al.*, 2014). Upon phosphorylation, GSK3β activity is inhibited what causes its substrate, eukaryotic initiation factor 2B (eIF2B), to become dephosphorylated and activated (Frame & Cohen, 2001). Thus, the lowered levels of pSer^{9}GSK3ß might have increased the inhibitory action of GSK3β on protein synthesis. In contrast to our expectations, the reduction of pSer^{9}GSK3ß was more marked in the protein group. This could have caused a greater inhibition of protein synthesis in this group despite the higher levels of BCAA and leucine present following the intervention (Calbet *et al.*, 2017). Consequently, the balance between protein synthesis and breakdown was not influenced significantly by the ingestion of whey protein, and both groups lost a similar proportion of lean mass (Calbet *et al.*, 2017).

Since 4E-BP1 inhibits protein synthesis, the increase of total 4E-BP1 observed here might have attenuated protein synthesis. However, this potential effect was counteracted by a commensurate increase in Thr$^{37/46}$4E-BP1 after CRE. Interestingly, there was an inverse association between the levels of total 4E-BP1 on the one hand, and testosterone and free testosterone on the other hand, as well as between the levels of pSer^{9}GSK3β and total testosterone. This suggests that testosterone may down-regulate 4E-BP1 and up-regulate GSK3β to facilitate protein synthesis, as has been reported in animal and cell culture studies (White *et al.*, 2013). Likewise, the rise in the level of pSer^{51}eIF2α from PRE to CRE, which impedes translation initiation, was associated with the change in serum cortisol. Altogether, these findings confirm that the serum cortisol/testosterone ratio plays a critical role in the regulation of protein synthesis.

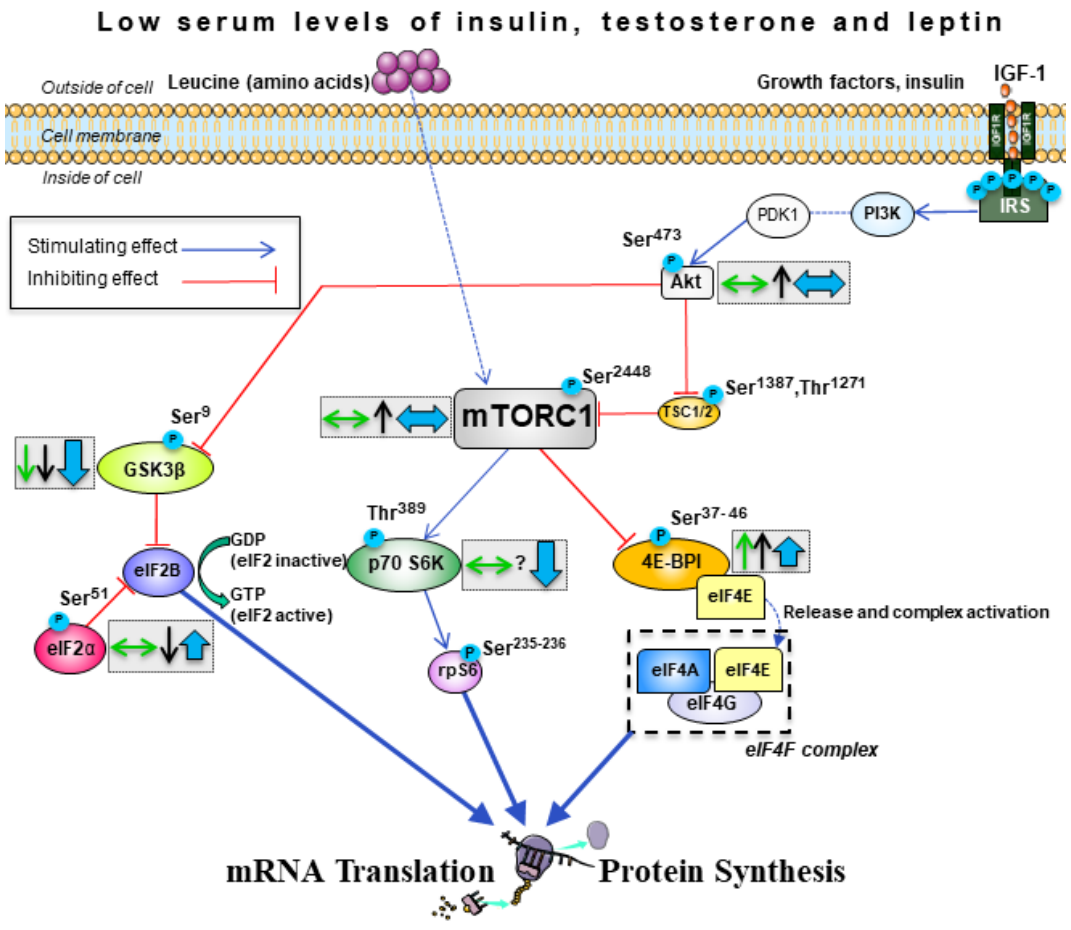


Figure 13. Schematic representation of the proposed mechanisms regulating translation initiation and protein synthesis in human skeletal muscle under a severe energy deficit.

In a state with low serum concentrations of insulin, testosterone and leptin, anabolic signalling activated by insulin-like growth factor-1 signalling (IGF1), acting via its receptor (IGFR) and the insulin receptor substrate 1 (IRS1), is negatively regulated blunting Akt activation. Akt and other protein kinases modulate the GTPase activity of tuberous sclerosis proteins 1 and 2 (TSC1/2), repressing their GTP activity and thus stimulating mTOR-derived signalling. Such stimulation is likely to be lacking during severe energy deficit as reflected by the unchanged levels of phosphorylated Akt observed here. The amino acid induction of protein synthesis via mTOR activation (through various intermediary proteins) is blunted in such a high energy-deficient state, likely due to the proteolytic effect resulting from the increased cortisol and the reduced testosterone plasma concentrations. mTOR regulates protein synthesis by phosphorylating p70 ribosomal protein S6 kinase (p70S6K) and eIF4E-binding protein 1 (4E-BP1). A partial activation of the pathway is seen with the presence of: 1) increased levels of phosphorylated 4E-BP1, which allows the release of the mRNA cap-binding protein eIF4E, required for the formation of the eIF4F complex, essential for the global increase in translation and 2) a reduction in the phosphorylation of the alpha subunit of eIF2 (eIF2α) on serine 51 induces eIF2B activation and thereby partial stimulation of translation initiation. However, p70S6k remains dephosphorylated (inactivated) impeding the phosphorylation of its target (ribosomal protein S6; rpS6), leading to downregulation of mRNA translation. Phosphorylation of the GDP-GTP exchange factor eIF2B by glycogen synthase kinase 3β (GSK3β) leads to its inhibition downregulating translation initiation. Upon anabolic stimulation GSK3β is phosphorylated (inactivated) by Akt allowing translation initiation, however, the severe energy deficit observed here blunts Akt-mediated inactivation of GSK3β.

The arrows inside dashed grey boxes presented besides protein markers depict overall outcomes in the present investigation. Green (total form) and black (phosphorylated form) thin arrows represent the overall direction of the results (increase/decrease) after the intervention (mean of all muscles). Blue thick arrows represent the overall effect on protein synthesis (stimulation/inhibition). The length of the arrow is representative of the magnitude of the change. Dashed blue connecting lines indicate indirect effect (mediator proteins not shown).

11.2. THE PRESERVATION OF MUSCLE MASS BY EXERCISE CANNOT BE EXPLAINED BY CHANGES IN PROTEIN SYNTHESIS SIGNALLING

Despite failing to enhance protein synthesis signalling, exercise resulted in a remarkable preservation of muscle mass in an exercise-dose dependent manner (Calbet *et al.*, 2017). Therefore, our data point towards an attenuation of protein breakdown as the potential main mechanisms by which exercise spares muscle mass during a severe energy deficit. Unexpectedly, administration of whey protein, which is rich in leucine and stimulates protein synthesis while attenuating protein breakdown in humans (Pennings *et al.*, 2011), did not preserve lean mass, regardless of the level of exercise. The amount of protein ingested (0.8 g/kg body weight), as well as the timing (every 8 h), should have maximally stimulated protein synthesis in both the rested and exercised muscles (Witard *et al.*, 2014). It remains unknown whether some stimulation of protein synthesis could have been achieved by administering 1.6 or even 2.4 g/kg body weight of dietary protein per day, as reported during low-calorie diets (Carbone *et al.*, 2012; Pasiakos *et al.*, 2013). However, Hector et al. have recently reported a reduction of protein synthesis during a 10-day LCD (40%-reduced energy intake), which was not altered by doubling the daily protein intake to 2.4 g/kg body weight (Hector *et al.*, 2018). Thus, the present and Hector et al. studies indicate that during moderate (Hector *et al.*, 2018) and severe energy deficits the skeletal muscle becomes refractory to the stimulation of protein synthesis by the ingestion of proteins, even when 3 times the RDA for protein is administered (Hector *et al.*, 2018).

One possible explanation for the lack of differences between our dietary groups involves their similar catabolic response during CRE. In fact, we have demonstrated that the amount of lean mass lost can be predicted from the change in the catabolic index (cortisol/free testosterone) (Calbet *et al.*, 2017), which rose similarly in both groups. We can only speculate as to why the ingestion of whey protein was associated here with an elevated catabolic index. One plausible explanation is the requirement for more pronounced activation of the hypothalamic/pituitary/adrenal axis when whey protein alone is ingested in order to avoid hypoglycaemia (Watts & Donovan, 2010). In rodents, a diet with high proteins to carbohydrates ratio content promotes the expression of tumour necrosis factor alpha (TNF-α) mRNA (Diaz-Rua *et al.*, 2017), which via activation of NF-κB, may facilitate muscle proteolysis.

11.3. THE EXERCISED ARM MUSCLES ARE LIKELY MORE SENSITIVE THAN THE LEG MUSCLES TO STIMULATION OF PROTEIN SYNTHESIS BY CIRCULATING AMINO ACIDS DURING SEVERE ENERGY DEFICIT

It has been reported that exercise potentiates the anabolic response to amino acids (Blomstrand *et al.*, 2006). Accordingly, a positive association was observed between the changes from PRE to CRE in pSer^{2448}mTor in the trained arm and the changes in plasma BCAA and leucine. Infusion of BCAA or leucine alone is known to enhance 4E-BP1 phosphorylation in human skeletal muscle (Liu *et al.*, 2001). In the present investigation, the serum levels of all three BCAA were elevated after CRE in both groups, although more markedly in the subjects ingesting whey

protein, despite the fact that the blood samples were drawn 12 hours after the last ingestion of approximately 26 g of whey protein. Despite a greater BCAA and leucine plasma concentration during the CRE in the group supplemented with proteins, 4E-BP1 phosphorylation was similarly increased in both groups, without clear differences between the exercised and non-exercised muscles. However, the protein synthesis-signalling cascade was blunted due to the reduced pSer^{9}GSK3β, probably caused by the increased serum cortisol/testosterone ratio present in all muscles. Thus, these findings indicate that when the energy deficit becomes severe, the anabolic effect of amino acids is not potentiated by the contractile activity hold by the exercised muscles.

11.4. THE INCREASE IN CORTISOL LEVELS DURING FASTING IS ASSOCIATED WITH BLUNTED PROTEIN SYNTHESIS SIGNALLING

The elevation of serum cortisol levels in both groups following CRE may have contributed to the inhibition of protein synthesis by lessening the increase in total 4E-BP1, leaving p70S6K in a dephosphorylated state (Shah *et al.*, 2000a) and reducing pSer^{51}eIF2α (Rannels *et al.*, 1980). Moreover, glucocorticoids inhibit the uptake of amino acids by muscle cells (Kostyo & Redmond, 1966); stimulate KLF-15 (Kruppel-like factor 15), which activates the transcription of the BCAT (branched-chain amino acid aminotransferase) gene (Shimizu *et al.*, 2011), encoding an enzyme involved in BCAAs catabolism; and inhibit the phosphorylation of 4E-BP1 and S6K1 mediated by insulin and IGF-I (insulin-like growth factor I) (Shah *et al.*, 2000b). Glucocorticoids may also inhibit

phosphorylation of PI3K and Akt (Zheng *et al.*, 2010), upstream kinases of mTor. The inhibitory phosphorylation of FoxOs is dependent on Akt phosphorylation which excludes FoxOs from the nucleus therefore downregulating their transcriptional activity (Brunet *et al.*, 1999). Indeed, mice in which Akt is constitutively activated are resistant to the negative effects of glucocorticoids on protein synthesis (Frost & Lang, 2012) and muscle mass (Schakman *et al.*, 2008).

11.5. SERUM LEPTIN CONCENTRATION IS POSITIVELY ASSOCIATED WITH PROTEIN SYNTHESIS SIGNALLING IN HUMAN SKELETAL MUSCLE

As another novelty, we have found a close association between serum leptin concentration and the mean protein expression of total Akt, total GSK3β and pSer^{9}GSK3β in the three analysed muscles. Moreover, there was also a close association between the changes in serum leptin concentration and the changes in pSer^{9}GSK3β. Although these associations are compatible with the proposed anabolic effect of leptin in skeletal muscle (Arounleut *et al.*, 2013; Perez-Suarez *et al.*, 2017), the prolonged administration of leptin to amenorrhoeic athletes has not been associated with significant changes in lean mass (Brinkoetter *et al.*, 2011). Moreover, no activation of protein synthesis signalling was observed in human skeletal muscle 20 min after the intravenous administration of metreleptin (Moon *et al.*, 2015). Since leptin receptors were up-regulated (Perez-Suarez *et al.*, 2017) and the serum concentration of leptin increased from CRE to CD (i.e., with re-feeding), we think that leptin may have a role in easing a potential anabolic response of skeletal muscles upon re-feeding.

11.6. SEVERE ENERGY DEFICIT ELICITS UPREGULATION OF GENES INVOLVED IN THE UBIQUITIN PROTEASOME SYSTEM, PRIMARILY IN THE MUSCLES SUBJECTED TO A HIGH EXERCISE VOLUME

Low levels of insulin and elevated levels of glucocorticoids trigger the activation of proteolysis and several models of muscle loss (Mitch *et al.*, 1999). Accordingly, following the four-day intervention, *TRIM63/MuRF-1*, a crucial gene encoding a ubiquitin ligase was upregulated in the legs. Similarly, *PSMD4*, a gene encoding proteasomal subunits was increased in this muscle group. These genes have been shown to be upregulated during atrophic conditions (Combaret *et al.*, 2004; Glass, 2005). In fact, an increase in protein degradation through proteasome is dependent on the increased activation of *TRIM63/MuRF-1* (Sandri *et al.*, 2004). Other genes associated with activation of ubiquitin proteasome were also upregulated, although their exact role in muscle remains to be elucidated (e.g. *TRIM7, MYC, PARP1, CDKN1A)*. The Akt/FoxO signalling pathway regulates the expression of *TRIM63/MuRF1* whose transcription is upregulated under Akt protein levels are decreased, promoting nuclear exclusion of FoxO. Upregulation of *TRIM63/MuRF1* is regulated by the activation of the inhibitor of kappa, alpha (IκBα)/nuclear factor kappa B (NFκB) signalling pathway. The unaltered protein expression levels of Akt together with the increased IκBα/NFκB signalling (data not shown) seen after the four-day intervention are compatible with the upregulation of the ubiquitin proteasome system.

11.7. SEVERE ENERGY DEFICIT ELICITS THE EXPRESSION OF GENES INVOLVED IN AUTOPHAGY INDUCTION AND BLOCKADE OF AUTOPHAGIC FLUX

The results of the current investigation show changes in the expression of genes involved directly or indirectly in the autophagy-lysosomal system following four days of severe energy restriction concomitantly with prolonged low-intensity exercise. The upregulation of *BNIP3* is reported to increase autophagy induction and particularly mitophagy (selective degradation of mitochondria by autophagy (Tolkovsky, 2009)) (Hamacher-Brady *et al.*, 2006). Indeed, *BNIP3* upregulation alone is sufficient to induce autophagosome formation in skeletal muscle (Mammucari *et al.*, 2007). The overexpression of this gene was supported by a ~95% increase of protein levels which was similar in all muscles (data not shown). *SH3GLB1*, a gene involved in membrane biogenesis for the formation of autophagosomes (Khan *et al.*, 2014) was also overexpressed in the legs after CRE.

After CRE, *p62/SQSTM1* expression was increased in the legs. The levels of p62/SQSTM1 protein are considered a marker of the rate of lysosomal degradation and accumulation of this protein is associated with autophagic-flux inhibition (Komatsu *et al.*, 2007) despite some controversy (Fritzen *et al.*, 2016b). The changes in gene expression here obtained are supported at the protein level by a ~95% and ~25% increase in legs and arms, respectively (data not shown). In line with this, *TP53INP2* expression was downregulated following CRE. *TP53INP2* is required in mammalian systems for autophagome formation and completion recognised to interact with LC3 and its ortholog GABARAP (GABA Type A

Receptor-Associated Protein) (Nowak *et al.*, 2009), which may be indicative of a block in autophagic flux. Other genes directly or indirectly involved in autophagy were up- or down-regulated, although their biological function, particularly in skeletal muscle, remains unknown (e.g. *ITGA3, PARP1, CTSD).*

Our results are in agreement with experiments studying *in vivo* gene expression changes in response to food deprivation and denervation in rodents resulting in muscle atrophy (Mammucari *et al.*, 2007; Zhao *et al.*, 2007) as well as in humans following strenuous long-lasting endurance exercise protocols (Jamart *et al.*, 2012a), highlighting a similar pattern of catabolic state in skeletal muscle. Interestingly, in contrast to rodent studies, during prolonged endurance exercise (~>2 h), an increase in autophagy induction but blocked autophagic flux has been reported in skeletal muscle (Glynn *et al.*, 2010; Møller *et al.*, 2015; Schwalm *et al.*, 2015; Fritzen *et al.*, 2016a; Moberg *et al.*, 2017). The latter is in agreement with the changes in gene expression reported here together with our unpublished protein expression (Martin-Rincon *et al.*, 2016; Martin-Rincon *et al.*, 2017; Martin-Rincon *et al.*, 2018b). Autophagy is known to promote cellular survival by controlling and appropriate level of amino acid pools and providing maintaining energy levels (Botti-Millet *et al.*, 2016). Therefore, we speculate with a plausible protein-sparing mechanism present in humans under high levels of cellular stress driven by energy deficiency and enormous levels of contractile activity

Taken together, our results show an upregulation in the ubiquitin-proteasome and autophagy-lysosomal gene expression in human skeletal muscle following four days of combined caloric restriction and prolonged exercise. To the

best of our knowledge, no study has characterized to date the changes in gene expression of the principal proteolytic pathways regulating muscle mass under conditions of severe energy deficiency using differentiating between patterns in exercised and non-exercised muscle groups. The increased expression of transcripts was primarily present locally in the legs, suggestive that both systems are strongly activated by large amounts of contractile activity but not to the same extent in non-exercise or moderately exercised muscle groups under conditions of energy deficiency. Such increases have been associated previously with a state of muscle catabolism (Masiero *et al.*, 2009; Sandri, 2013).

11.8. LIMITATIONS

Although muscle glycogen concentrations were not assessed, the fact that the total concentration of proteins in skeletal muscle was increased by 4.9% after CRE is compatible with a marked reduction of muscle glycogen and the associated water. In fact, we have estimated that the increase in protein concentration observed here after CRE could be explained by 11-12 g/kg wet muscle reduction in muscle glycogen (Fernandez-Elias *et al.*, 2015). Muscle protein concentration returned to baseline values after the three days on a control diet, compatible with at least partial recovery of muscle glycogen stores, and therefore our lean mass assessment obtained three days after the end of CRE should reflect more trustfully the actual changes in muscle mass, than the measurements performed after CRE. Lastly, we could not find a molecular explanation for the mechanism by which exercise attenuated the loss of muscle mass. However, since all muscles were likely exposed to the same internal neuroendocrine milieu, we think that this molecular

mechanism should be specifically triggered by contractile activity inside the active muscle fibers. Data recently obtained and not included in the current investigation point towards an attenuation of autophagy-flux markers and reduced levels of IκBα/NFκB signalling in the arms when compared to legs as plausible molecular mechanisms driving the muscle-sparing effect of exercise during severe energy deficiency.

Signalling in muscle biopsies is highly dependent on temporal factors. In the current study, all biopsies were taken in the fasted state on the morning following the preceding phase (i.e. CRE and CD). Potential signals driving acutely by exercise and or the ingestion of the protein or carbohydrate solutions during CRE could have being overlooked.

CONCLUSIONS

12. CONCLUSIONS

1. Exercise exerts a muscle mass sparing effect during a severe energy deficit in an exercise-dose dependent manner, and therefore more pronounced in the active muscles and particularly in those subjected to a higher exercise volume.

2. The combination of prolonged low-intensity exercise and caloric restriction results in a catabolic state characterised by reduced plasma concentrations of testosterone, leptin and insulin which cause a loss of fat mass and fat-free mass.

3. During a severe energy deficit, protein synthesis and translation initiation are blunted primarily by a reduced phosphorylation of GSK3β at Ser^{9}.

4. The skeletal muscle is refractory to the protein synthesis effect of whey protein ingestion during a severe energy deficit, regardless of the performance of moderate or large amounts of contractile activity.

5. The combination of prolonged exercise and caloric restriction upregulates the expression of atrogenes and autophagy-related genes, suggestive of a state of catabolism, with these effects being primarily present in the muscles subjected to large amounts of exercise.

6. The ingestion of neither protein nor carbohydrate alter the modulation of atrogenes and autophagy-related genes observed in response to a severe energy deficit, in spite of the presence of large-to-moderate volumes of contractile activity.

FUTURE PERSPECTIVES

13. FUTURE PERSPECTIVES

Further studies should assess whether combining the ingestion of carbohydrates and protein, increasing the protein intake to higher doses and/or including other types of exercise could hold additional effects on the modulation of protein turnover during a severe energy deficit. Specifically, the response of a combination of very low calorie diets with resistance exercise seems to be of particular interest, as this type of training is the most effective to achieve muscle hypertrophy.

