Original article

MOLECULAR METABOLISM

mTORC2 and AMPK differentially regulate muscle triglyceride content via Perilipin 3

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ABSTRACT

Objective: We have recently shown that acute inhibition of both mTOR complexes (mTORC1 and mTORC2) increases whole-body lipid utilization, while mTORC1 inhibition had no effect. Therefore, we tested the hypothesis that mTORC2 regulates lipid metabolism in skeletal muscle.
Methods: Body composition, substrate utilization and muscle lipid storage were measured in mice lacking mTORC2 activity in skeletal muscle (specific knockout of RICTOR (Ric mKO)). We further examined the RICTOR/mTORC2-controlled muscle metabolome and proteome; and performed follow-up studies in other genetic mouse models and in cell culture.
Results: Ric mKO mice exhibited a greater reliance on fat as an energy substrate, a re-partitioning of lean to