Future experiments should be undertaken to provide further insight into the relevance of the inhibition and/or stimulation of GSK3β Ser^{9} phosphorylation to ease the anabolic response to amino acids in human skeletal muscle under a situation of severe energy deficient as well as during less pronounced energy deficits.

The upregulation of the two major proteolytic pathways in the current investigation further opens an avenue of research on the modulation of the main catabolic signals driving the loss of muscle mass during severe energy deficiency. Assessment of the proteomic responses involved will provide with a better understanding of their role in the loss of muscle mass and the sparing effect exerted by exercise under these conditions and also during less extreme protocols of energy deficiency sustained over longer periods. Simultaneous determinations of muscle protein synthetic and breakdown rates with stable isotopes have not been carried out in humans. Future studies including the assessment of protein turnover with stable isotopes and muscle signalling cascades could help to delineate the dynamics of these processes in humans.

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14. REFERENCES

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APPENDIX

ARTICLE I

Protein synthesis signaling in skeletal muscle is refractory to whey protein ingestion during a severe energy deficit evoked by prolonged exercise and caloric restriction

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Abstract

Background: Exercise and protein ingestion preserve muscle mass during moderate energy deficits.

Objective: To determine the molecular mechanisms by which exercise and protein ingestion may spare muscle mass during severe energy deficit (5500 kcal/day)

Design: Fifteen overweight, but otherwise healthy men underwent a pre-test (PRE); caloric restriction (3.2 kcals/kg body weight/day) + exercise (45 min one-arm cranking + 8h walking) for 4 days (CRE); followed by a control diet (CD) for 3 days, with a caloric content similar to pre-intervention while exercise was reduced to less than 10.000 steps per day. During CRE, participants ingested either whey protein (PRO, n=8) or sucrose (SU, n=7) (0.8 g/kg body weight/day). Muscle biopsies were obtained from the trained and untrained deltoid, and vastus lateralis.

Results: Following CRE and CD, serum concentrations of leptin, insulin, and testosterone were reduced, whereas cortisol and the catabolic index (cortisol/total testosterone) increased. The Akt/mTor/p70S6K pathway and total eIF2α were unchanged, while total 4E-BP1 and $Thr^{37/46}$4E-BP1 were higher. After CRE, plasma BCAA and EAA were elevated, with a greater response in PRO group, and total GSK3β, $pSer^{9}$GSK3β, $pSer^{51}$eIF2α and $pSer^{51}$eIF2α/total eIF2α were reduced, with a greater response of $pSer^{9}$GSK3β in the PRO group. The

changes in signaling were associated with the changes in leptin, insulin, amino acids, cortisol, cortisol/total testosterone and lean mass.

Conclusions: During severe energy deficit pSer^{9}GSK3β levels are reduced and human skeletal muscle becomes refractory to the anabolic effects of whey protein ingestion, regardless of contractile activity. These effects are associated with the changes in lean mass and serum insulin, testosterone and cortisol concentrations.

Introduction

Although weight-loss programs are designed to eliminate fat mass (FM), 10-53% of the weight lost is fat-free mass (FFM) [1], with greater loses of FFM after interventions involving very-low-calorie diets (VLCD, <800 kcal/d) or surgical procedures [1]. In the case of low-calorie diets (LCD), loss of FFM can be attenuated by concomitant exercise and by increasing the ratio of proteins to carbohydrates in the diet [2], although this effect appears to be limited to prolonged interventions eliciting a moderate energy deficit.

Essential amino acids, and in particular leucine, are powerful stimulators of protein synthesis [3], an effect that may be potentiated by exercise [3]. However, severe energy deficit, such as during starvation, elicits a neuroendocrine response characterized by high cortisol and reduced testosterone levels in serum (raising the catabolic cortisol/testosterone index) [4]. This is accompanied by reduced insulin levels and increased peripheral insulin resistance [5], which may together attenuate the anabolic response to amino acids [6]. The mechanism by which exercise and amino acids interact to stimulate protein synthesis during severe energy deficiency remains unknown [7].

Serum leptin concentration is also markedly reduced during starvation [8]. Skeletal muscle expresses functional leptin receptors whose signaling responses share some common steps with insulin and growth factors [9]. However, the potential role that leptin may have on the regulation of muscle mass during starvation remains unknown in humans. Protein synthesis in muscle is stimulated by the

pathway involving the mammalian target of rapamycin (mTor) protein kinase, which is activated through phosphorylation by Akt (protein kinase B). Activated mTor forms two distinct complexes, TORC1 and TORC2. TORC1 is sensitive to inhibition by rapamycin and propagates downstream signaling by phosphorylating p70 ribosomal S6 kinase (p70S6K, also referred to as S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (also referred to as PHAS-1), resulting in their activation and inhibition, respectively [10].

Phosphorylation of 4E-BP1 and p70S6K by mTor are essential for protein synthesis and hypertrophy in muscle [10]. Akt can promote protein synthesis by inhibiting glycogen synthase kinase 3 beta (GSK3β), impeding its inhibitory action on protein synthesis [11]. Protein synthesis requires sufficient and appropriate amino acid supply, and amino acid deprivation induces the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), thereby impeding translation initiation [12]. However, little is presently known concerning the regulation of these signaling cascades by muscle contractions and plasma amino acids in humans under a severe energy deficit.

The current investigation was designed to assess the body composition and major signaling pathways regulating protein synthesis in three different skeletal muscles of overweight volunteers undergoing a severe energy deficit. For this purpose, a VLCD involving intake of solely sucrose or whey protein was combined with 45 min of one-arm cranking and eight hours of walking each day. Accordingly, in each subject one deltoid muscle remained inactive, the

contralateral deltoid was exercised moderately, and the *vastus lateralis* was subjected to prolonged exercise. Our hypothesis was that a whey protein diet will attenuate the loss of muscle mass by activating the Akt/mTor/p70S6K/4E-BP1 pathway and, moreover, that this effect will be more pronounced in the muscles performing more exercise and potentiated by elevated levels of circulating leucine and essential amino acids (EAA).

Methods

Participants

A detailed description of our study population and general procedures as well as part of the neuroendocrine effects, have been reported previously [13, 14]. Table 1 summarizes the background characteristics of our 15 overweight men assigned randomly to ingest a diet consisting solely of sucrose (SU; n=7) or whey protein (Whey PRO; n=8) during caloric restriction phase (CR). After being informed of potential risks and benefits, all subjects provided their written consent to participate. Ethical approval was obtained from the Regional Ethical Review Board of Umeå University (Umeå, Sweden), as well as the ethical committee of the University of Las Palmas de Gran Canaria (Canary Islands, Spain).

Experimental protocol

The protocol consisted of a pre-test phase (PRE), caloric restriction combined with exercise for 4 days (CRE), followed by a control diet with reduced exercise for 3 days (CD) (Fig. 1). During the PRE, and at the end of the CRE and CD phases, body composition was assessed (Lunar iDXA, GE Healthcare, Madison, WI, USA) [15]; 20 mL blood samples were drawn (in the supine position); and muscle biopsies were then taken. All of these procedures were performed in the morning following a 12-h overnight fast.

During CRE, participants ingested either whey protein (PRO, n=8) or sucrose (SU, n=7) (0.8 g/kg body weight/day). The solution of whey protein also contained Na^+ (308 mg/L) and K^+ (370 mg/L), as did the sucrose solution (160 mg of Na^+ and 100 mg of K^+ per L). The subjects drank 0.5 L of this solution in the morning (immediately before arm-cranking) and again at midday and at 20.00 h. In addition, both groups were allowed to drink a hypotonic rehydrating solution containing Na^+ (160 mg/L), Cl^- (200 mg/L), K^+ (100 mg/L), citrate (700 mg/L), and sucrose (3g/L) *ad libitum*.

Every day during the CD phase, the participants ate three standardized meals containing their normal daily intake of energy (as assessed by weighing all food ingested during the 7-day pre-test period) and were not allowed to walk more than 10,000 steps. This phase was designed to allow replenishment of body water and stabilization of body weight.

Assessment of physical activity, nutrition and body composition

The short version of the International Physical Activity Questionnaire was employed to assess daily energy expenditure due to physical activity [16]. During the 7-day PRE phase, the participants kept a dietary record and their food intake was analyzed (Dietist XP, Kost & Näringsdata, Bromma, Sweden). During the CD phase, all participants were provided a diet with the same energy content as that recorded during PRE, and the food ingested weighed. Energy intake was also calculated employing the Dietist XP program. During this phase, the sucrose and whey protein groups ingested 2256 ± 513 and 2086 ± 450 kcal/d (means ± SD), respectively.

Biochemical, hormonal and muscle signaling related variables

Detailed information on the procedures used to determine biochemical variables, plasma amino acids, hormones, protein concentration in the muscle biopsies and signaling kinases can be found in the "Materials and Methods online supplement".

Statistical analyses

All values were examined for normal distribution using the Shapiro-Wilks test and, when necessary, transformed logarithmically before analysis. A repeated-measures ANOVA with time and the two different diets (sucrose versus whey protein) was applied for analysis of the mean responses. When a significant

effect of diet or interaction between diet and time was indicated, pairwise comparisons at specific time-points were adjusted for multiple comparisons with the Holm–Bonferroni procedure. The relationship between variables was examined by simple linear regression. The changes in the levels of phosphorylated kinases from PRE to CRE ((CRE-PRE) x 100/PRE) (average of all three muscles) were analyzed for potential correlations with the plasma concentrations of hormones and amino acids. Unless otherwise stated, the values reported are means ± standard deviations. Statistical significance was set at a P-value ≤ 0.05 and all statistical analyses were performed using SPSS v.21 (SPSS Inc., Chicago, IL, USA).

Results

Body composition and lean mass

The changes in body composition, performance, blood lipids and neuroendocrine status have been reported previously [13, 14, 17]. After CRE, lean mass was reduced by 6 and 4% in the arms and the legs, respectively (both, $P<0.001$; $P<0.05$ for time x extremity interaction), with no significant difference between the two diets ($P=0.34$). Accordingly, after the three days on the control diet, the relative lean mass losses from the legs and trained arm were 57% and 29% less than the losses from the control arm ($P<0.05$) [14]. At baseline, the arms and leg muscles had a similar protein concentration (181.7±15.4 and 188.8±12.9 g.kg wet muscle^{-1}, mean of both arms and the vastus lateralis, respectively,

P=0.19). After CRE the protein concentration in the muscle biopsies (mean of the three muscles) was increased by 4.9% (P=0.010), remaining non-significantly elevated (2.2%, P=0.34 compared to baseline values) after the three days on a control diet (P=0.84 for extremity x time interaction).

Metabolic and hormonal changes

The changes in plasma concentrations of amino acids and hormones have also been previously documented [14]. In brief, plasma concentrations of asparagine, the three branched-chain amino acids (BCAA) and the EAA were elevated after CRE, with more pronounced increases in the whey protein group [14]. Serum concentrations of insulin and free testosterone, as well as the free androgen index (FAI) were reduced similarly in both groups, whereas cortisol and the catabolic index (cortisol/total testosterone and cortisol/free testosterone) rose after CRE and CD [14], without significant differences between groups (see Table 4 in reference 14).

Signaling related to protein synthesis in skeletal muscle

Representative immunoblots are presented in supplementary figure 1. In comparison to the PRE period, the level of total Akt (Fig. 2A) was increased to a similar extent in both groups following CD, but only in the arm muscles ($P<0.05$ for extremity x time interaction). However, neither $pSer^{473}$Akt (Fig. 2B) nor the ratio $pSer^{473}$Akt /total Akt (Fig. 2C) were altered significantly during the intervention (Fig 2).

The changes observed in the total level of mTor were not statistically significant (Fig. 2D), while pSer^{2448}mTor (Fig. 2E) was elevated following CD compared with PRE and CRE ($P<0.05$ for time effect), primarily due to the increase in the trained arm ($P<0.05$). This response was more marked in the sucrose than in the protein-supplemented group ($P<0.05$ for extremity x supplementation). pSer^{2448}mTor did not change significantly in the legs. Concomitantly, pSer^{2448}mTor/total mTor (Fig. 2F) and total p70S6K (Fig. 2G) were unchanged, while pThr^{389}p70S6K was undetectable.

Regardless of the type of supplementation, the mean levels of total 4E-BP1 (Fig. 3A) and Thr$^{37/46}$4E-BP1 (Fig. 3B), a downstream target of mTor, were elevated after CRE and remained so after CD, although Thr$^{37/46}$4E-BP1/total 4E-BP1 did not change significantly (Fig. 3C).

The total GSK3β (Fig. 3D) was reduced in all muscles after CRE and remained so after CD. Ser^{9}GSK3β phosphorylation (Fig. 3E) was also lowered after CRE, but returned to PRE levels after CD, resulting in higher pSer^{9}GSK3β/total GSK3β ratio (Fig. 3F). These changes in pSer^{9}GSK3β were more pronounced in the protein than the sucrose group ($P<0.05$ for extremity x supplementation). Total eIF2α (Fig. 3G) did not change significantly, while pSer^{51}eIF2α (Fig. 3H) and the pSer^{51}eIF2α/total eIF2α ratio (Fig. 3I) were reduced in all muscles after CRE, and remained so after CD.

Relationships between changes in plasma amino acids, hormones, lean mass and muscle signaling

A detailed description of the associations observed between signaling, amino acids and hormones is reported in the supplementary results. Briefly, associations were observed between Akt, pSer^{473}Akt, mTor, 4E-BP1, GSK3β, pSer^{9}GSK3β, eIF2α and pSer^{51}eIF2α on the one hand, and leptin, insulin, EAA (or BCAA), and cortisol/total testosterone ratio, on the other hand, when both groups were pooled together. Likewise, the change in the Thr$^{37/46}$4E-BP1/total 4E-BP1 ratio from PRE to CRE and from PRE to CD was associated with the corresponding change in cortisol (r=0.37, P=0.043; both groups pooled together, n=30).

The mean regional lean mass was correlated positively with both the mean Thr$^{37/46}$4E-BP1/total 4E-BP1 (r=0.85, P=0.004, n=9) and the mean pSer^{9}GSK3β/total GSK3β (r=0.75, P=0.021, n=9, each point is the mean of the 15 subjects). An association was also observed between the change from PRE to CD in lean mass (all muscles) and the corresponding changes in pSer^{473}Akt (r=-0.33, P=0.028, n=45) and pSer^{473}Akt/total Akt (r=-0.34, P=0.023, n=45).

Discussion

In contrast to our hypothesis, protein ingestion during a severe energy deficit did not facilitate protein synthesis signaling. Although protein synthesis in skeletal muscle is known to be stimulated by amino acids to a greater extent when the muscle has been exercised, the current study shows that when the energy deficit is severe, protein synthesis becomes resistant to the anabolic

effect of amino acids in manner that cannot be counteracted by moderate or large amounts of contractile activity. This study also shows that during a severe energy deficit the serum concentration of the anabolic hormones insulin and testosterone are reduced, blunting the translation initiation due to insufficient inactivation of GSK3β through phosphorylation at serine 9. Ser^{9}GSK3β phosphorylation is catalyzed by Akt, whose own phosphorylation in skeletal muscle is stimulated by insulin, testosterone and leptin [18-20] (Fig. 4). The associations observed here between the reductions in serum insulin, leptin and $pSer^{9}$GSK3β support a mechanistic link between the drop in these hormones and the lack of activation of protein synthesis, despite the fact that the circulating concentrations of amino acids were increased in both groups. Consequently, circulating amino acids cannot be incorporated into muscle proteins during a severe energy deficit and are left available for gluconeogenesis, which is essential to maintain the glycaemia under these conditions. This interpretation is supported by cell experiments showing that insulin, via Akt activation, phosphorylates GSK3β [21], while leptin can signal in skeletal muscle through ERK1/2, which is also able to elicit Ser^{9} GSK3β phosphorylation [22]. Moreover, we found that $pSer^{9}$GSK3β was also associated with total testosterone, which via Akt activation elicits GSK3β phosphorylation [20].

The acute effects of severe energy deficit on the signaling cascades that regulate protein synthesis

No significant changes in the Akt/mTor/p70S6K signaling pathway were observed here after four days of a severe energy deficit close to 5500 kcal/d, regardless of the level of contractile activity or protein supplementation. However, since our measurements were performed in a fasted state and after a 12-h rest period, a transient activation of Akt/mTor/p70S6K during the first hours after the ingestion of supplements may have escaped detection. In fact, the levels of total 4E-BP1 and $Thr^{37/46}$4E-BP1, a downstream target of mTor, were elevated in all muscles after CRE, indicating that this part of the pathway leading to translation initiation was activated. This conclusion is also supported by the reductions of $pSer^{51}$eIF2α and $pSer^{51}$eIF2α/total eIF2α observed in all muscles after CRE, which should have facilitated translation initiation [12].

However, this stimulating effect elicited by the elevated $Thr^{37/46}$4E-BP1 and the reduced $pSer^{51}$eIF2 α and $pSer^{51}$eIF2α/total eIF2α ratio was counteracted by the decrease of $pSer^{9}$GSK3ß after CRE. GSK3β intervenes in the regulation of protein synthesis, cell proliferation and survival [23]. Upon phosphorylation, GSK3β activity is inhibited what causes its substrate, eukaryotic initiation factor 2B (eIF2B), to become dephosphorylated and activated [11]. Thus, the lowered levels of $pSer^{9}$GSK3ß might have increased the inhibitory action of GSK3β on protein synthesis. In contrast to our expectations, the reduction of $pSer^{9}$GSK3ß was more marked in the protein group, which could have caused a greater inhibition of protein synthesis in this group despite the increased levels of BCAA and leucine [14]. Consequently, the balance between protein synthesis

and breakdown was not influenced significantly by the ingestion of whey protein, and both groups lost a similar proportion of lean mass [14].

Since 4E-BP1 inhibits protein synthesis, the increase of total 4E-BP1 observed here might have attenuated protein synthesis. However, this potential effect was counteracted by a commensurate increase in $Thr^{37/46}$4E-BP1 after CRE. Interestingly, there was an inverse association between the levels of total 4E-BP1 on the one hand, and testosterone and free testosterone on the other hand, as well as between the levels of $pSer^{9}$GSK3β and total testosterone. This suggests that testosterone may down-regulate 4E-BP1 and up-regulate GSK3β to facilitate protein synthesis, as has been reported in animal and cell culture studies [20]. Likewise, the rise in the level of $pSer^{51}$eIF2α from PRE to CRE, which impedes translation initiation, was associated with the change in serum cortisol. Altogether, these findings confirm that the serum cortisol/testosterone ratio plays a critical role in the regulation of protein synthesis.

The preservation of muscle mass by exercise cannot be explained by changes in protein synthesis signaling

Despite failing to enhance protein synthesis signaling, exercise resulted in a remarkable preservation of muscle mass [14]. Therefore, our data point towards an attenuation of protein breakdown as the potential main mechanisms by which exercise spares muscle mass during a severe energy deficit. Unexpectedly, administration of whey protein, which is rich in leucine and stimulates protein

synthesis while attenuating protein breakdown in humans [24], did not preserve lean mass, regardless of the level of exercise. The amount of protein ingested (0.8 g/kg body weight), as well as the timing (every 8 h), should have maximally stimulated protein synthesis in both the rested and exercised muscles [25]. It remains unknown whether some stimulation of protein synthesis could have been achieved by administering 1.6 or even 2.4 g/kg body weight of dietary protein per day, as reported during low-calorie diets [2, 7]. However, Hector et al. have recently reported a reduction of protein synthesis during a 10-day low-calorie diet (40%-reduced energy intake), which was not altered by doubling the daily protein intake to 2.4 g/kg body weight [26]. Thus, the present and Hector et al. studies indicate that during moderate [26] and severe energy deficits the skeletal muscle becomes refractory to the stimulation of protein synthesis by the ingestion of proteins, even when 3 times the RDA for protein is administered [26].

One possible explanation for the lack of differences between our dietary groups involves their similar catabolic response during CRE. In fact, we have demonstrated that the amount of lean mass lost can be predicted from the change in the catabolic index (cortisol/free testosterone) [14], which rose similarly in both groups. We can only speculate as to why the ingestion of whey protein was associated here with an elevated catabolic index. One plausible explanation is the requirement for more pronounced activation of the hypothalamic/pituitary/adrenal axis when whey protein alone is ingested in

order to avoid hypoglycemia [27]. In rodents, a diet with high proteins to carbohydrates ratio content promotes the expression of TNF-α mRNA [28], which via activation of NF-κβ, may facilitate muscle proteolysis.

The exercised arm muscles are likely more sensitive than the leg muscles to stimulation of protein synthesis by circulating amino acids during severe energy deficit.

It has been reported that exercise potentiates the anabolic response to amino acids [3]. Accordingly, a positive association was observed between the changes from PRE to CRE in pSer^{2448}mTor in the trained arm and the changes in plasma BCAA and leucine. Infusion of BCAA or leucine alone is known to enhance 4E-BP1 phosphorylation in human skeletal muscle [29]. In the present investigation, the serum levels of all three BCAA were elevated after CRE in both groups, although more markedly in the subjects ingesting whey protein, despite the fact that the blood samples were drawn 12 hours after the last ingestion of approximately 26 g of whey protein. Despite a greater BCAA and leucine plasma concentration during the CRE in the group supplemented with proteins, 4E-BP1 phosphorylation was similarly increased in both groups, without clear differences between the exercised and non-exercised muscles. However, the protein synthesis-signaling cascade was blunted due to the reduced pSer^{9}GSK3β, probably caused by the increased serum cortisol/testosterone ratio, which affected all muscles. These findings indicate that during severe energy deficit,

the anabolic effect of amino acids is not potentiated by contractile activity, in the exercised muscles.

The increase in cortisol levels during fasting is associated with blunted protein synthesis signaling

The elevation of serum cortisol levels in both groups following CRE may have contributed to the inhibition of protein synthesis by lessening the increase in total 4E-BP1, leaving p70S6K in a dephosphorylated state [30] and reducing pSer^{51}eIF2α [31]. Moreover, glucocorticoids inhibit the uptake of amino acids by muscle cells [32]; stimulate KLF-15 (Kruppel-like factor 15), which activates the transcription of the BCAT (branched-chain amino acid aminotransferase) gene [33], encoding an enzyme involved in BCAAs catabolism; and inhibit the phosphorylation of 4E-BP1 and S6K1 mediated by insulin and IGF-I (insulin-like growth factor I) [34]. Glucocorticoids may also inhibit phosphorylation of PI3K (phosphatidylinositol 3-kinase) and Akt [35], upstream kinases of mTor and Forkhead box protein O (FOXO). Indeed, mice in which Akt is constitutively activated are resistant to the negative effects of glucocorticoids on protein synthesis [36] and muscle mass [37].

Serum leptin concentration is positively associated with protein synthesis signaling in human skeletal muscle

As another novelty, we have found a close association between serum leptin concentration and the mean protein expression of total Akt, total GSK3β and pSer^{9}GSK3β in the three analyzed muscles. Moreover, there was also a close association between the changes in serum leptin concentration and the changes in pSer^{9}GSK3β. Although these associations are compatible with the proposed anabolic effect of leptin in skeletal muscle [17, 38], the prolonged administration of leptin to amenorrheic athletes has not been associated with significant changes in lean mass [39]. Moreover, no activation of protein synthesis signaling was observed in human skeletal muscle 20 min after the intravenous administration of metreleptin [40]. Since leptin receptors were up-regulated [17] and the serum concentration of leptin increased from CRE to CD (i.e., with re-feeding), we think that leptin may have a role in easing a potential anabolic response of skeletal muscles upon re-feeding.

Limitations

Although muscle glycogen concentrations were not assessed, the fact that the total concentration of proteins in skeletal muscle was increased by 4.9% after CRE is compatible with a marked reduction of muscle glycogen and the associated water. In fact, we have estimated that the increase in protein concentration observed here after CRE could be explained by 11-12 g/kg wet muscle reduction in muscle glycogen [41]. Muscle protein concentration returned to baseline values after the three days on a control diet, compatible with at least

partial recovery of muscle glycogen stores, and therefore our lean mass assessment obtained three days after the end of CRE should reflect more trustfully the actual changes in muscle mass, than the measurements performed after CRE. Lastly, we could not find a molecular explanation for the mechanism by which exercise attenuated the loss of muscle mass. However, since all muscles were likely exposed to the same internal neuroendocrine milieu, we think that this molecular mechanism should be specifically triggered by contractile activity inside the active muscle fibers.

In summary, our present findings demonstrate that during severe energy deficit the skeletal muscles become refractory to the anabolic effects of whey protein ingestion. Increased circulating amino acids after the ingestion of proteins during severe energy deficit fail to potentiate the anabolic stimulus of exercise because the reduction of serum insulin and testosterone concentration (together with the increase in cortisol) impede a full activation of the protein synthesis signaling cascade by diminishing Ser^{9}GSK3β, which blunts translation initiation. Future studies should examine whether inhibition of pSer^{9}GSK3β dephosphorylation and/or stimulation of GSK3β Ser^{9} phosphorylation facilitates an anabolic response to amino acids in humans under severe energy deficit. Finally, this study clearly shows that preservation of skeletal muscle mass by exercise during periods of severe energy deficit is not mediated by an enhanced protein synthesis signaling.

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Table 1. Pre-test physical characteristics and fitness

	Diet	
	Sucrose (n=7)	Whey protein (n=8)
Age (years)	38.7 ± 8.2	43.0 ± 8.0
Height (cm)	181 ± 5.5	180 ± 4.2
Weight (kg)	98 ± 12.0	100 ± 14.9
BMI (kg/m^2)	29.9 ± 3.1	30.9 ± 4.2
Lean mass (kg)	63.1 ± 3.1	65.4 ± 6.0
Fat mass (kg)	31.5 ± 9.1	31.4 ± 9.2
Body fat (%)	31.6 ± 5.3	30.9 ± 4.1
VO_2max (L/min)	3.8 ± 0.3	3.9 ± 0.3
Daily energy intake (kcal)	2256 ± 513	2086 ± 489
Physical activity (IPAQ) (kcal/d)	612 ± 315	601 ± 289

IPAQ: International Physical Activity Questionnaire. The values presented are means ± SD.

Figure legends

Figure 1. Schematic illustration of the experimental protocol. PRE: pre-test; CRE: 4 days of caloric restriction (3.2 kcal/day) and exercise (45 min of single-arm cranking and 8 hours of walking each day); CD: three days on a diet isoenergetic with that observed during the PRE phase with reduced exercise (<10,000 steps per day); DXA: dual-energy x-ray absorptiometry; IPAQ: international physical activity questionnaire.

Figure 2. Protein expression levels of total and phosphorylated Akt (A-C) and mTOR (D-F) as well as total p70S6K (G) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * $P < 0.05$ compared to PRE; † $P < 0.05$ compared to CRE; $ $P < 0.05$ compared to the legs; § $P < 0.05$ compared to the legs.

Figure 3. Protein expression levels of total and phosphorylated 4E-BP1 (A-C), GSK3β (D-F) and eIF2α (G-I) in skeletal muscle following the different experimental stages with ingestion of sucrose or whey protein during severe

energy deficit. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs; Means: (C. Arm + T. Arm + Legs)/3; Arms: (C. Arm + T. Arm)/2; Exercised: (T. Arm + Legs)/2. The values shown are means ± standard errors (n = 7 and 8, for the sucrose and protein groups, respectively) expressed in arbitrary units (a.u.). * $P < 0.05$ compared to PRE; † $P < 0.05$ compared to CRE; $ $P < 0.05$ compared to the legs; § $P < 0.05$ compared to the legs.

Figure 4. Schematic representation of the proposed mechanisms regulating translation initiation and protein synthesis in human skeletal muscle under a severe energy deficit. In a state with low serum concentrations of insulin, testosterone and leptin, anabolic signaling activated by insulin-like growth factor-1 signaling (IGF1), acting via its receptor (IGFR) and the insulin receptor substrate 1 (IRS1), is negatively regulated blunting Akt activation. Akt and other protein kinases modulate the GTPase activity of tuberous sclerosis proteins 1 and 2 (TSC1/2), repressing their GTP activity and thus stimulating mTOR-derived signaling. Such stimulation is likely to be lacking during severe energy deficit as reflected by the unchanged levels of phosphorylated Akt observed here. The amino acid induction of protein synthesis via mTOR activation (through various intermediary proteins) is blunted in such a high energy-deficient state, likely due to the proteolytic effect resulting from the increased cortisol and the reduced testosterone plasma concentrations. mTOR regulates protein synthesis by phosphorylating p70 ribosomal protein S6 kinase (p70S6K) and eIF4E-binding protein 1 (4E-BP1). A partial activation of the pathway is seen with the presence of: 1) increased levels of phosphorylated 4E-BP1, which allows the release of the mRNA cap-binding protein eIF4E, required for the formation of the eIF4F complex, essential for the global increase in translation and 2) a reduction in the phosphorylation of the alpha subunit of eIF2 (eIF2α) on serine 51 induces eIF2B activation and thereby partial stimulation of translation initiation. However, p70S6k remains dephosphorylated (inactivated) impeding the phosphorylation of its target (ribosomal protein S6; rpS6), leading to downregulation of mRNA

translation. Phosphorylation of the GDP-GTP exchange factor eIF2B by glycogen synthase kinase 3β (GSK3β) leads to its inhibition downregulating translation initiation. Upon anabolic stimulation GSK3β is phosphorylated (inactivated) by Akt allowing translation initiation, however, the severe energy deficit observed here blunts Akt-mediated inactivation of GSK3β.

The arrows inside dashed grey boxes presented besides protein markers depict overall outcomes in the present investigation. Green (total form) and black (phosphorylated form) thin arrows represent the overall direction of the results (increase/decrease) after the intervention (mean of all muscles). Blue thick arrows represent the overall effect on protein synthesis (stimulation/inhibition). The length of the arrow is representative of the magnitude of the change. Dashed blue connecting lines indicate indirect effect (mediator proteins not shown).

Supplementary figure. Representative images of protein expression levels (Western Blot) for each protein of interest, its regulatory phosphorylations and total protein amount loaded (Reactive Brown staining) for a single subject from the sucrose group. From top to bottom: Akt, pSer^{473}Akt, mTor, pSer^{2448}mTor, p706SK, 4E-BP1, pThr$^{37/46}$4E-BP1, GSK3β, pSer^{9}-GSK3β, eIF2α, pSer^{51}eIF2α, and α-tubulin. A non-intervention human muscle sample (C) was included in quadruplicate as a loading control. For a detailed explanation of the various phases, see Figure 1. PRE, before the intervention; CRE, caloric restriction and exercise; CD, control diet; C. Arm, control arm; T. Arm, training arm; Legs, legs.

Based on the applied molecular standards, approximated molecular weights are indicated by arrows on the right.

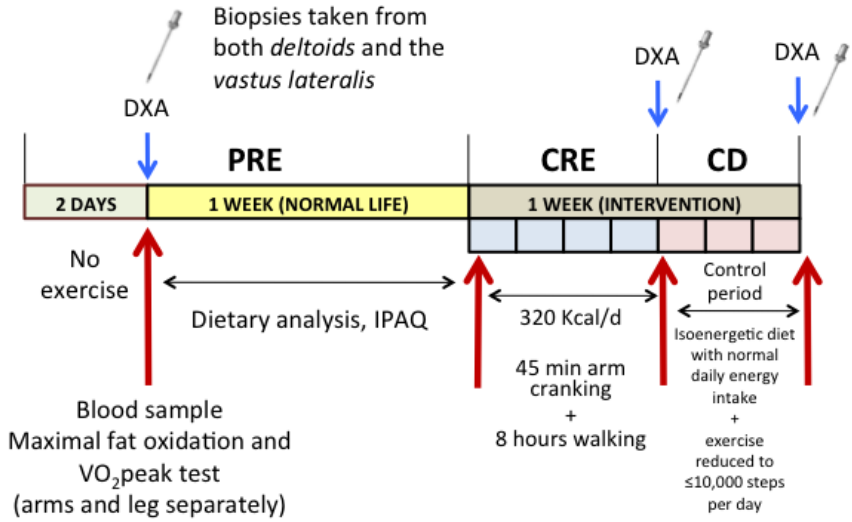


Figure 1

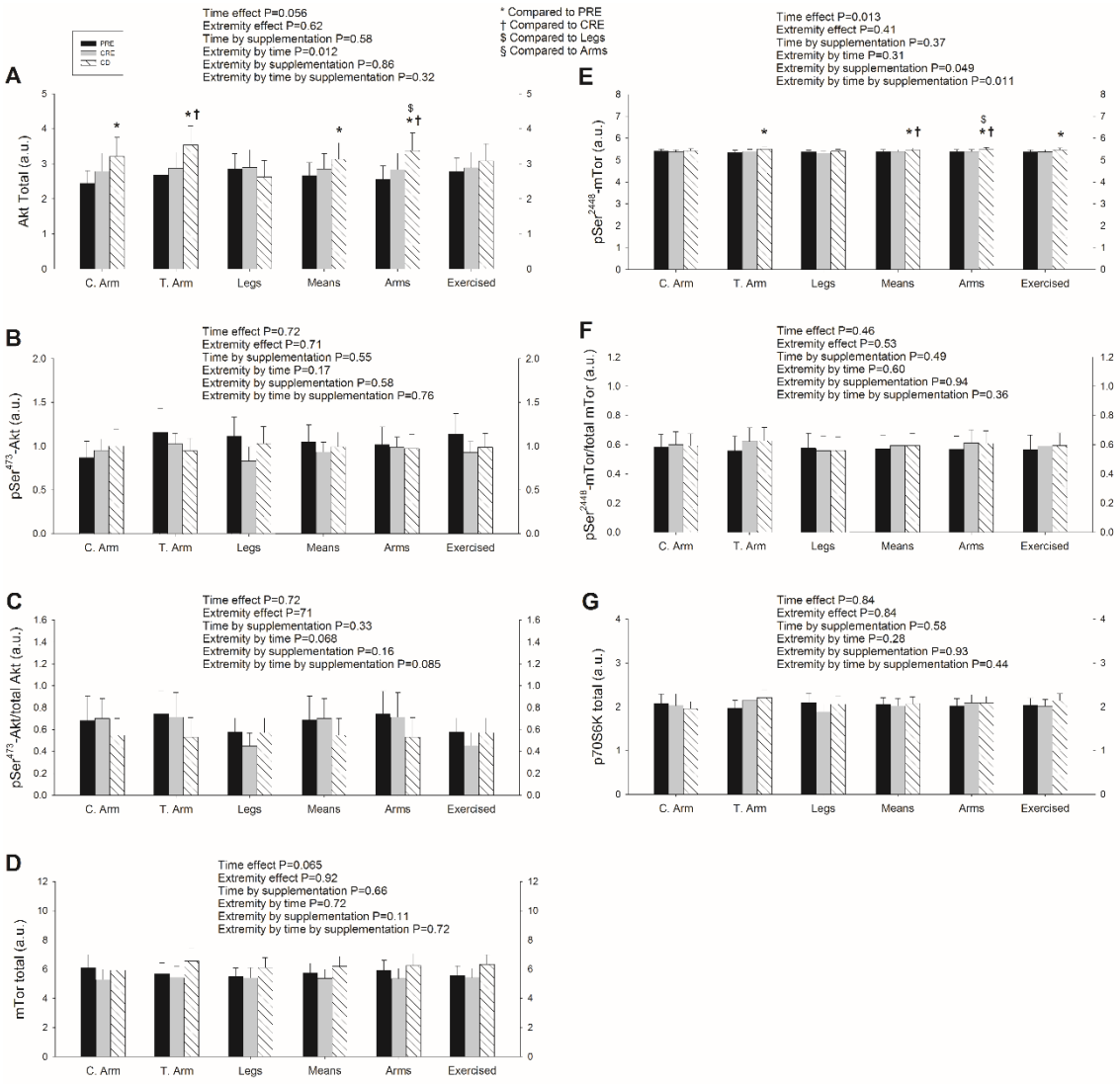

A
Time effect P=0.056
Extremity effect P=0.62
Time by supplementation P=0.58
Extremity by time P=0.012
Extremity by supplementation P=0.86
Extremity by time by supplementation P=0.32
* Compared to PRE
† Compared to CRE
$ Compared to Legs
§ Compared to Arms
PRE
CRE
CD
Akt Total (a.u.)
C. Arm
T. Arm
Legs
Means
Arms
Exercised
B
Time effect P=0.72
Extremity effect P=0.71
Time by supplementation P=0.55
Extremity by time P=0.17
Extremity by supplementation P=0.58
Extremity by time by supplementation P=0.76
pSer473-Akt (a.u.)
C
Time effect P=0.72
Extremity effect P=71
Time by supplementation P=0.33
Extremity by time P=0.068
Extremity by supplementation P=0.16
Extremity by time by supplementation P=0.085
pSer473-Akt/total Akt (a.u.)
D
Time effect P=0.065
Extremity effect P=0.92
Time by supplementation P=0.66
Extremity by time P=0.72
Extremity by supplementation P=0.11
Extremity by time by supplementation P=0.72
mTor total (a.u.)
E
Time effect P=0.013
Extremity effect P=0.41
Time by supplementation P=0.37
Extremity by time P=0.31
Extremity by supplementation P=0.049
Extremity by time by supplementation P=0.011
pSer2448-mTor (a.u.)
F
Time effect P=0.46
Extremity effect P=0.53
Time by supplementation P=0.49
Extremity by time P=0.60
Extremity by supplementation P=0.94
Extremity by time by supplementation P=0.36
pSer2448-mTor/total mTor (a.u.)
G
Time effect P=0.84
Extremity effect P=0.84
Time by supplementation P=0.58
Extremity by time P=0.28
Extremity by supplementation P=0.93
Extremity by time by supplementation P=0.44
p70S6K total (a.u.)

Figure 2

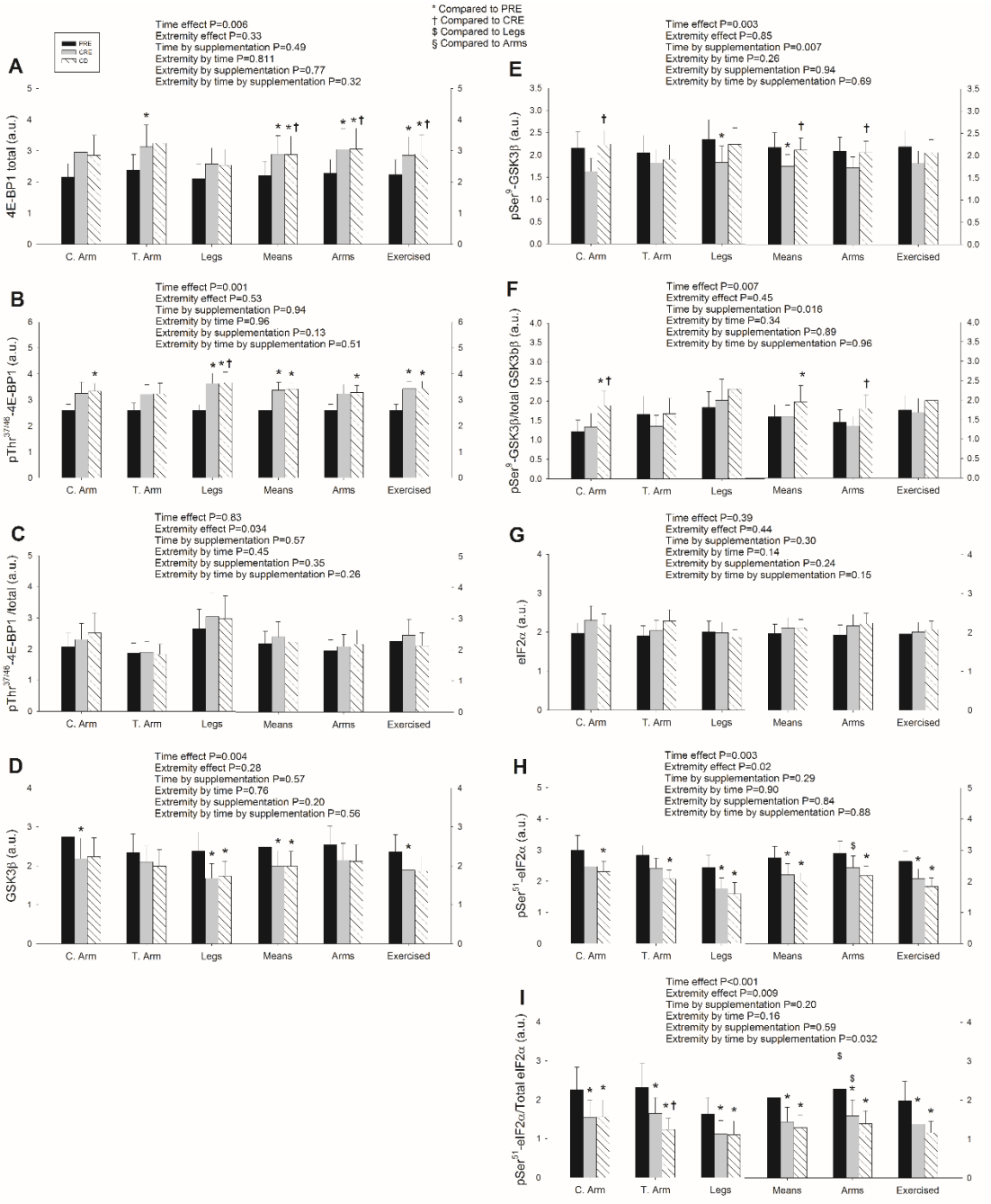

* Compared to PRE
† Compared to CRE
$ Compared to Legs
§ Compared to Arms
PRE
CRE
CD
A
Time effect P=0.006
Extremity effect P=0.33
Time by supplementation P=0.49
Extremity by time P=0.811
Extremity by supplementation P=0.77
Extremity by time by supplementation P=0.32
4E-BP1 total (a.u.)
B
Time effect P=0.001
Extremity effect P=0.53
Time by supplementation P=0.94
Extremity by time P=0.96
Extremity by supplementation P=0.13
Extremity by time by supplementation P=0.51
pThr37/46-4E-BP1 (a.u.)
C
Time effect P=0.83
Extremity effect P=0.034
Time by supplementation P=0.57
Extremity by time P=0.45
Extremity by supplementation P=0.35
Extremity by time by supplementation P=0.26
pThr37/46-4E-BP1 /total (a.u.)
D
Time effect P=0.004
Extremity effect P=0.28
Time by supplementation P=0.57
Extremity by time P=0.76
Extremity by supplementation P=0.20
Extremity by time by supplementation P=0.56
GSK3β (a.u.)
E
Time effect P=0.003
Extremity effect P=0.85
Time by supplementation P=0.007
Extremity by time P=0.26
Extremity by supplementation P=0.94
Extremity by time by supplementation P=0.69
pSer9-GSK3β (a.u.)
F
Time effect P=0.007
Extremity effect P=0.45
Time by supplementation P=0.016
Extremity by time P=0.34
Extremity by supplementation P=0.89
Extremity by time by supplementation P=0.96
pSer9-GSK3β/total GSK3β (a.u.)
G
Time effect P=0.39
Extremity effect P=0.44
Time by supplementation P=0.30
Extremity by time P=0.14
Extremity by supplementation P=0.24
Extremity by time by supplementation P=0.15
eIF2α (a.u.)
H
Time effect P=0.003
Extremity effect P=0.02
Time by supplementation P=0.29
Extremity by time P=0.90
Extremity by supplementation P=0.84
Extremity by time by supplementation P=0.88
pSer51-eIF2α (a.u.)
I
Time effect P<0.001
Extremity effect P=0.009
Time by supplementation P=0.20
Extremity by time P=0.16
Extremity by supplementation P=0.59
Extremity by time by supplementation P=0.032
pSer51-eIF2α/Total eIF2α (a.u.)
C. Arm
T. Arm
Legs
Means
Arms
Exercised

Figure 3

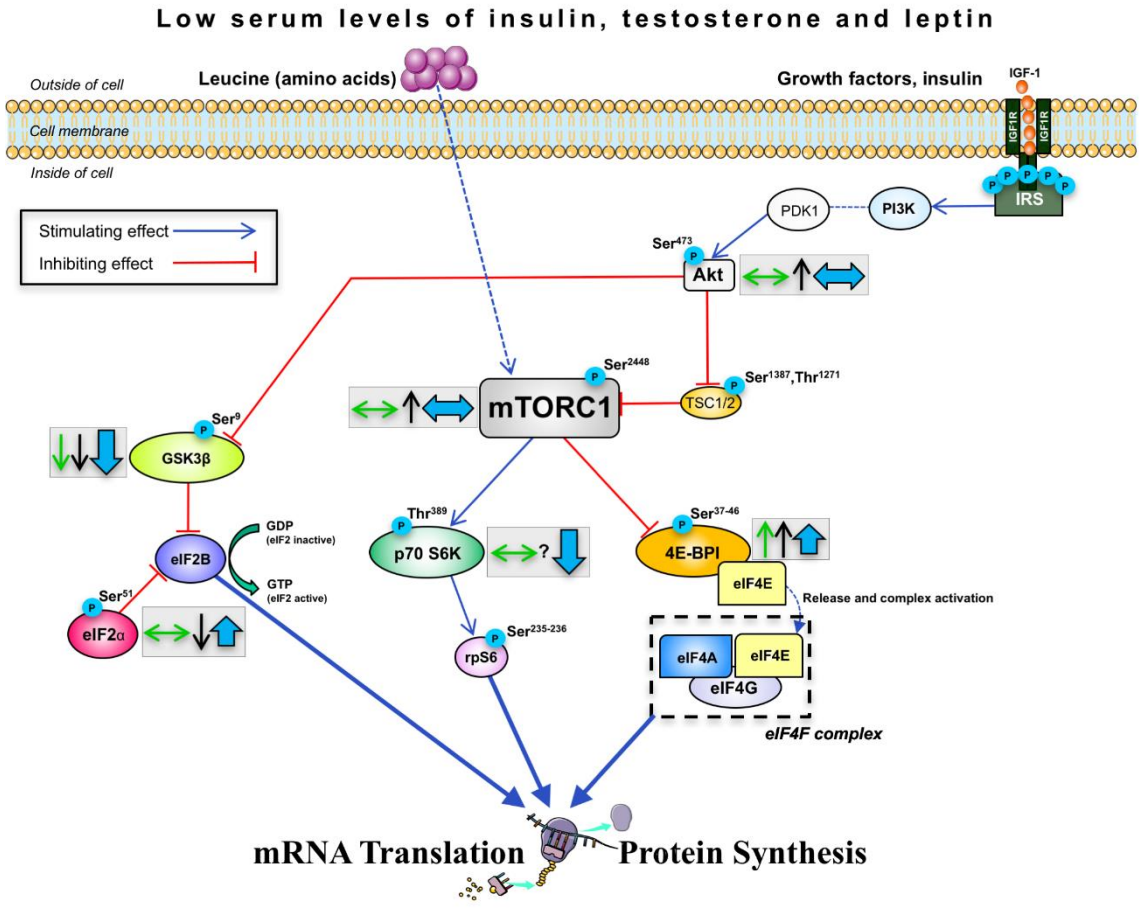
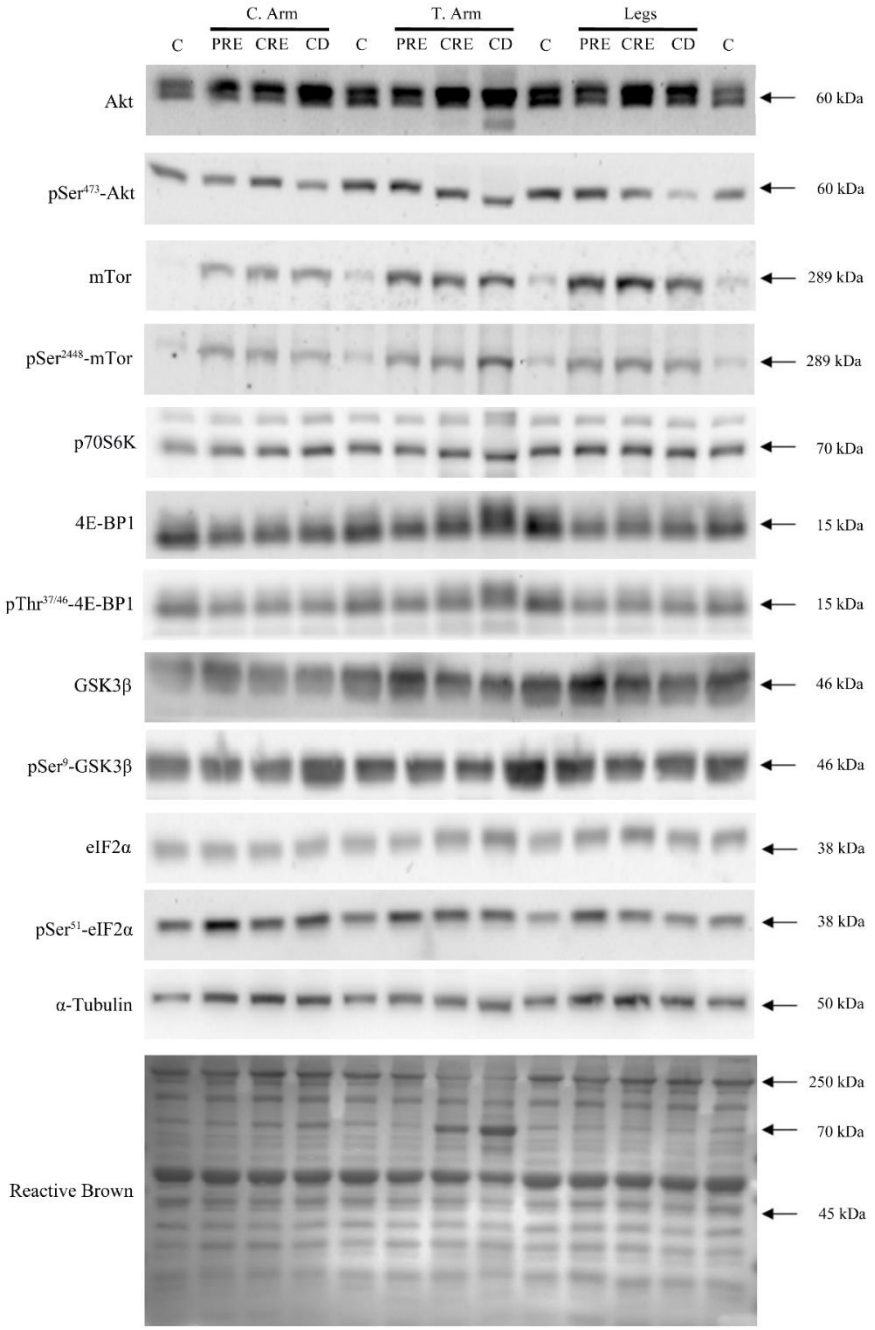

Low serum levels of insulin, testosterone and leptin
Outside of cell
Leucine (amino acids)
Growth factors, insulin
IGF-1
Cell membrane
IGF1R
IGF1R
Inside of cell
IRS
PDK1
PI3K
Stimulating effect
Inhibiting effect
Ser473
Akt
Ser2448
mTORC1
Ser1387, Thr1271
TSC1/2
Ser9
GSK3β
Thr389
p70 S6K
?
Ser37-46
4E-BPI
eIF4E
Release and complex activation
GDP
(eIF2 inactive)
GTP
(eIF2 active)
eIF2B
Ser51
eIF2α
Ser235-236
rpS6
eIF4A
eIF4E
eIF4G
eIF4F complex
mRNA Translation
Protein Synthesis

Figure 4



Suppl figure

Materials and methods online supplement

Biochemical and hormonal analyses

After a 12-h overnight fast, 30 mL blood samples were drawn from an antecubital vein directly into Vacutainer Tubes (REF: 368499; 368498). Some samples were collected in tubes containing EDTA and centrifuged for 5 min at 4000 RPM and 4 °C, to obtain plasma; while others were centrifuged for 10 min at 4000 RPM and 4 °C to prepare serum. All of these samples were aliquoted on tubes precooled on ice water and rapidly stored at -80 º C until further analysis.

The concentration of glucose in serum was measured with the hexokinase procedure, utilizing kits from ABX Pentra (Horibia Medical, Montpellier, France). Serum insulin was quantified by an electrochemiluminescence immunoassay (ECLIA) performed with reagent kits and a Modular Analytics Analyzer E170 (Roche Diagnostics SL, Barcelona, Spain), at a sensitivity of 0.2 µU/mL and with corresponding intra- and inter-assay coefficients of variation of 2.0 and 2.6%, respectively. Serum leptin was determined by the enzyme-linked immunosorbent assay (ELx800 Universal Microplate Reader, Bio-Tek Instruments, Inc, Winooski, Vermont, USA), employing reagent kits from Linco Research (#EZHL-80SK, St Charles, Missouri, USA). In this case, the sensitivity was 0.05 ng/mL and the intra and inter-assay coefficients of variation were 3.8% and 4.4%, respectively (Calbet *et al.*, 2015). Cortisol and total testosterone were measured with chemiluminescence enzyme immunoassays (Immulite 2000 Cortisol, Ref. L2KCO2, Immulite 2000 Total

Testosterone, Ref. L2KTW2; Siemens) exhibiting sensitivities of 5.5 and 0.5 nmol/L and intra- and inter-assay coefficients of variation of 6.2 and 7.3%, and 8.2 and 9.1%, respectively. Free testosterone was determined by a radioimmunoassay (Coat-A-Count Free Testosterone, Ref. TKTF1; Siemens) with a sensitivity of 0.5 pmol/L and intra- and inter-assay coefficients of variation of less than 8%. Sexual hormone-binding globulin (SHBG) was assessed with a chemiluminescence enzyme immunoassay (Immulite SHBG, Ref. L2KSH2; Siemens) characterized by a sensitivity of 0.02 nmol/L and intra- and inter-assay coefficients of variation of 2.7 and 5.2%, respectively. The free androgen index (FAI) was calculated as [TT (nmol/L) / SHBG (nmol/L)] x 100.

Following automated precolumn derivatization of plasma amino acids with o-phthalaldehyde, the resulting derivatives were separated by reversed-phase HPLC (on a 5-µm Resolve C18 column; Waters), and quantified by fluorescence detection. The derivatization reagent was prepared by dissolving 50 mg o-phthalaldehyde in 1 mL of methanol and then adding 9 mL potassium borate buffer (0.4 mol/L, pH 10) and a drop of 2-mercaptoethanol. Solvent A consisted of phosphate buffer (0.1 mol/L, pH 7.0)/methanol/tetrahydrofuran (92:2:2) and Solvent B methanol/water (65:35). The HPLC system contained two Model 510 pumps, a PCM Pump Control Module, a WISP 710 autosampler and a Model 470 fluorescence detector, all from Waters (Barcelona, Spain). Measurements were performed with an excitation wavelength of 338 nm and

emission wavelength of 425 nm and the data collected and processed by a Model 860 Waters Networking Computer System.

Materials

The Complete protease inhibitor cocktail and PhosSTOP phosphatases inhibitor cocktail were obtained from Roche Diagnostics (Mannheim, Germany; #04693116001 and #04906845001, respectively). All primary antibodies used were from Cell Signaling Technology (Danvers, MA, USA). The HRP-conjugated secondary anti-rabbit antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA; catalog sc-2030). The Immun-Blot PVDF membranes, the Inmmun-StarTM WesternCTM and the Protein Plus Precision All Blue Standards 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. The corresponding catalog numbers of the primary antibodies from Cell Signaling were as follows: anti-phospho-Akt (Ser^{473}), no. 9271; anti-phospho-eIF2α (Ser^{51}), no. 9721; anti-phospho-GSK-3β (Ser^{9}), no. 5558, anti-phospho-mTor (Ser^{2448}), no. 2971; anti-phospho-p70s6k (Thr^{389}), no. 9205; anti- phospho-4E-BP1 ($Thr^{37/46}$), no. 9459; anti-Akt, no.9272; anti-eIF2α, no. 9722; anti-GSK-3β, no. 9325, anti-mTor, no. 2972; anti-p70s6k, no. 9202; and the anti-4E-BP1, no. 9452. The monoclonal mouse anti-α-tubulin antibody (no. T-5168-ML) was obtained from Biosigma (Sigma, St. Louis, MO).

Extraction of total protein and Western blotting

Three muscle biopsies were taken from each deltoid muscle (middle portion) and from the middle portion of the *vastus lateralis* muscle as previously reported (Perez-Suarez *et al.*, 2017). After disinfection of the skin, 1-2 ml local anesthetic (Lidocaine 2%) was injected into the skin and subcutaneous tissue, taking care not to penetrate below the superficial fascia. Thereafter, a 6-7 mm incision was made and the biopsy Bergstrom-type needle inserted. The muscle sample (~100 mg) was dissected free of any debris and fat tissue present, and immediately frozen in liquid nitrogen at -80°C until further analysis.

Extracts of muscle protein were prepared as previously described and total protein content quantified using the bicinchoninic acid assay (Smith *et al.*, 1985). In brief, 30 mg of muscle were homogenized in urea lysis buffer (6 M urea, 1% SDS and 1X Complete Protease Inhibitor and phosphatases PhosphoStop 1X) and the lysate then centrifuged for 15 min at 20,000 rpm at 4° C. The resulting supernatant containing the protein fraction, was diluted with electrophoresis loading buffer (62.50 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, bromophenol blue). Then, 30-35 µg of protein were loaded onto each lane, depending on linearity test. To compensate for variability between gels, the 9 samples from each subject and 4 control samples (human muscle) were equally loaded onto the same gel. When the coefficient of variation for the control samples was >20% the blot was repeated. The sample

protein bands were normalized to the mean value of the band densities of the 4 controls.

Membranes were blocked for 1 h in 4% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (BSA-blocking buffer) and incubated overnight with primary antibodies. For all targeted proteins, the membranes were initially incubated with the phospho-specific antibodies and, after detection, the antibodies were stripped off using a buffer containing (Restore^{TM} PLUS Western Blot Stripping Buffer from ThermoFisher Scientific, Surfact Anionic), and subsequently reblocked and reincubated with antibodies against total protein. All antibodies were diluted in 4% BSA-blocking buffer with the exception of phospho-eIF2α (Ser^{51}) which was diluted in 5% Blotto blocking buffer. After incubation with primary antibodies, the membranes were incubated with an HRP-conjugated anti-rabbit antibody (diluted 1:5000 in Blotto blocking buffer) and subsequent visualization with Immmuno Western CTM-Star™ (Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, UK) using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) until saturation of the signal. Finally, the bands in the exposition immediately prior to saturation of the signal were quantified with the Quantity One © image analyzer (Bio-Rad Laboratories, Hercules, CA, USA). To control for differences in loading and transfer efficiency, the membranes were subsequently stained with reactive brown and these parameters quantified. Likewise, membranes were incubated with a monoclonal mouse anti-α-tubulin antibody (Blotto-blocking buffer). No

significant changes were observed in α-tubulin protein levels nor in Reactive Brown total protein amount (data not shown).

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Supplementary results

Relationships between changes in plasma levels of amino acids and hormones and muscle signaling

Akt. There was a linear relationship between Akt protein expression (mean of the three muscles) and the serum concentrations of leptin (r=0.41, P=0.005; n=45), insulin (r=0.33, P=0.026; n=45), HOMA (r=0.33, P=0.028; n=45), cortisol/total testosterone (r=0.32, P=0.033; n=45), cortisol/free testosterone (r=0.36, P=0.015; n=45), total testosterone (r=-0.40, P=0.007; n=45), free testosterone (r=-0.42, P=0.004; n=45). The increase in Akt protein expression from PRE to CD (mean of the three muscles) was inversely associated with the corresponding change in insulin (r=-0.72, P=0.003; n=15) and leucine (r=-0.58, P=0.024; n=15). Likewise there was a negative association between the increase in Akt protein expression from PRE to CRE and from PRE to CD with the corresponding changes in cortisol (r=-0.39, P=0.032; n=30), cortisol/total testosterone (r=-0.43, P=0.018; n=30), and cortisol/free testosterone (r=-0.40, P=0.028; n=30). $pSer^{473}$Akt level (mean of the three muscles) was inversely associated with the serum concentration of EAA (r=-0.36, P=0.015; n=45) and the cortisol /total testosterone (r=-0.31, P=0.038; n=45).

mTor. The expression level of mTor (mean of the three muscles) was inversely associated with the serum concentration of EAA (r=-0.32, P=0.033; n=45). There was a negative association between the increase in mTor protein expression from PRE to CRE and from PRE to CD with the corresponding changes in cortisol

(r=-0.40, P=0.028; n=30), cortisol/total testosterone (r=-0.38, P=0.041; n=30). A positive association was observed between the changes from PRE to CRE in pSer^{2448}mTor in the trained arm and the changes in BCAA (r=0.62, P=0.014; n=15), leucine (r=0.73, P=0.002; n=15) and betahydroxybutyrate (r=0.75, P=0.001; n=15). The corresponding correlation between the changes (mean of the three muscles) of pSer^{2448}mTor with BCAA (r=0.55, P=0.035; n=15) and leucine (r=0.65, P=0.008; n=15) was also statistically significant.

The expression level of total 4E-BP1 was associated with total testosterone (r=-0.29, P=0.049; n=45) and free testosterone (r=-0.42, P=0.005; n=45). The changes from PRE to CRE in Total 4E-BP1 protein expression levels (mean of the three muscles) were associated with the changes in insulin (r=-0.56, P=0.031; n=15), and also when the changes from PRE to CD were included (r=-0.38, P=0.041; n=30), as well as with the changes from PRE to CD in cortisol (r=-0.55, P=0.035; n=15) and cortisol/total testosterone (r=-0.53, P=0.041; n=15). The changes in 4E-BP1 expression from PRE to CRE and from PRE to CD were associated with the changes in cortisol (r=-0.48, P=0.007; n=30). The increase from PRE to CD in Thr$^{37/46}$4E-BP1 (mean of the three muscles) was associated to the corresponding changes in the serum concentrations of BCAA (r=-0.56, P=0.030; n=15). Likewise, the changes in Thr$^{37/46}$4E-BP1/total 4E-BP1 from PRE to CRE and from PRE to CD were associated with the corresponding changes in cortisol (r=0.37, P=0.043; n=30).

Total GSK3β (mean of the three muscles) was associated with the serum concentration of leptin (r=0.43, P=0.003; n=45), insulin (r=0.46, P=0.001; n=45), and EAA (r=-0.33, P=0.026; n=45). pSer^{9}GSK3β was associated with the serum concentration of leptin (r=0.39, P=0.008; n=45) and insulin (r=0.48, P=0.001; n=45). The increase of pSer^{9}GSK3β (mean of the three muscles) from PRE to CRE and from PRE to CD was associated with the corresponding increase of leptin (r=0.65, P<0.001; n=30), insulin (r=0.38, P=0.040; n=30) and total testosterone (r=0.40, P=0.027; n=30). Likewise, the changes of pSer^{9}GSK3β/total GSK3β (mean of the three muscles) from PRE to CRE and from PRE to CD were associated with the changes in leptin (r=0.52, P=0.003; n=30), insulin (r=0.37, P=0.044; n=30), BCAA (r=-0.39, P=0.032; n=30), and total testosterone (r=0.52, P=0.003; n=30).

Total eIF2α (mean of the three muscles) was associated with the serum concentration of leptin (r=0.29, P=0.053; n=45), insulin (r=0.34, P.

=0.024; n=45), and EAA (r=-0.33, P=0.027; n=45). The change in total eIF2α from PRE to CRE (mean of the three muscles) was associated to the change in BCAA (r=0.56, P=0.029; n=15) and leucine (r=0.58, P=0.023; n=15). pSer^{51}eIF2α (mean of the three muscles) was also associated with the serum concentration of leptin (r=0.34, P=0.021; n=45). The change in pSer^{51}eIF2α from PRE to CRE (mean of the three muscles) was associated with the change in leptin (r=0.58, P=0.023; n=15; for the trained arm only r=0.86, P<0.001; n=15) and cortisol (r=0.65, P=0.009; n=15), while the change from PRE to CD was

associated to the change in insulin (r=0.50 P=0.055; n=15). The expression levels of pSer^{51}eIF2α/Total eIF2α were associated to the serum concentration of free testosterone (r=0.30 P=0.042; n=45). The change from PRE to CRE in pSer^{51}eIF2α/Total eIF2α (mean of the three muscles) was associated to the change in leptin (r=0.51, P=0.050; n=15), while the change from PRE to CD was associated with the change in insulin (r=0.72, P=0.003; n=15).

Relationships between changes in muscle signaling responses and lean mass

There was a positive association between the mean lean mass and the mean Thr$^{37/46}$4E-BP1/total 4E-BP1 (r=0.85, P=0.004, n=9) and the mean pSer9GSK3β/total GSK3β (r=0.75, P=0.021, n=9, each point is the mean of the 15 subjects). In the trained arm, there was an association between the changes in lean mass from PRE to CRE and the changes in Akt protein expression (r=0.59, P=0.022, n=15), and from PRE to CD with the changes in pSer^{51}eIF2α (r=0.51, P=0.051, n=15) and pSer^{51}eIF2α/total eIF2α (r=-0.58, P=0.024, n=15). An association was also observed between the changes from PRE to CD in lean mass (all muscles) and the corresponding changes in pSer^{473}Akt (r=-0.33, P=0.028, n=45) and the pSer^{473}Akt/total Akt (r=-0.34, P=0.023, n=45). In the control arm, the change in lean mass from PRE to CRE was associated with the change in pSer^{9}GSK3β/total GSK3β (r=0.69, P=0.004, n=15).

In the legs, there was a negative association between the changes from PRE to CRE in lean mass and the changes in p70S6k (r=-0.57, P=0.027, n=15). Likewise,

there was an association between the changes from PRE to CD in lean mass and the corresponding changes in total mTor (r=-0.56, P=0.03, n=15) and the ratio $pSer^{2448}$mTor/total mTor (r=0.52, P=0.050, n=15), $Thr^{37/46}$4E-BP1 (r=-0.70, P=0.004, n=15).

ARTICLE II

SCIENTIFIC REPORTS

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N^1-methylnicotinamide is a signalling molecule produced in skeletal muscle coordinating energy metabolism

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Obesity is a major health problem, and although caloric restriction and exercise are successful strategies to lose adipose tissue in obese individuals, a simultaneous decrease in skeletal muscle mass, negatively effects metabolism and muscle function. To deeper understand molecular events occurring in muscle during weight-loss, we measured the expressional change in human skeletal muscle following a combination of severe caloric restriction and exercise over 4 days in 15 Swedish men. Key metabolic genes were regulated after the intervention, indicating a shift from carbohydrate to fat metabolism. Nicotinamide N-methyltransferase (NNMT) was the most consistently upregulated gene following the energy-deficit exercise. Circulating levels of N^1-methylnicotinamide (MNA), the product of NNMT activity, were doubled after the intervention. The fasting-fed state was an important determinant of plasma MNA levels, peaking at ~18 h of fasting and being lowest ~3 h after a meal. In culture, MNA was secreted by isolated human myotubes and stimulated lipolysis directly, with no effect on glucagon or insulin secretion. We propose that MNA is a novel myokine that enhances the utilization of energy stores in response to low muscle energy availability. Future research should focus on applying MNA as a biomarker to identify individuals with metabolic disturbances at an early stage.

The number of individuals who are overweight or obese is increasing worldwide and obesity elevates the risk of many serious diseases, including certain forms of cancer, cardiovascular disease and type 2 diabetes (T2D). Failure to address a continued increase in obesity will thus have negative effects on life expectancy[1]. The total estimated cost of diabetes diagnosed in the U.S. in 2012 was $245 billion, which is an increase by more than 40% in 5 years[2]. However, obesity can be counteracted by reducing energy intake and increasing energy expenditure thereby achieving a negative energy balance. Several studies support the use of exercise in combination with dietary changes as a weight-loss strategy[3–5]. An important reason to include exercise in a weight-loss program is its effect on preserving fat-free mass (FFM)[4,6], which is essential when combating obesity[6]. Skeletal muscle produces

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myokines which *e.g.* can regulate mitochondrial efficiency and thereby contribute to decreasing fat mass and insulin resistance[7]. Many weight-loss programs successfully achieve short-term weight loss, but the major challenge is that overweight/obese individuals fail to sustain the weight reduction over time[8,9].

Most successful strategies rely on lifestyle interventions ranging from several weeks to months. However, we have recently shown that a clinically relevant reduction in fat mass can be achieved and maintained in overweight men by an intense 4-day intervention, combining prolonged low-intensity exercise (9 h/day) and severe caloric restriction[10]. Following the 4-day intervention, total body mass was reduced by 4.9 kg, of which ~40% (2.1 kg) was fat mass. More importantly, the reduction in body mass was sustained (4.4 kg) four weeks after the intervention and the relative reduction in fat mass was increased to 90% (3.8 kg) of the total decrease in body mass. The majority of this reduction (~70%) was accounted for by loss of trunk fat, leading to a diminished waist circumference (~7 cm). A significant reduction in body weight (2.4 kg) was maintained a year after the intervention, most of which (~80%) was due to loss of fat mass. This weight reduction was accompanied by improvements in blood lipids, *e.g.* reduced total cholesterol and low-density lipoprotein levels.

Although loss of adipose tissue is beneficial, a simultaneous decrease in skeletal muscle mass has negative effects on metabolism as well as muscle function and performance[11,12]. In order to minimize such negative effects of exercise during an energy deficit state, a deeper understanding on a detailed molecular level is needed. Thus, the aim of the present study was to examine changes in gene expression in skeletal muscle in response to exercise combined with severe energy deficit.

Results

Changes in skeletal muscle mRNA expression in response to severe energy deficit. Fifteen overweight Swedish men were exposed to a combination of caloric restriction and high-volume-low-intensity exercise for 4 days yielding a ~5000 kcal/day negative energy balance and an average total decrease in fat mass of 2.1 kg[10]. During the intervention, one arm was exercised each morning for 45 minutes followed by ~8 h of hiking. After the intervention, the participants were again examined after 3 days of isoenergetic diet and reduced exercise (maximum 10000 steps/day). Skeletal muscle biopsies were taken (3 at each time point) in the fasted state in the morning at 3 time points during the study from both the exercised and non-exercised arm muscles (*deltoid*) and from one leg muscle (*vastus lateralis*), *i.e.* before (PRE) and after (WCR, *i.e.* Walking + Caloric Restriction) the 4-day intervention and after the reexamination 3 days later (DIET). Characteristics of the participants are presented in Supplementary Table S1. To study changes in muscle gene expression in response to low energy availability, we performed a microarray analysis using the Illumina Beadarray system. During the WCR period the diet consisted of 0.8 g/kg body mass of whey protein ($n = 8$) or sucrose ($n = 7$), split into three doses (morning, midday and afternoon). There was no significant difference in muscle gene expression due to diet (whey protein or sucrose), *i.e.* no genes were observed with a significant change in expression between the two diets in response to the intervention ($p_{adj} > 0.05$), therefore the two diet groups were merged for subsequent analyses ($n = 15$). In the non-exercised arm, 39 genes were differentially expressed between PRE and WCR (false discovery rate (FDR) < 5%), 14 showed higher and 25 lower expression at WCR. In the exercised arm, 44 genes were differentially expressed (FDR < 5%), 22 genes showed higher and 22 lower expression (WCR vs. PRE, FDR < 5%). In the leg, 421 genes were differentially expressed (FDR < 5%), 207 increased and 214 decreased (WCR vs. PRE)—(Supplementary Table S2). This larger number of differentially expressed genes in the leg could reflect the higher amount of work performed by this muscle group during the intervention. The 10 most differentially expressed genes (increased and decreased) at WCR compared with PRE in the exercised, non-exercised arm and leg muscles are reported in Table 1. Genes differentially expressed in more than one tissue are presented in Supplementary Fig. S1, *e.g.* 19 genes were found in all three muscle tissues. The most consistent decreased expression was observed for the transferrin receptor (*TFRC*) gene (0.17, 0.15 and 0.19; $p_{adj} < 0.001$) for non-exercised, exercised arm and leg muscles respectively (WCR vs. PRE). In contrast, Nicotinamide N-methyltransferase (*NNMT*) was upregulated 5.4, 4.2 and 2.9-fold at WCR compared with PRE ($p_{adj} < 0.05$) in non-exercised and exercised arm and leg muscles, respectively.

The only gene found to be differentially expressed between DIET and WCR (FDR < 5%) in the non-exercised arm was carnitine palmitoyltransferase 2 (*CPT2*). In the exercised arm, three genes, *SCHIP1*, *CPT2* and high-mobility group protein B2 (*HMGB2*), were differentially expressed (DIET vs. WCR, FDR < 5%). Of these, *HMGB2* and *CPT2* were regulated in the opposite direction, *i.e.* increased expression after the 4-day intervention and reduced after the 3-day follow up. In the leg, 147 genes were found differentially expressed between DIET and WCR (FDR < 5%). Out of these, 121 (82%) were regulated in the opposite direction in WCR vs. PRE, *i.e.* a large proportion of the identified changes in gene expression induced by the 4-day intervention was reversed by the 3-day period with isoenergetic diet and limited exercise. All differentially expressed genes (FDR < 5%) in WCR vs. PRE and DIET vs. WCR are reported in Supplementary Tables S2 and S3, respectively.

A severely energy deficient state leads to increased muscle expression of NNMT and elevated circulating levels of N^1-methylnicotinamide. The most consistently increased expression at WCR versus PRE was seen for the *NNMT* gene, *i.e.* the gene with the largest increase in expression in all 3 muscle tissues. The increased expression of NNMT was confirmed at the protein level using western blot with a ~12-fold ($p < 0.001$), ~8-fold ($p < 0.01$) and ~19-fold ($p < 0.001$) increase for non-exercised and exercised arm and leg muscles respectively ($n = 15$, Fig. 1a). NNMT is a methyltransferase, catalyzing N-methylation of nicotinamide (NA) to produce N^1-methylnicotinamide (MNA) and has recently been shown to regulate energy expenditure with elevated expression in white adipose tissue (WAT) of obese and diabetic mice[13]. We therefore investigated if the severely energy deficient state, induced by the diet and exercise intervention, would result in a difference in circulating plasma levels of nicotinamide (pNA) and/or N^1-methylnicotinamide (pMNA) using liquid chromatography coupled to mass spectrometry (LC-MS). Blood samples were drawn in the fasted state (12 h) at 5

Non-Exercised arm				Exercised arm				LEG			
Gene Symbol	Entrez GeneID	Fold change	FDR adjusted P-value	Gene Symbol	Entrez GeneID	Fold change	FDR adjusted P-value	Gene Symbol	Entrez GeneID	Fold change	FDR adjusted P-value
Top 10 up-regulated genes											
NNMT	4837	5.35	0.00623	*HMGCS2*	3158	5.51	0.03552	*ACTC1*	70	5.77	0.00023
HMGCS2	3158	4.64	0.02902	*NNMT*	4837	4.15	0.03968	*MT2A*	4502	4.47	0.00010
PDK4	5166	4.43	0.00526	*HMOX1*	3162	3.77	0.02117	*ANGPTL4*	51129	3.90	0.00101
FCN3	8547	2.87	0.01057	*ANGPTL4*	51129	3.69	0.02170	*CDKN1A*	1026	3.01	0.01522
TPPP3	51673	2.83	0.00085	*C13ORF39*	196541	3.67	0.01396	*NNMT*	4837	2.93	0.00851
RHOD	29984	2.50	0.02892	*TPPP3*	51673	2.70	0.00332	*HMOX1*	3162	2.92	0.00186
UCP2	7351	2.22	0.00563	*UCP2*	7351	2.66	0.00022	*SERPINA3*	12	2.81	0.00149
GLRX	2745	2.10	0.01057	*FCN3*	8547	2.53	0.01693	*PDK4*	5166	2.77	0.00636
CEBPD	1052	2.03	0.02448	*RHOD*	29984	2.38	0.04033	*LOC644150*	644150	2.71	4.30E-06
MPP6	51678	1.78	0.01057	*IRF7*	3665	2.25	0.00959	*FCN3*	8547	2.56	0.00016
Top 10 down-regulated genes											
TFRC	7037	0.17	0.00085	*TFRC*	7037	0.15	6.04E-07	*TFRC*	7037	0.19	2.56E-05
TMEM70	54968	0.32	0.00488	*TMEM70*	54968	0.30	0.01396	*C5ORF13*	9315	0.22	8.45E-09
WDR62	284403	0.34	0.00623	*G0S2*	50486	0.36	0.04413	*ITGB1BP3*	27231	0.22	0.00073
OR7E37P	100506759	0.37	3.54E-05	*OR7E37P*	100506759	0.39	5.06E-07	*SLC16A3*	9123	0.23	1.68E-05
FLJ25404	146378	0.37	0.01057	*C5ORF13*	9315	0.40	0.00311	*ZNF197*	10168	0.25	0.00023
EXTL1	2134	0.38	0.00488	*WDR62*	284403	0.41	0.01396	*WDR62*	284403	0.26	7.19E-05
MASP1	5648	0.38	0.02448	*EXTL1*	2134	0.41	0.00109	*OR7E37P*	100506759	0.30	8.45E-09
C5ORF13	9315	0.39	0.01057	*ATPGD1*	57571	0.45	0.00146	*ATPGD1*	57571	0.30	2.60E-07
ATPGD1	57571	0.43	0.00450	*CA14*	23632	0.45	0.00446	*LOC342934*	N/A	0.31	0.00074
CA14	23632	0.44	0.00344	*MAP6D1*	79929	0.48	0.00446	*ANK1*	286	0.31	4.15E-12

Table 1. Top 10 genes ranked on fold change (up or down) in skeletal muscle from non-exercised arm (*deltoid*), exercised arm and leg (*vastus lateralis*) following a 4-day intervention (PRE vs. WCR).

different time points. No difference in pNA concentration was observed (Fig. 1b), but the concentration of pMNA was ~2-fold increased at WCR compared to before the intervention, *i.e.* compared to the average pMNA concentration at time points −7 and 0 days ($0.24 \pm 0.024\,\mu M$ vs. $0.12 \pm 0.010\,\mu M$, $p < 0.01$, $n = 15$, Fig. 1b).

The nutritional state regulates pMNA levels. To test if the nutritional state alone, regardless of exercise, influences the circulating pMNA level, C57BL6/J mice were subjected to either a 4 h or 12 h fasting period. The food was withdrawn in the morning ($n = 6$) and one set of mice was sacrificed after 4 h ($n = 4$) and a second set after 12 h ($n = 6$). In the fed state (time = 0), the pMNA concentration was $0.15 \pm 0.019\,\mu M$ and increased after 4 h to 0.32 ± 0.018 ($p < 0.01$, Supplementary Fig. S2). After 12 h of fasting the level was $0.22 \pm 0.012\,\mu M$.

To test if feeding regulates the pMNA level also in humans, 18 healthy Danish volunteers were subjected to a 36 h fast followed by a meal. The study has previously been described[14] and characteristics of the included individuals are presented in Supplementary Table S4. Blood was drawn at 12, 18, 27 and 36 h of fasting and at 1.5 and 3 h after the meal. The pMNA level initially increased with duration of fasting ($0.12 \pm 0.019\,\mu M$ at 12 h, $p < 0.05$, compared to 0.07 ± 0.009 *i.e.* the mean of [36 h, 1.5 h and 3 h post meal]), reaching a highest concentration at 18 h of fasting ($0.20 \pm 0.029\,\mu M$, $p < 0.001$, compared to 0.07 ± 0.009 *i.e.* the mean of [36 h, 1.5 h and 3 h post meal])—(Fig. 2). Extending the fasting period further led to a reduction of the pMNA concentration, reaching a lower-limit of $0.071 \pm 0.0099\,\mu M$ after 27 h of fasting (Fig. 2). Eating a meal after 36 h of fasting, when the pMNA concentration was $0.068 \pm 0.010\,\mu M$, did not lower pMNA further ($0.078 \pm 0.016\,\mu M$ and $0.063 \pm 0.012\,\mu M$ at 1.5 h and 3 h post meal, respectively, Fig. 2). In another experiment 13 healthy Danish volunteers were subjected to a 15 h fast followed by a meal. Characteristics of the participants are presented in Supplementary Table S5. Six of the volunteers participated in both experiments. After 10 h of fasting the pMNA concentration was $0.18 \pm 0.063\,\mu M$. A meal, given after 15 h of fasting, yielded a lower pMNA concentration of $0.096 \pm 0.016\,\mu M$ 1.5 h after the meal (Supplementary Fig. S3). This level decreased further at 3 h after the meal to $0.063 \pm 0.012\,\mu M$, $p < 0.05$ compared to 10 h of fasting (Supplementary Fig. S3).

Increased pMNA stimulates lipolysis, but not glucagon or insulin secretion. Having established an effect of energy availability on the circulating pMNA level, we hypothesized that MNA could coordinate metabolism as a cross-tissue signalling molecule able to mobilize energy substrates from stores in WAT and the liver as MNA has been shown to stimulate glucose release from isolated mouse hepatocytes[15]. An effect on lipolysis has not been shown. Therefore, we also investigated the impact of increased MNA on lipolysis in isolated primary rat adipocytes. Glycerol release was measured both at non-stimulated and isoproterenol-stimulated (10 nM) conditions with addition of 1, 10 or 100 mM MNA. In the basal state, 100 mM MNA increased glycerol release ~14-fold compared to control (0.10 ± 0.0045 vs. $0.0072 \pm 0.0011\,\mu mol/30\,min/ml$, $p < 0.001$, $n = 6$–10, Fig. 3a). In the isoproterenol-stimulated state, 100 mM MNA increased glycerol release ~1.5-fold compared to isoproterenol

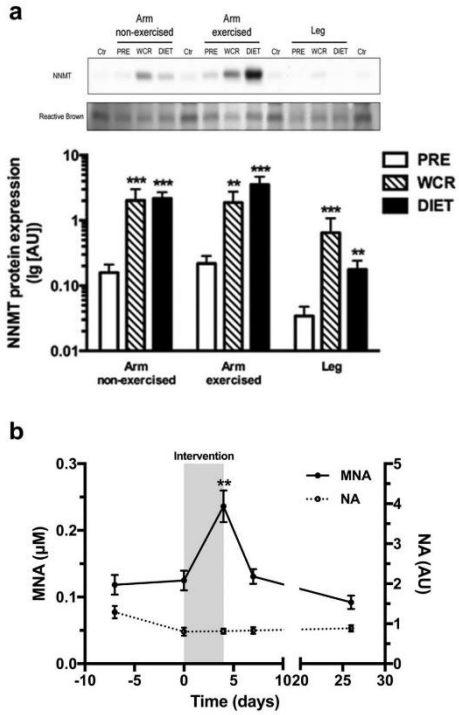

Figure 1. NNMT protein levels in skeletal muscle and MNA/NA plasma concentrations in humans during a 4-day low-caloric-high-volume exercise intervention. (**a**) Protein expression of NNMT in human skeletal muscle measured using Western blot before (PRE), after (WCR) and at 3 days post (DIET) a 4-day low-caloric-high-volume exercise intervention. Expression in the non-exercised arm, exercised arm and leg are given separately. A quality control sample was included on all gels (Ctr). Top square image shows a representative NNMT band (~30 kDa) (blot was cut on either side of the band before staining with antibody). Lower image is cropped from the full-length blot stained with reactive brown (for image of full-length blot, see Supplementary Fig. S6), and shows the region of the blot that was cut and stained with NNMT antibody. $^{**}p < 0.01$, $^{***}p < 0.001$ using Wilcoxon signed-rank tests compared to PRE, ($n = 15$). No significant difference between WCR and DIET was observed. (**b**) Circulating levels of plasma nicotinamide (NA) and N1-methylnicotinamide (MNA) at five time point before and after the 4-days intervention, ($n = 15$). The intervention period is indicated with grey shading. Blood was drawn after 12 h of fasting at each time point. $^{**}p < 0.01$ using Wilcoxon signed-rank tests compared to the average level of the two time points before the intervention. No significant difference in NA concentration was observed. Data is given as mean ± SEM.

alone (0.19 ± 0.0043 vs. 0.13 ± 0.014 μmol/30 min/ml, $p < 0.01$, $n = 6–10$, Fig. 3a). Isoproterenol alone stimulated glycerol release ~18-fold ($p < 0.001$, $n = 10$, Fig. 3a). To test if MNA exerts its effects primarily on WAT and the liver or if its regulation of lipolysis and glucose production could be mediated via glucagon and/or insulin, hormone secretion was measured from human pancreatic islets of Langerhans stimulated with 1–100 mM of MNA. However, no effect of MNA on either glucagon or insulin release was found (Fig. 3b,c).

Isolated human myotube cells have the ability to secrete MNA. Next, we analyzed the expression of NNMT in muscle cells, and more importantly if muscle cells are able to secrete MNA. Human myotubes were cultured for 48 and 72 h in two different media, *i.e.* α-MEM or F10. Two culture conditions were chosen for replication purposes and the main difference in composition between the two media was the amount of included amino acids and NA, *e.g.* α-MEM medium contains ~1.7-fold higher NA concentration compared to F10. The mRNA expression level of NNMT and the concentration of MNA in the culture medium were measured. No difference in myotube NNMT mRNA expression was seen between the two time points (48 and 72 h) in either of the two media

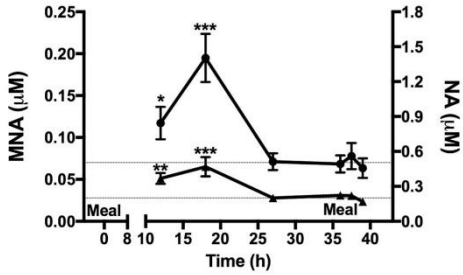

Figure 2. Plasma MNA and NA levels in humans during fasting and feeding. Circulating levels of plasma MNA and NA after prolonged fasting (36 h) and refeeding. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Wilcoxon signed-rank tests. Time points 12 h, 18 h and 27 h of fasting are compared to the mean level of time points 36 h fasting, 1.5 h post feeding and 3 h post feeding, $n = 18$. Data is given as mean ± SEM.

(10.65 ± 1.51 AU vs. 11.99 ± 1.53 AU [α-MEM] and 13.39 ± 1.94 vs. 15.34 ± 4.28 [F10], or between the two media at either time point (10.65 ± 1.51 AU vs. 13.39 ± 1.94 [48 h] and 11.99 ± 1.53 AU vs. 15.34 ± 4.28 [72 h], $n = 5$ in 1–3 independent experiments, Fig. 4a). However, the concentration of MNA in the medium was increased by 1.7-fold and 1.9-fold at 72 h vs. 48 h in both α-MEM medium (1.06 ± 0.17 vs. 0.61 ± 0.11 μM/μg RNA_{tot}, $p < 0.05$, $n = 5$ in 1–3 independent experiments) and in F10 medium (1.95 ± 0.27 vs. 1.04 ± 0.19 μM/μg RNA_{tot}, $p < 0.01$, $n = 5$ in 1–3 independent experiments), respectively (Fig. 4b). Interestingly, increased levels of MNA was observed in F10 compared to α-MEM medium, showing a 1.8-fold increase at 72 h of culture (1.95 ± 0.27 vs. 1.06 ± 0.17 μM/μg RNA_{tot}, $p = 0.056$ with a Mann-Whitney U test, $n = 5$ in 1–3 independent experiments, Fig. 4b), indicating that the concentration of amino acids may influence muscle MNA secretion. No difference in the concentration of NA was seen between the two time points in either medium (Supplementary Fig. S4). To screen for metabolites correlating with the release of MNA, we measured the concentration of 47 metabolites in the media using LC-MS after culturing human myotubes as described above, *i.e.* with combination of different durations (48 and 72 h) and media (α-MEM and F10) (Supplementary Fig. S5). 2-Hydroxybutanoic acid (*i.e.* α-Hydroxybutyrate, α-HB) was the most correlated metabolite with MNA in α-MEM medium (α-MEM: $r^2 = 0.93$ [48 h] and 0.64 [72 h]; F10: $r^2 = 0.009$ [48 h] and 0.010 [72 h] (Fig. 4c)).

Discussion

In this study, we examined changes in skeletal muscle gene expression after caloric restriction in combination with an exercise intervention. The expression of key genes involved in metabolic regulation (*e.g. CPT2*, *PDK4* and *PFKFB3*) was altered after the 4-day intervention, indicating a shift from carbohydrate to fat metabolism. As expected, we observed an increased expression of *PDK4* after the 4-day intervention, indicating a low carbohydrate oxidation rate, as PDK4 is an inhibitor of the pyruvate dehydrogenase complex (PDC) and thereby the transport of pyruvate into the mitochondria. An increased lipid oxidation rate was also supported by the increased expression of *CPT2*, an important part of the transport system of fatty acids into the mitochondria. The results also indicate increased muscle wasting, *i.e.* increased expression of genes involved in the ubiquitine proteasome system (*e.g. TRIM63* and *PSMD4*), as well as in the autophagy-lysosomal proteolytic system (*e.g. BNIP3*, *CTSD* and *SQSTM1*). A majority of the changes observed were however reversed by 3 days of isoenergetic diet and limited exercise.

The major finding was that the expression of *NNMT* in skeletal muscle was consistently increased after the intervention with a concomitant increase of pMNA. In connection, nicotinamide riboside kinase 2 (ITGB1BP3) and poly [ADP-ribose] polymerase 1 (PARP1), proteins involved in the NAD^+-salvage pathway, were found to have lower expression after the intervention. Fasting alone increased the pMNA concentration and eating a meal, when pMNA was high, reduced pMNA to approximately 0.07 μM after 3 h. Interestingly, while the concentration of NA was not significantly changed at the severely energy deficient state (Fig. 1b), a concomitant increase in NA was seen with the increase of MNA in response to fasting alone (Fig. 2). NNMT has recently been shown to regulate energy expenditure with elevated expression in WAT in obese and diabetic mice[13]. Knockdown of NNMT in WAT and liver in mice protects against diet-induced obesity by increasing cellular energy expenditure. However, these experiments were not performed in tissue-specific mouse models and a possible effect of skeletal muscle NNMT knockdown was not considered[13]. Chronic MNA treatment lowers fasting glucose levels and prolongs the survival of rats with streptozotocin-induced diabetes[16]. Patients with T2D show approximately two fold higher expression of NNMT in WAT compared to healthy controls[17] and elevated levels of MNA both in the circulation[17,18] and in urine[19]. The pMNA level is positively correlated with BMI[17,19], inversely with insulin sensitivity[17] and bariatric surgery has been shown to reduce both WAT NNMT expression and the level of pMNA[17]. In liver, NNMT is a positive regulator of gluconeogenesis, via a sirtuin 1-dependent mechanism and MNA stimulates gluconeogenesis in primary hepatocytes[15]. In addition, here we show that MNA also has the ability to stimulate lipolysis in isolated rat adipocytes, but has no effect on the release of insulin or the starvation hormone glucagon from human islets of Langerhans. Out of the 47 measured metabolites in media from cultured human myotubes,

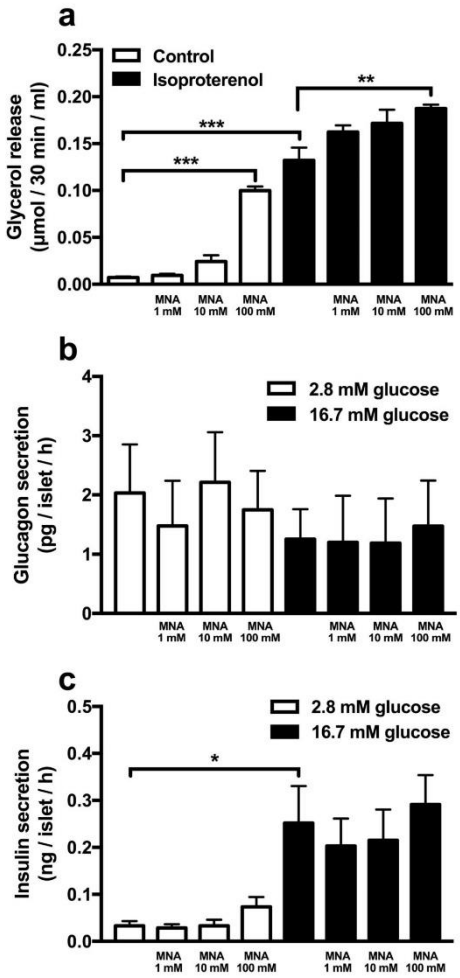

Figure 3. Measurements of the effect of MNA on rat lipolysis and human glucagon and insulin secretion. (**a**) Lipolysis in isolated rat adipocytes. Primary adipocytes were pre-incubated with MNA (1–100 mM) for 30 min, and then stimulated with isoproterenol (Iso, 10 nM) for additional 30 min. Glycerol release was measured in the medium both in the unstimulated and Iso-stimulated state, n = 6–10 independent experiments of pools of 2–3 rats per experiments. (**b**) Glucagon secretion from human islets of Langerhans, n = 3–5. (**c**) Insulin secretion from human islets of Langerhans, n = 4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Kruskal–Wallis tests with Dunn's correction for multiple comparisons within each group (Ctrl and Iso). Data is given as mean ± SEM.

the MNA concentration was most strongly correlated with that of α-HB. α-HB has been suggested to be an early biomarker of insulin resistance and a predictor of glucose intolerance[20,21]. The results presented here clearly demonstrate that the NNMT-MNA axis plays an important role as a regulator of energy metabolism, supported by previous studies[13,15,17]. In addition, data presented in literature[13,16–19] also show that a perturbation of this mechanism is seen in obesity and T2D. Taken together, these results suggest that pMNA could be a potential biomarker for metabolic flexibility[22,23] and possibly be used as an early marker for metabolic disease.

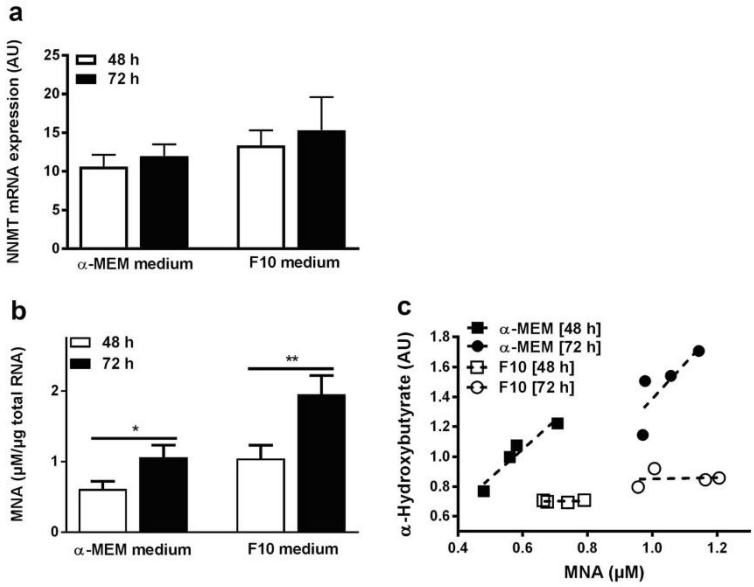

Figure 4. NNMT mRNA expression, and the release of MNA and α-Hydroxybutyrate from human myotubes. (**a**) The mRNA expression level of NNMT in human myotube cells measured using QPCR. The myotubes were cultured in either α-MEM or F10 medium for 48 h or 72 h. n = 5 in 1–3 experiments. (**b**) Concentration of MNA in human myotube culture medium. n = 4–5 in 1–3 experiments. The MNA concentration is expressed versus total RNA content to normalize for cell density. (**c**) Concentration of MNA and α-Hydroxybutyrate in the culture medium after 48 h or 72 h incubation in either α-MEM or F10 medium. n = 4 in 1–3 independent experiments. *$p < 0.05$, **$p < 0.01$ using Paired t tests. Data is given as mean ± SEM.

Here we show an increase of muscle NNMT protein and pMNA concentrations after exercise and caloric restriction. It has previously been reported that a 12-week exercise intervention led to a reduction of WAT *NNMT* expression in prediabetic and diabetic individuals, but no change in healthy controls[17]. The effect on pMNA was not measured. In contrast, a single bout of endurance exercise increased liver NNMT activity and pMNA[24]. However, the increased pMNA level observed after exercise could not be explained by the regulation of liver NNMT, as liver NNMT activity was not increased after exercise in IL-6 knockout mice, but yet led to an increase of pMNA[24]. Although adipocytes have been shown to secrete MNA[25], the increased pMNA seen after exercise cannot be explained by expressional regulation of *NNMT* in WAT or the liver. The data presented here suggest that this elevated pMNA concentration could be explained by release of pMNA from skeletal muscle under exercise and/or fasting conditions. However, in the metabolically unhealthy, *i.e.* obese, prediabetic or T2D states, a correlation between WAT *NNMT* expression and the fasting pMNA level have been described[17], indicating that a dysregulation of the NNMT-MNA axis in WAT may play a greater role under such conditions. How regulation of muscle NNMT activity influences metabolism in prediabetic and diabetic individuals remains to be studied, as all individuals examined here were non-diabetic. However, the duration of fasting should be considered in all comparisons, as our results showed an initial increase and subsequent decrease in pMNA levels after fasting.

Caloric restriction and weight reduction have many positive effects on metabolism and also increase liver *NNMT* expression in mice[15,26]. Here we show a similar regulation of *NNMT* expression in human skeletal muscle. This is in contrast to WAT *NNMT* expression, which is reduced by weight reduction[17]. Thus, the regulatory mechanisms of *NNMT* expression as well as the mechanisms underlying the downstream effects are most likely different in these tissues. In line with the regulation in liver[15], we postulate that the downstream effects of muscle NNMT activity are not mediated by a shift in the methyl donor balance, as in WAT[13], but rather by its product MNA. The muscle energy-sensing mechanism is however unclear, but possibly involves a signal emanating from the balance between anaerobic and aerobic metabolism, or a shift from carbohydrate to lipid oxidation. Caloric restriction elicits increased activity of proteins in the sirtuin family (*e.g.* Sirt1)[27], which in turn results in lysine deacetylation and breakdown of nicotinamide adenine dinucleotide (NAD+) to NA and 1′-O-acetyl-ADP-ribose[28] or 2′- and 3′-O-acetyl-ADP-ribose[29]. An increased activity of NNMT during such low-energy states could serve to maintain a high degree of deacetylase activity and fat oxidation by removing NA and thereby its feedback-inhibition of Sirt1[30]. NNMT may thus serve as a sensor for the change in the redox

system of the cell. The level of circulating amino acids could possibly play a part in the muscle energy-sensing mechanism, as indicated by the experiments in myotubes showing higher concentration of MNA in the amino acid poor F10 medium (Fig. 4b).

In summary, we have shown that a high-volume-low-intensity exercise intervention in combination with caloric restriction elevates the expression of *NNMT* in human skeletal muscle, and that this increase is mirrored by a rise in the fasting level of pMNA. The pMNA level can also be regulated by the nutritional state alone, *i.e.* fasting leads to an initial increase followed by a decline, while eating a meal when the pMNA level is high lowers this level. MNA can be secreted from human myotubes and stimulates lipolysis, but has no effect on glucagon or insulin release. On the basis of these findings and the existing literature, we propose that MNA is a myokine that signals directly to WAT and the liver to mobilize energetic substrates when the availability of energy is low in muscle. Future research should focus on applying MNA as a biomarker for skeletal muscle insulin sensitivity to identify individuals with metabolic disturbances at an early stage.

Methods

Participants. The Ostersund study[10] was designed to get insight into the mechanisms that regulate muscle metabolism, muscle mass and fat mass during severe energy deficit in humans. To isolate the potential effects due to exercise from those elicited by the neuro-endocrine systemic adaptations, muscle biopsies were obtained from three different skeletal muscles subjected to different levels of exercise. The inclusion criteria were: 1) an age of 18–55 years, 2) stable body weight for at least 3 months prior to the start of the experiments, 3) a BMI $\geq 25\,kg/m^2$, 4) a waist circumference >102 cm, and 5) 20–40% body fat. The exclusion criteria were: 1) orthopedic limitations incompatible with prolonged walking or exercise, 2) smoking and 3) chronic disease of any kind. Sixteen men were recruited initially, but one retired due to incompatibility with his working duties. The 15 remaining participants were assigned randomly to a diet consisting of either sucrose (n = 7) or whey protein (n = 8) during study phase III. Characteristics of participants are presented in Supplementary Table S1. The study was divided into five different phases: I = baseline assessment (PRE), II = intervention (WCR), III = recovery (DIET), IV = follow up 4 weeks post intervention (4W) and V = follow up 1 year post intervention (1Y). The 4-day intervention during phase II consisted of walking, arm cranking exercise + caloric restriction. The 3-day recovery during phase III consisted of isocaloric diet and reduced exercise (maximum 10000 steps per day). During phase I and at the end of phases II, III and IV, body composition (Lunar iDXA, GE Healthcare, Madison, WI; and Bioimpedance, InBody 720, Biospace Co. Ltd, Seoul, Korea) and VO_{2max} using a metabolic cart (Jaeger Oxycon Pro, Viasys Healthcare, Hoechberg, Germany) during an incremental exercise test to exhaustion on a cycle ergometer (Monark 839E, Ergomedic, Sweden) were assessed. In addition, during phases I-IV 30-ml blood samples were drawn, following a 12 h overnight fast. In phase I, blood samples were obtained on two occasions, one week apart. During phase V, only body composition was assessed. A full description of the experimental protocol can be found elsewhere[10].

The 36 h fasting study has previously been published[14]. Briefly, 18 men (age = 24.6 ± 1.2 years and BMI = 22.9 ± 3.2 kg/m^2) were recruited from the Danish National Birth Registry as healthy, non-diabetic, with no diabetes in two family generations. Characteristics of the participants are presented in Supplementary Table S4. All were subjected to 36 h of fasting and sampling. 8–16 weeks after the 36 h fasting study, 6 of these subjects participated in a second study together with 7 other matched males born with a low birth weight. Characteristics of the participants are presented in Supplementary Table S5. The 13 participants received a control diet and were subjected to a 15 h fast. During both study settings, the participants were allowed ad-libitum water. For 72 h prior to the study interventions, the participants received a control diet of precooked meals for standardization of energy intake (10 MJ per day, 50% carbohydrate, 35% fat, 15% protein). Furthermore, the participants were not allowed to perform exercise or consume alcohol or soft drinks in these three days. All blood samples were immediately distributed into tubes, placed on ice, and centrifuged (Eppendorf Centrifuge 5810R, Eppendorf AG, Hamburg, Germany). Plasma was obtained and stored at −80 C until later analysis.

Microarray analysis of muscle biopsies. Fifteen overweight Swedish men participated in a 4-day intervention combining severe caloric restriction with prolonged exercise, as previously described. Muscle biopsies were obtained from right and left *deltoid* muscle and from *vastus lateralis* of either leg. RNA was extracted from 10–20 mg of muscle biopsies using a TissueLyser II (Qiagen) and the RNeasy Fibrous Tissue mini kit (Qiagen). RNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (A260/A280 > 1.8 and A260/A230 > 1.0 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was evaluated using the 2100 Bioanalyzer Instrument (Agilent Technologies). Input RNA, 100 ng for the leg and 200 ng for the arm samples, was used for subsequent biotinylation and hybridization. TotalPrep™-96 RNA Amplification Kit (Thermo Fisher Scientific) was used to generate biotinylated, amplified cRNA for subsequent hybridization of 750 ng (150 ng/μl) on the Human HT-12 v4 Expression BeadChip (Illumina) and scanning on the iScan system (Illumina). The raw data was read and quantified using Illumina Genome Studio version 1.1.1. Summary level expression data was read using the Bioconductor beadarray package. Differential expression analysis was performed using the Bioconductor limma package. P-values were corrected using the False Discovery Rate method, with an acceptance of < 0.05.

Total protein extraction, electrophoresis, and Western blot analysis. Protein extracts from the muscle biopsies were prepared as previously described[31], and total protein content was quantified using the bicinchoninic acid assay[32]. 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. The total protein to load and the optimal antibody concentration was determined by linear regression from a gradient of protein extracts at concentrations ranging from 20 to 45 μg. The linear relation between total protein concentration loaded and quantitative intensity of the bands was calculated.

After confirming linearity in this range, equal amounts (30 μg) of each sample were electrophoresed with 10% SDS-PAGE as previously described[33] and transferred to an Immun-Blot PVDF Membrane for Protein Blotting (Bio-Rad Laboratories). To control for differences in loading and transfer efficiency across membranes, membranes were stained with Reactive Brown 10 (0.07%) and destained with water for 10 sec to remove background. Loading was similar for control and experimental samples (data not shown). After this, membranes were destained for 3 × 10 min in NaOH (0.1 N). To determine NNMT total protein amount, the membrane, cut on either side of the region for the NNMT band (~30 kDa), was incubated with an antibody directed against NNMT (NNMT (G-4), a mouse monoclonal antibody raised against NNMT of human origin, sc-376048, Santa Cruz Biotechnology), diluted (1:1000) in 4% BSA, in Tris-buffered saline with 0.1% Tween 20 (TBS-T; BSA-blocking buffer). Antibody-specific labelling was revealed by incubation with goat anti-mouse IgG-HRP (sc-2031, Santa Cruz Biotechnology), diluted (1:5000) in 5% blocking buffer, and visualized with the ClarityTM Western ECL Substrate kit (Bio-Rad Laboratories) using the ChemiDoc XRS system (Bio-Rad Laboratories) and analysed with Quantity One (Bio-Rad Laboratories). Densitometry analyses were carried out immediately. Samples from each subject were run on the same gel. In addition, in all gels, a 30 μg control sample obtained from the extraction of 5 g of pooled human skeletal muscle from different healthy donors was loaded 4 times per gel as an internal control.

Measurements of pMNA and NA using LC-MS. Measurements of 1-methylnicotinamide were performed in human serum, mouse EDTA plasma and cell media using a UHPLC-QTOF-MS System (Agilent Technologies 1290 LC, 6550 MS, Agilent Technologies, Santa Clara, CA, USA). All samples were stored at −80 °C and thawed on ice before metabolite extraction was performed by addition of six volumes of extraction solvent, consisting of 80:20 LC-MS grade methanol/water containing the stable isotope labelled 1-methylnicotinamide-d4 (Toronto Research Chemicals Inc., Toronto, Canada). Samples were incubated at 4 °C, with mixing at 1250 rpm during 1 hour, before precipitated proteins were removed by centrifugation for 20 minutes at 14000 × g. The supernatant was transferred to glass vials. Sample preparation for measurement of α-HB in cell media was performed in the same manner. Liquid chromatography separation was performed by injecting 2 μl sample on an Acquity UPLC BEH Amide column (1.7 μm, 2.1 * 100 mm; Waters Corporation, Milford, MA, USA) or on an ACE C18 column (1.7 μm, 2.1 * 100 mm; Advanced Chromatography Technologies Ltd., Aberdeen, UK) for measurements of 1-methylnicotinamide and α-HB respectively. Separation on Acquity UPLC BEH Amide was performed by gradient elution using two mobile phases (mobile phase A: H_2O with 10 mM ammonium formate and 0.1% formic acid; mobile phase B: acetonitrile with 0.1% formic acid) using a flow rate of 0.4 ml/min and subsequent mass spectrometric analysis was performed in positive electrospray ionization. Gradient elution on ACE C18 (mobile phase A: H_2O with 0.1% formic acid; mobile phase B: acetonitrile with 0.1% formic acid) was performed with at flow rate of 0.3 ml/min and subsequent mass spectrometric analysis utilized negative electrospray ionization. The auto sampler was kept at 16 °C. The sheath gas temperature was set at 350 °C and the sheath gas flow at 12 l/min. The drying gas flow was 14 l/min and was delivered at 200 °C. Mass spectra were acquired at a rate of 1 spectra/s and the mass range was 70–1000 m/z (mass-to-charge ratio). Samples were analysed in batches of maximum 45 samples, where pooled QC samples were injected every 5 samples and in the beginning of each batch to ensure high repeatability and to condition the LC-column respectively.

Adipocyte isolation and lipolysis assay. To measure lipolysis, rat adipocytes were isolated from epididymal fat tissue, as described previously[34]. Cells were suspended (10% suspension) in Krebs-Ringer (KRH) medium containing 25 mM Hepes pH 7.4, 200 nM adenosine and 1% BSA (w/v), preincubated with MNA (1–100 mM) for 30 min, and then stimulated with isoproterenol (Iso, 10 nM) for additional 30 min as indicated in the figures (at 37 °C, with shaking, 150 cycles/min). After 30 min, samples were placed on ice for 20 min, and 200 μl of the cell medium was subsequently removed for enzymatic determination of the glycerol content, as described previously[35].

Glucagon and insulin secretion measurements in isolated human islets of Langerhans. Islets from 6 human donors (gender = F3/M3, age 55 ± 7, BMI 26.5 ± 2.0 kg/m^2, HbA1c 5.9 ± 0.1, days in culture 2.8 ± 0.6) were provided by the Nordic Network for Clinical Islet Transplantation (Uppsala, Sweden). Human islets were hand-picked to ensure high purity. Insulin and glucagon secretion was measured in static batch incubations described previously[36]. Briefly, batches with 12 islets (in quadruplicates) were pre-incubated in Krebs Ringer solution (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 25 mM $NaHCO_3$, pH 7.4, supplemented with carbogen and 1 mg/ml of bovine serum albumin) supplemented with 1 mM glucose for 30 min, followed by 1 h incubation at 2.8 and 16.7 mM glucose in absence or presence of MNA (1–100 mM) as indicated. Insulin secretion was measured using radioimmunoassay kit (Euro-Diagnostica, Malmö, Sweden) and glucagon secretion was measured with ELISA (Mercodia, Uppsala, Sweden).

Culturing of human myotubes. Human primary Skeletal Muscle Derived Cells (SkMDC) from five different donors were purchased from Cook Myosite (Pittsburg, US). The myoblast cells were cultured and differentiated into myotubes following instructions supplied by the vendor. Briefly, cells were maintained in growth basal medium with growth supplements (MK-2288; Cook Myosite), 10% fetal calf (FCS) serum (Thermo Fisher Scientific) and antibiotics (0.4% penicillin/streptomycin (P/S). Cells were passaged at ~50% confluence. To induce differentiation, cells were plated at ~45000 cells/cm^2 in 6-well plates. Growth media was replaced with differentiation media (MD-9999; Cook Myosite) with 2% FCS and 0.4% P/S, and the cultures were incubated for 3 days, during which time myotube differentiation occurred (monitored using the expression of differentiation markers, *i.e.* Myosin Heavy Chain 2 and Myocyte-specific enhancer factor 2C). After differentiation, the multinucleated myotubes were either cultured in α-Minimum Essential Medium (α-MEM) or Ham's F10 Nutrient Mix (F10)

Supplementary Information

N^1-methylnicotinamide is a signalling molecule produced in skeletal muscle coordinating energy metabolism

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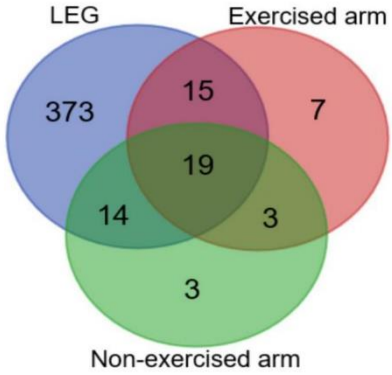
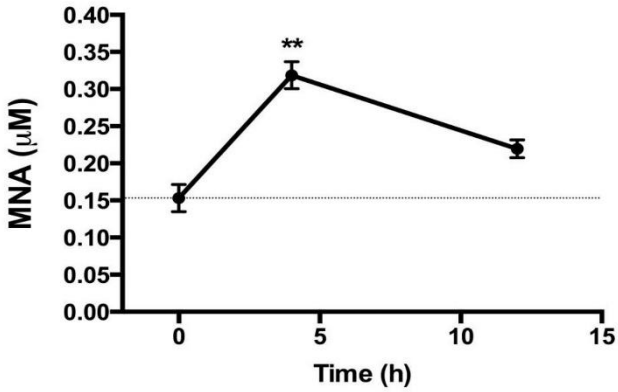
Supplementary Figures



Tissues with differentialy expressed genes	Total number of genes	Genes
Exercised arm LEG Non-exercised arm	19	EXTL1 RHOD LOC390557 GADL1 LDHA FCN3 CA14 C5ORF13 TPPP3 CCPG1 OR7E37P ASS1 TFRC DLEU1 WDR62 NNMT GLRX ATPGD1 NOL3
Exercised arm LEG	15	HMOX1 ANGPTL4 IRF7 MAP6D1 GPX3 TRIM7 HS.553217 HEXB C13ORF39 CHMP1B ANK1 HMGB2 YPEL3 STAT5A CPT2
LEG Non-exercised arm	14	PKM2 CCDC69 CEBPD DNAJB5 FLJ25404 PPM1J LOC642005 MASP1 TPI1 SLC38A3 MPP6 PDK4 TP53INP2 SCHIP1
Exercised arm Non-exercised arm	3	HMGCS2 TMEM70 UCP2

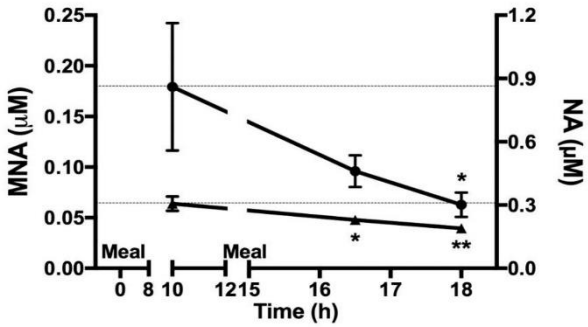
Supplementary Fig. S1. Genes differentially expressed at PRE versus WCR

The number and gene symbol names of genes found differentially expressed before (PRE) versus after (WCR) the 4-day intervention of caloric restriction and high-volume-low-intensity exercise in more than one tissue.



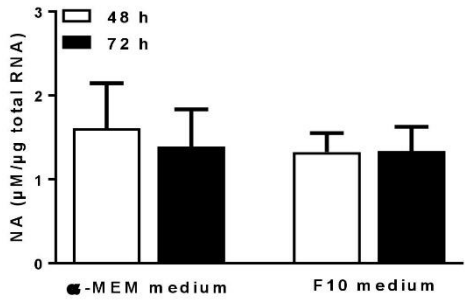
Supplementary Fig. S2. Plasma N1-methylnicotinamide (MNA) levels in mice during fasting and feeding

Circulating levels of plasma MNA after 4 and 12 h fasting in mice. ** $p < 0.01$ using Kruskal–Wallis tests with Dunn´s correction for multiple comparisons versus fed (time = 0). n = 4 – 6. Data is given as mean ± SEM.



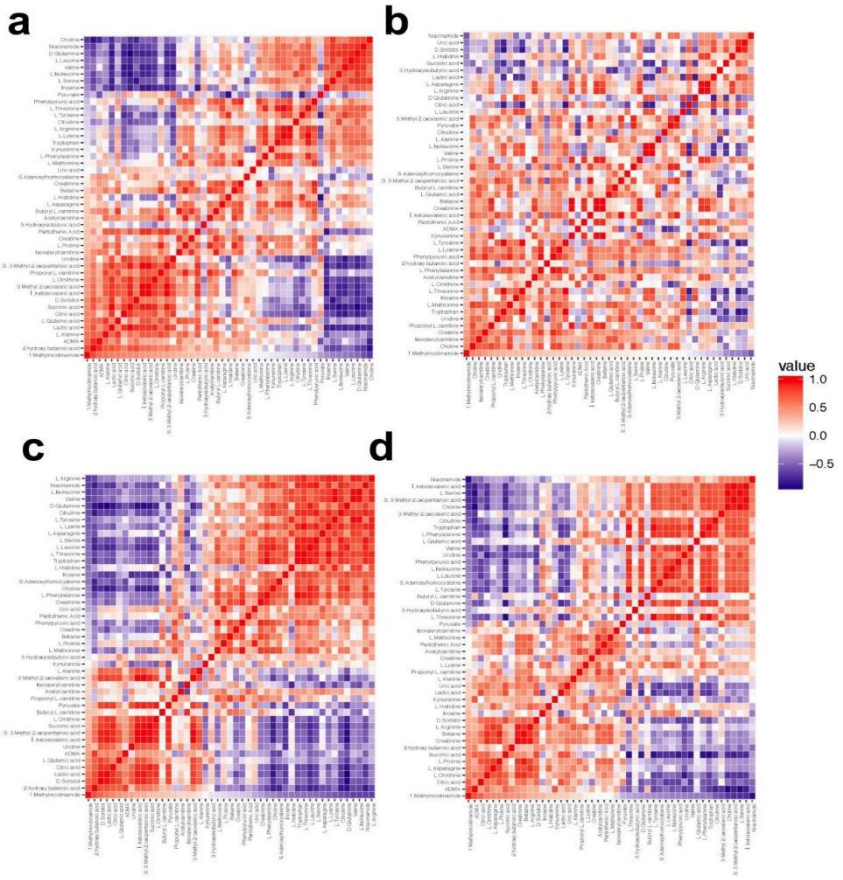
Supplementary Fig. S3. Plasma MNA and NA levels in humans during fasting and feeding

Circulating levels of plasma MNA and nicotinamide (NA) after 15 h of fasting and refeeding. * $p < 0.05$, ** $p < 0.01$ using Wilcoxon signed-rank tests. Time points 1.5 h and 3 h post feeding is compared to 10 h of fasting, n = 13. Data is given as mean ± SEM.



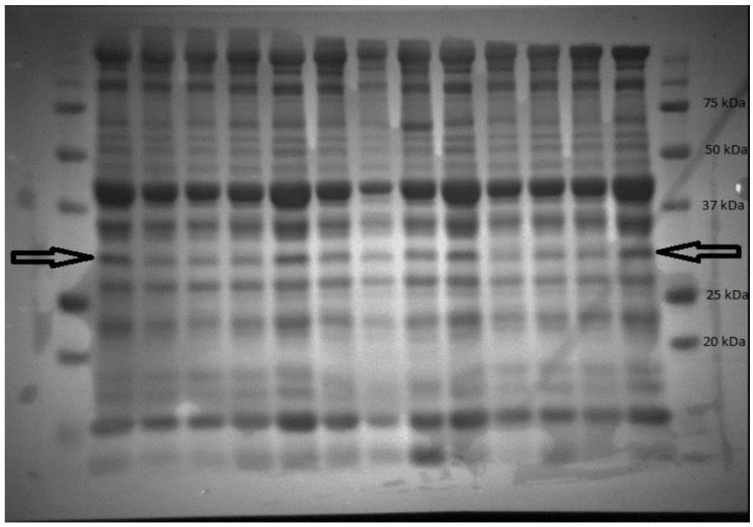
Supplementary Fig. S4. Nicotinamide (NA) release from human myotubes.

Concentration of NA in human myotube culture medium. n = 5 in 1-3 experiments. The NA concentration is expressed versus total RNA content to normalize for cell density. Data is given as mean ± SEM.



Supplementary Fig. S5. Correlation matrix of 47 metabolite levels in human myotubes culture media

Human myotubes cultured in α-MEM (**a** and **c**) or F10 (**b** and **d**) media for 48 h (**a** and **b**) or 72 h (**c** and **d**). The correlation coefficients are displayed in colour code with red = positive and blue = negative correlation. The average of n = 4 in 1-3 experiments are shown.



Supplementary Fig. S6. Full unedited blot for Figure 1a stained with Reactive Brown

To control for differences in loading and transfer efficiency across membranes, membranes were stained with Reactive Brown 10. Arrows indicate region (~30kDa) that was cut and stained with NNMT antibody.

Supplementary Tables

Supplementary Table S1

The pretest physical characteristics (means ± SD) of the participants of the exercise and caloric restriction intervention (n = 15).

	DIET	
	Sucrose (n=7)	Whey protein (n=8)
Age (years)	38.7 ± 8.2	43.0 ± 8.0
BMI (kg/m^2)	29.9 ± 3.1	30.9 ± 4.2
Lean mass (kg)	63.1 ± 3.1	65.4 ± 6.0
Fat mass (kg)	31.5 ± 9.1	31.4 ± 9.2
Body fat (%)	31.6 ± 5.3	30.9 ± 4.1
RMR (kcal/day)	1780 ± 272	1970 ±348
VO_{2max} (mL/kg/min)	38.8 ± 6.0	39.7 ± 5.5
Systolic BP (mmHg)	135 ± 7	132 ± 13
Diastolic BP (mmHg)	88 ± 5	89 ± 9

Abbreviations:
BMI, Body Mass Index; RMR, Resting Metabolic Rate; BP, Blood Pressure

Supplementary Table S4

Physical characteristics of the included individuals in the 36h fasting study (means ± SD) (n=18).

Age (years)	24.6 ± 1.20
BMI (kg/m^2)	22.9 ± 3.23
Lean mass (kg)	65.3 ± 7.03
Fat mass (kg)	13.4 ± 5.46
Body fat (%)	16.5 ± 4.77
RMR (kcal/day)	1681 ± 146

Abbreviations:
BMI, Body Mass Index; RMR, Resting Metabolic Rate; BP, Bood Pressure

Supplementary Table S5

Characteristics of the included individuals in the 15h fasting study (means ± SD) (n=13).

Age (years)	25.3 ± 1.21
BMI (kg/m^2)	22.0 ± 3.31
Lean mass (kg)	63.0 ± 4.41
Fat mass (kg)	11.3 ± 4.57
Body fat (%)	14.7 ± 4.43
RMR (kcal/day)	1548 ± 155

Abbreviations:
BMI, Body Mass Index; RMR, Resting Metabolic Rate; BP, Bood Pressure

ARTICLE III

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REVIEW ARTICLE

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Exercise-mediated modulation of autophagy in skeletal muscle

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Although exercise exerts multiple beneficial health effects, it may also damage cellular structures. Damaged elements are continuously degraded and its constituents recycled to produce renovated structures through a process called autophagy, which is essential for the adaptation to training. Autophagy is particularly active in skeletal muscle, where it can be evaluated using specific molecular markers of activation (unc-51-like kinase 1 [ULK1] phosphorylation) and specific proteins indicating increased autophagosome content (increased total LC3, LC3-II, LC3-II/LC3-I ratio). Studies in humans are technically limited but have provided evidence suggesting the activation of autophagy in skeletal muscle through AMP-activated protein kinase (AMPK) and its downstream target ULK1. Autophagy activation is more likely when the intensity is elevated and the exercise performed in the fasted state. The autophagy-gene program and autophagosome content are upregulated after ultraendurance running competitions. However, autophagosome content is reduced after endurance exercise at moderate intensities (50% and 70% of VO_2max) for 60-120 minutes. Autophagosome content is decreased within the first few hours after resistance training. The effects of regular endurance and strength training on basal autophagy remain to be established in humans. One study has reported that acute severe hypoxia increases autophagosome content in human skeletal muscle, which is reverted by 20 minutes of low-intensity exercise. Experiments with transgenic mice have shown that autophagy is necessary for skeletal muscle adaptation to training. Little is known on how genetic factors, environment, nutrition, drugs and diseases may interact with exercise to modulate autophagy at rest and during exercise in humans.

KEYWORDS
endurance training, nutrient intake, protein degradation, protein turnover, resistance training, signaling

1 | INTRODUCTION

Exercise is known to exert multiple beneficial effects and is recommended for the improvement of health and the prevention and treatment of many pathologic conditions.[1,2] Nevertheless, exercise, as well as nutrient deprivation, hypoxia, infection, high temperature or exposure to toxic chemicals and radiation, may damage cellular structures.[3] In all living organisms, damaged elements are continuously removed and renovated,[4] facilitating remodeling and adaptation to training in skeletal muscles. In this process, dysfunctional components should be identified, degraded and its constituents recycled as building blocks for the synthesis of new components of improved quality. Autophagy (from the Greek *auto*, "self" and *phagein*, "to eat") constitutes, in eukaryotic cells, the principal mechanism for the degradation of unneeded components such as damaged organelles, cytoplasmic fractions and protein aggregates.[5] Autophagy is not

only a critical biological process to avoid disease,[5-7] but it is also required for a physiologic adaptation to regular exercise (angiogenesis, mitochondrial biogenesis, insulin sensitivity and muscle hypertrophy).[8,9,11]

Until now, three main autophagy pathways have been identified. The best-understood mechanism, macroautophagy (hereafter referred to as autophagy), consists of a dynamic process in which a double-membrane cytoplasmic vesicle called autophagosome selectively engulfs dysfunctional or damaged proteins, organelles and fractions of cytoplasm. Subsequently, the completed autophagosome fuses with the lysosome (forming the autolysosome) for an eventual degradation of the sequestered components via lysosomal hydrolases. Chaperone-mediated autophagy utilizes specific chaperones such as hsc70, which identifies and targets cytosolic substrate proteins containing KFERQ-like motifs in their amino acid sequence to be transported to the lysosome for degradation.[12] A third pathway named microautophagy comprises the engulfment of cytoplasmic residues by lysosomal membrane invaginations. The three main autophagy pathways end with lysosome digestion and release of the molecular components of engulfed materials, mostly amino acids, and presumably lipids and sugars, that may follow complete degradation or be recycled by the cell for the *de novo* synthesis of molecules and cellular structures.

An appropriate level of basal autophagy, also referred to as quality control autophagy, is crucial for a correct disposal of cellular and organellar damaged structures to maintain cell homeostasis.[13] Upregulation or downregulation of autophagy is one of the main mechanisms of cellular adaptation to stress. However, excessive or insufficient autophagy (basal or in response to cellular stress) has been identified as the principal cause of several diseases.[14-17] When cellular stress is increased, for example during hypoxia,[18] limited nutrient availability[19] or exercise,[9] autophagy flux (defined as a measure of autophagic degradation activity[20]) is elevated to provide energy substrates and to adapt cellular structures to the newly elevated demands. Skeletal muscle is one of the tissues with highest basal autophagy flux and greater capacity to increase autophagy flux.[19] The maintenance of skeletal muscle health and function requires an adequate level of autophagy and a finely tuned balance between mitophagy (selective removal of mitochondria by autophagy[21]) and mitochondrial biogenesis.[22,23]

This review examines the current evidence on the influence of exercise on skeletal muscle macroautophagy. Given the limited evidence obtained in humans, some animal findings have also been considered in this review. More specifically, we discuss the effects of exercise modality, duration and intensity on autophagy responses. The influence of regular exercise on skeletal muscle autophagy and the importance of autophagy for skeletal muscle adaptation to training are also addressed.

2 | MOLECULAR MARKERS OF AUTOPHAGIC FLUX IN SKELETAL MUSCLE

At least five different sequential events can be distinguished in autophagy: (a) induction (or activation), (b) formation of the sequestering phagophores (a double-membrane structure), (c) formation of autophagosomes (spheroid organelles still surrounded by a double membrane), (d) fusion of autophagosomes with lysosomes to form autolysosomes (organelles with a single membrane) and (e) hydrolysis of the cargo in the autolysosomes and release of molecular constituents. The term "autophagic flux" is used when referring to the entire process of autophagy (ie from cargo sequestration to its eventual degradation), as opposed to the measurement of the numbers or volume of autophagic elements at any step[24] (Figure 1). Currently, there are a considerable number of valid methods to assess autophagic flux in a wide range of living organisms.[24] Recent advances have enabled the *in vivo* assessment of autophagic flux in mammalian systems, principally by using autophagy inhibitors and inducers. Nevertheless, this remains unfeasible in humans[20] and alternative procedures with limited validity are often applied. For example, a commonly used approach to determine the rate of cargo degradation is the measurement of the rate of general protein breakdown by autophagy. Frequently autophagic flux is indirectly assessed by determining the changes in molecular markers of the different steps involved in autophagy, mainly adenosine monophosphate-activated kinase (AMPK), the unc-51-like kinase (ULK), the Forkhead Box (Fox) O (FoxO), the microtubule-associated protein 1A/1B-light chain 3 (Atg8/LC3) and the sequestosome 1 (p62/SQSTM1) (Figure 2). This approach has limitations since certain stimuli may result in changes of specific molecular markers of autophagy, unrelated to real modifications in autophagic flux.[25]

AMP-activated protein kinase (AMPK), which plays a critical role in energy homeostasis,[26-28] is also considered a crucial activator of autophagy[24] and mitophagy.[29] AMPK is activated by phosphorylation of the 172-threonine residue of the α chain when the AMP/ATP ratio is increased.[30] Exercise-elicited AMPKα Thr^{172} phosphorylation depends on the training status and the characteristics of the exercise.[31,32] Animal studies indicate that AMPK activation is necessary for exercise to induce autophagy[33,34] while myristoylation of AMPKß is needed to induce mitophagy.[29] AMPK promotes autophagy by directly phosphorylating and activating ULK. ULK activation is essential for the initiation of autophagosome formation.[35,36] Among the five isoforms of ULK, ULK1 is considered the predominant isoform involved in autophagy.[24] ULK1 together with the autophagy-related 13 (Atg 13) and the focal adhesion kinase family-interacting protein of 200 kDa (FIP200) form an indispensable complex

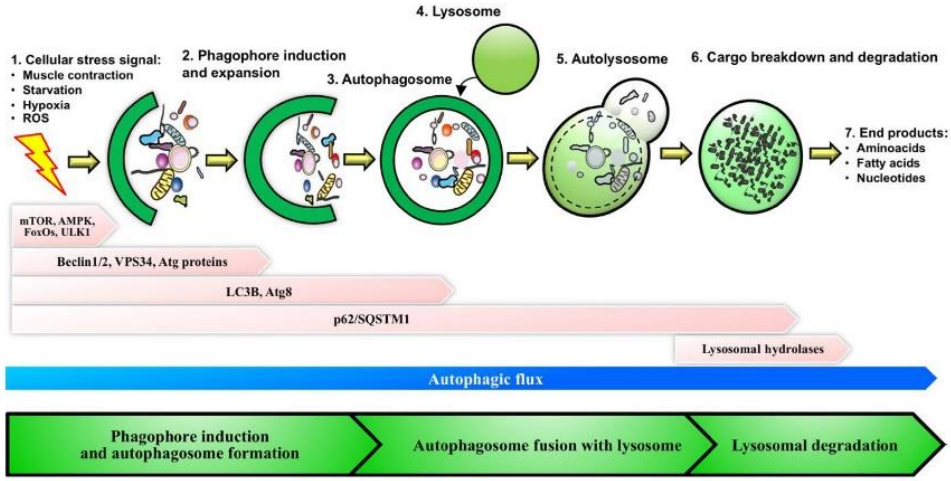

FIGURE 1 Schematic representation of the steps involved in autophagy. Autophagy begins with the induction and nucleation of the phagophore (isolation membrane) from the initiation complex. Phagophore undergoes expansion and closes on itself forming the autophagosome while enclosing cellular components. The completed autophagosome fuses with lysosomes to form an autolysosome. Finally, internal cellular components of the autolysosome are degraded alongside its inner membrane by lysosomal hydrolases into the final end-products (essentially amino acids, fatty acids and nucleotides), which can be recycled

for autophagy induction.[35,37] Although the exact AMPK-mediated ULK1 phosphorylation site(s) remains unclear Ser^{317}, ^{467}, ^{555}, ^{574}, ^{637} and ^{777}, as well as Thr^{574} have been implicated.[24,38,39] Nevertheless, it has also been reported that Ser^{555} phosphorylation of ULK1 is not sufficient to increase autophagosome content in rodent muscle and human myotubes.[40]

In contrast, phosphorylation of ULK1 at the 757-serine residue by the mammalian target of rapamycin complex 1 (mTORC1) prevents ULK1 activation.[39] AMPK inhibits mTORC1 complex by direct phosphorylation[41,42] and also by phosphorylating and activating Tuberous Sclerosis Complex 2 (TSC2).[43] Thus, AMPK acts simultaneously on two processes that synergize to activate autophagy: directly, by activating ULK1, and indirectly by impeding mTOR-dependent inhibition of ULK1.

When activated, ULK1 phosphorylates and activates the protein beclin-1 (encoded by gene *BECN1*) on Ser^{14} (mice nomenclature; Ser^{15} in humans), which seems necessary for complete activation of autophagy.[36] Nucleation and expansion require of a fine functioning of a complex formed by beclin-1, vacuole protein sortin 34 (Vps34) and 15 (Vps15), as well as activating molecule in BECN1 regulated autophagy protein 1 (AMBRA1), which interacts with the aforementioned ULK1/Atg13/FIP200 complex.[44] B-cell lymphoma-2 (BCL2) is an anti-apoptotic and anti-autophagy protein that inhibits autophagy by binding to the BH3 domain of the autophagy protein beclin-1 at the endoplasmic reticulum.[45] By binding to beclin-1, BCL2 blocks beclin-1 binding to Vps34 impeding autophagy initiation.[46,47] Induction of autophagy requires the disruption of the BCL2-beclin-1 complex. The important role played by ULK1 in autophagy has been confirmed using loss-of-function genetic models showing that lack of ULK1 impedes the conversion of LC3-I to LC3-II in mice.[39]

ULK1 effects may be reinforced by FoxOs (FoxO1, FoxO3 and FoxO4), among which FoxO3 upregulates autophagy-genes expression in skeletal muscle.[48] FoxO3 activation is facilitated in catabolic conditions such as ultraendurance exercise[50,51] and by nutrient deprivation and lack of growth factors (IGF-1, insulin, etc.), resulting in reduced intracellular Akt (also known as protein kinase B, PKB) activity.[52] However, during sprint,[53] endurance[54] and resistance exercise,[54] Akt is phosphorylated and activated, which may negatively regulate autophagy by phosphorylating threonine residues of FoxO3.[48,51] $PhosphoThr^{32}$-FoxO3 remains in the cytosol[55] and only translocates to the nucleus to stimulate the autophagy-gene program when dephosphorylated.[56,57] Akt can also inhibit autophagy by blunting Thr^{172}-AMPK phosphorylation via Ser^{485}-AMPKα1/Ser^{491}-AMPKα2 phosphorylation.[30,53,58,59] The exercise-induced activation of Akt is more prominent when the exercise is performed in the fed than the fasted state, likely due to the additive effect of the postprandial increase in insulin and exercise on Akt

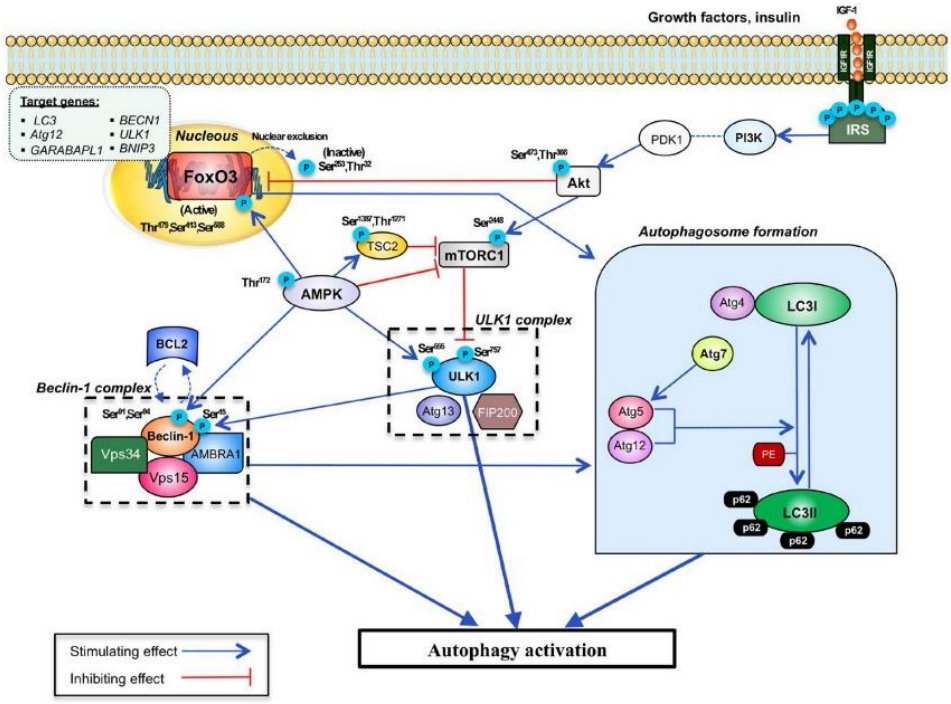

FIGURE 2 Signaling pathways regulating autophagy activation in skeletal muscle. Exercise-induced stress in muscle fibers activate a complex cascade of molecular events initially driven by FoxO3, AMPK and mTOR, depending upon the nutrient state and the type of exercise (modality, intensity and duration). AMPK induces autophagy activation by directly phosphorylating ULK1. Anabolic stimuli activate insulin signaling leading to mTOR activation, which acts as an autophagy inhibitor via downregulation of the ULK1-complex. AMPK blocks mTOR directly or by activating TSC2 via phosphorylation. FoxO3 coordinates a transcriptional program that promotes several autophagy genes. FoxO3 is phosphorylated and inhibited by Akt. Phosphorylated FoxO3 by Akt leads to exclusion from the nucleus. FoxO3 is upregulated by AMPK during prolonged energy deficit. Phagophore formation and expansion are controlled by the fine integration of proteins present in the beclin-1 complex. Exercise-mediated BCN2 phosphorylation induces beclin-1-to-BCN2 dissociation permitting autophagosome nucleation. Nucleation and expansion of the autophagosome requires LC3-I lipidation to LC3-II mediated by various conjugation reactions by Atg proteins. Sequestration of target cellular components for degradation is accomplished by ubiquitin, which selectively recruits and binds to p62. Recruited p62 directs the targeted material to be degraded within the growing autophagosome by binding to LC3-II on the autophagosomal membrane

activation.[53] Moreover, AMPK phosphorylates FoxO3a in Ser^{588} increasing its nuclear localization and activity.[60,61]

Prolonged endurance exercise is likely needed to promote FoxO3 signaling[49,50] although few hours after a single bout of bicycling for 120 minutes at 60% of VO_2max, *FoxO3* mRNA and protein levels were already increased.[62] In contrast to endurance exercise, FoxO3 protein levels remained unchanged after a single bout of resistance exercise.[62] The increase in FoxO3 protein after endurance exercise is also observed in the trained state.[62] The importance of FoxO3 for autophagy is further supported by suppression of autophagic flux in *FoxO3* knockout mice.[56]

The ubiquitin-like protein Atg8/LC3 can be found in phagophores, autophagosomes and, to a lesser extent, in autolysosomes. Native LC3, or proLC3, is proteolytically cleaved by autophagy-related 4 (Atg4) (a protease) releasing LC3-I, to which phosphatidylethanolamine is conjugated to generate the lipidated form of LC3 called LC3-II. LC3-II is the only protein marker that is reliably associated with completed autophagosomes (although it can also be found in phagophores)[24] and its levels correlate with autophagosome number.[63] During autophagy, there is an increased conversion of LC3-I to LC3-II and the ratio LC3-II/LC3-I is augmented. In the latest step of autophagy, the sequestosome

1 (p62/SQSTM1), an acceptor for ubiquitinated substrates, is reduced due to autolysosomal degradation.[64] Conversely, the accumulation of p62/SQSTM1 has been interpreted as a marker of autophagy inhibition.[35,65] Nevertheless, p62 changes should be interpreted cautiously because the level of p62 may also change by mechanisms unrelated to autophagy.[24,66] Moreover, using either the content of LC3-II or the ratio LC3-II/LC3-I as a marker of autophagic flux lacks specificity, as the content of autophagosomes depends on both the rate of formation and the rate of lysosomal degradation.[63,66] This is further complicated by the fact that the conversion of LC3-I to LC3-II is cell-type-specific and dependent on the stimulus used to induce autophagy.[24]

Due to these limitations, other methods including transmission electron microscopy and fluorescent techniques are used to assess autophagic flux in animals or cells.[24] In humans, however, it is not possible to employ molecular reagents, and the use of transmission electron microscopy should be limited to few measurements, which require repeated muscle biopsies. The assessment of mRNA encoding for autophagy proteins in combination with immunohistochemical techniques may help to obtain a more valid assessment of autophagy in human skeletal muscle.[67]

3 | ENDURANCE EXERCISE AND AUTOPHAGY IN SKELETAL MUSCLE

Salminen and Vihko[68] were the first reporting an augmented number of autophagic vacuoles following strenuous endurance exercise in rat skeletal muscle. Nonetheless, previous work had linked exercise to activation of autophagy in other tissues.[69] Since then, acute endurance exercise has been shown to activate autophagy at the gene and protein level in rodent skeletal muscle,[8,50,70-73] cardiac muscle,[74] as well as in other tissues[9] despite some controversy.[75] In humans, limited and controversial results have been reported to date.[40,50,67,76,77]

Jamart et al.[49] reported upregulation of autophagy following a 28-hours ultraendurance running (200 km), but this conclusion was based only on mRNA analysis. Muscle biopsies obtained after a 150 km running competition (18 hours) revealed a fivefold increase in LC3-II, indicating increased autophagosome content.[50] In contrast, a reduction of LC3-II has been observed after less strenuous endurance exercise protocols,[40,67,76] whereas LC3-II did not change after 30 minutes cycling at 70% of VO_2max[34] or 15 cycling at 40% of VO_2max with blood flow restriction.[34] Moller et al.[76] studied eight young recreational athletes before, and immediately after bicycling at 50% of VO_2max for 60 minutes, and 30 minutes after the end of exercise, on two occasions: (a) close to the end of a 36-hours fast, and (b) during continuous glucose infusion at 0.2 $kg \cdot h^{-1}$. Autophagy signaling through ULK1 was increased 30 minutes after the end of the exercise, while LC3-II and the LC3-II/LC3-I ratio were reduced at the end of exercise and remained at this level 30 minutes later (p62 was unchanged). Therefore, Moller et al.[76] study indicates a potential activation of autophagy, accompanied by a reduction of autophagosome content at the end of exercise, regardless of the nutrient background.

Schwalm et al.[67] studied the autophagy response to an acute bout of endurance exercise in well-trained athletes (minimal VO_2max of 50 $ml \cdot kg^{-1} \cdot min^{-1}$). The volunteers were studied under fasted and fed conditions and were divided into three groups: control (n=8), low-intensity (LI, n=8) and high-intensity (HI, n=7). Both groups cycled for 2 hours, the LI group at 55% and the HI at 70% of VO_2peak. As in Moller et al.,[76] ULK1 was phosphorylated at site Ser^{317} (mice nomenclature). This effect was greater when the high-intensity exercise was performed in the fasted state. In both the fed and the fasted states, LC3-II protein amount and LC3-II/LC3-I ratio were reduced after LI and HI, indicating decreased autophagosome content.[67] The autophagy transcriptional program was also activated, as shown by the increased LC3B, p62, GABA (A) receptor-associated protein like 1 (GabarapL1), and Cathepsin L mRNAs observed after HI but not after LI.[67] Interestingly, Schwalm et al.[67] study provide some evidence indicating that prolonged endurance exercise may be a more potent stimulus for autophagy than a short fasting period.

More recently, Fritzen et al.[40] studied skeletal muscle markers of autophagy after 70 minutes of one-leg knee extension exercise. Compared to the resting leg, the exercised leg showed decreased LC3-II levels and LC3-II/LC3-I ratio suggesting reduced autophagosome content (p62 protein levels remained unchanged).[40]

Masschelein et al.[18] suggest that the effect of exercise on autophagy may vary depending on the prevalent autophagy flux background. Three-hour exposure to severe hypoxia (F_IO_2=0.107) increased autophagosome content in 11 young MZ twins, as reflected by a 45% increase in LC3-II and a 29% increase in the LC3-II/LC3-I ratio in hypoxia compared with normoxia, accompanied by a 25% reduction of p62 protein level.[18] After the 3 hours in hypoxia, subjects performed a 20-minutes exercise bout on a cycle ergometer at 81.4±3.2% of VO_2max in hypoxia, which was compared to a control exercise performed on a previous day at the same absolute intensity but in normoxia (representing 50.7±2.3% of VO_2max in normoxia). The LC3-II and LC3-II/LC3-I ratio changes induced by the 3-hour passive exposure to hypoxia were reversed by exercise. However, p62 remained 15% lower than in normoxia, after exercise in hypoxia.

In contrast to human studies, most rodent experiments indicate that autophagy is stimulated during endurance exercise.[8,70,72,78] He et al.[8] convincingly demonstrated that

autophagy is already induced 30 minutes after the start of a 110 minutes long treadmill running session at 75% of their maximal running capacity. These authors used transgenic mice expressing a green fluorescent protein (GFP)-labeled marker of autophagosomes (GFP-LC3). The number of autophagosomes in skeletal and cardiac muscle was increased after 30 minutes and continued to rise until 80 minutes, plateauing thereafter (Figure 3). This was a generalized response observed in several muscle groups including predominantly slow and fast exercised muscles. These findings were supported by biochemical evidence as reflected by the increase in LC3-II and conversion of the non-lipidated form of LC3-I to LC3-II, and degradation of the autophagy substrate protein p62. Thus, this study provides validity, albeit in a rodent model, for the use of these molecular markers to assess changes in autophagosome number and flux in skeletal muscle indirectly. Interestingly, He et al.[8] also reported exercise-induced autophagy in islet β-cells and liver adipose tissue. Biochemical evidence (based on increased LC3-II) also suggested exercise-induced autophagy in adipose tissue. Moreover, this study showed that exercise-induced autophagy requires disruption of the BCL2-beclin-1 complex through a molecular mechanism that remains unknown.[8]

Some rodent studies indicate that a physiologic autophagy response to exercise is required to maintain a normal carbohydrate metabolism and skeletal muscle insulin sensitivity during exercise,[8] but this has not been confirmed by others using muscle-specific knockout mice.[79]

The underlying mechanisms for discrepancies between rodent and human experiments remain unknown. It has been speculated that rodents may use macroautophagy to release energy substrates while this mechanism is prevented in humans to favor the use of alternative metabolic pathways.[40] Although unlikely, a rise of autophagosome flux accompanied by a greater increase in autophagosome degradation, resulting in reduced autophagosome content, cannot be ruled out in human skeletal muscle after endurance exercise.

4 | EXERCISE PERFORMANCE AND AUTOPHAGY

Rodent loss-of-function genetic models have shown that defective autophagy may hamper exercise performance mainly due to pathologic alterations in skeletal muscle. These include loss of muscle force, reduced specific tension, increased oxidative stress, pathologic changes in mitochondrial structure and function, reduced capacity to cope with eccentric exercise-induced muscle damage, and defective adaptation to exercise.[23,48,80] Nonetheless, non-pathologic outcomes are seen in other tissues with autophagy deficiency, suggesting that skeletal muscle, compared to other tissues, may be particularly sensitive to defective autophagy.[65]

Maximal exercise performance is reduced in *BCL2*^{AAA} mice and in transgenic mice having decreased beclin-1 protein expression in skeletal muscle.[8] In contrast, no impairment of maximal exercise performance was observed in sedentary *Atg6*$^{+/-}$ mice, which are haplodeficient for the autophagy-related 6 (*Atg6/BECN1*), a class III PI3K necessary for autophagosome formation, compared to sedentary WT animals. Nonetheless, *Atg6*$^{+/-}$ mice fail to improve their endurance performance after 4 weeks of voluntary wheel running when compared to WT animals.[10] This lack of improvement in endurance was accompanied by a blunted adaptation to endurance training.

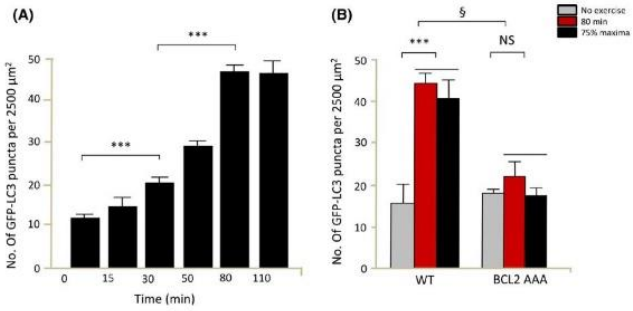

FIGURE 3 (A) Quantification of skeletal muscle (vastus lateralis) data from mice transgenically expressing a green fluorescent protein (GFP)-labeled marker of autophagosomes at serial time points after exercise (mean±SEM of 10 tissue sections) $^{**}P<.01$, $^{***}P<.001$. One-way Anova. Modified from He et al.[8] (B) Quantification of skeletal muscle data from GFP-LC3 transgenic mice (BCL2 AAA) or wild type before exercise, and following 80 min of exercise or 75% of maximal running capacity. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$, one-way ANOVA to compare between groups; § $^{*}P<.001$, two-way ANOVA to compare the magnitude of changes between different groups in mice of divergent genotypes; NS, not significant. Modified from He et al[8]

Muscle-specific deletion of autophagy-related 7 (*Atg7*), which impedes autophagosome formation, resulted in a loss of muscle mass and specific tension (force/cross-sectional area), which was exacerbated with ageing, and was accompanied by morphologic features of myopathy.[23] Moreover, *Atg7* knockout mice experience greater muscle damage with eccentric muscle contractions.[79] Similarly, peroxisome proliferator-activated receptor γ coactivator 1-α (*Pgc-1α*) knockout mice have reduced mitochondrial volume, lower mitochondrial turnover due to decreased mitophagy and mitochondrial biogenesis, and reduced exercise capacity.[73]

5 | INFLUENCE OF TRAINING ON BASAL AUTOPHAGY

Rodent experiments have shown that predominantly oxidative muscles have greater basal autophagy, and expression of autophagy and mitophagy proteins than predominantly glycolytic muscles.[10,21] Also in rodents, Lira et al.[10] showed that 4 weeks of voluntary wheel running led to a significantly increased basal autophagy (measured at least 28 hours after the last training session) in the *plantaris* muscle (mixed-fiber type). Although autophagy protein expression was increased, no significant changes were observed in basal autophagy and mitophagy protein content in the soleus muscle (predominantly oxidative).[10] The increased basal autophagy elicited by training may be in part mediated by Pgc-1α, since muscle-specific overexpression of *Pgc-1α* results in increased basal autophagy flux in mixed-fiber muscles, like plantaris in mice.[10] Pgc-1α overexpression upregulates the mitophagy protein BCL2/adenovirus E1B 19 kDa interacting protein 3 (Bnip3), although not at the transcriptional level, to counteract the increased mitochondrial biogenesis, because a mismatch between mitochondrial biogenesis and mitophagy would result in accumulation of dysfunctional mitochondria. The mechanism by which *Pgc-1α* overexpression upregulates autophagy flux in skeletal muscle remains to be elucidated.

6 | RESISTANCE TRAINING AND AUTOPHAGY IN SKELETAL MUSCLE

Only two studies have determined the effects of a resistance training session on autophagosome content in human skeletal muscle focusing on the first hours after the end of the resistance exercise bout[81,82] while the effects of resistance training on basal autophagy in humans have not been explored. This is in contrast with the reported reduced autophagosome content after resistance training in rodents.[83]

In young and older adults, autophagosome content was reduced 3, 6 and 24 hours after a single bout of resistance exercise consisting of 8 sets of 10 repetitions at 70% one-repetition maximum with 3 minutes of rest in between each set, but the fractional breakdown rate of muscle proteins remained unaltered because protein degradation via the ubiquitin proteasome system (UPS) was increased.[81] The same research team examined the effects of a supplement containing essential amino acids (EAA; 0.35 g·kg·Lean Mass^{-1}) and two different amounts of carbohydrate (CHO; one group 0.5 g·kg Lean Mass^{-1} and another group 1.4 g·kg Lean Mass^{-1}) on muscle protein synthesis and degradation in 12 subjects divided into two groups (n=6). Subjects received the supplement 1 hour after 10 sets of 10 repetitions of bilateral leg extension at 70% of their predetermined one-repetition maximum.[82] The biopsies obtained 1 hour after the ingestion of the supplements showed a significant reduction of LC3-II without significant changes in LC3-I, suggestive of reduced autophagosome content, without significant differences between groups.

7 | REDUCED BASAL AUTOPHAGY IN SKELETAL MUSCLE CONTRIBUTES TO SARCOPENIA BUT REGULAR EXERCISE ANTAGONIZES THIS EFFECT

It has been postulated that the loss of muscle mass and force with ageing (sarcopenia) may be caused by dysregulation of mitochondrial function, dynamics (fission/fusion) and turnover (mitophagy/biogenesis).[84] Regular exercise is the best treatment available to slowdown the development of sarcopenia with ageing,[85] improving mitochondrial quality and perhaps the number of quiescence satellite cells, a process that depends on basal autophagy.[86] Ageing is associated with reduced mitofusin 2 (MFN2), which in turn decreases autophagic flux in skeletal muscle.[87] Regular exercise increases MFN2 expression and by this way may normalize autophagy attenuating the stimulus for sarcopenia, according to the mitochondrial theory of sarcopenia.[88] Nevertheless, experiments in humans are required to determine the effects of exercise in basal skeletal muscle autophagy in elderly.

8 | CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Healthy adaptation to exercise training is only possible with a proper balance between the mechanisms for removal and recycling of damaged or dysfunctional cellular components and the formation of new cellular structures in skeletal muscles.[22,23] Future studies are needed to determine how

different exercise modalities may modulate autophagy in humans depending on sex, age, health status and training background. Little is known about the environmental effects on autophagy. Nothing is known about the interaction between the genotype and the exercise-induced autophagy in humans. Future studies should also examine how drugs which may modify autophagy, like metformin, could alter the adaptations to exercise. Thus, a lot remains to be discovered in this field.

ACKNOWLEDGEMENTS

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DISCLOSURE

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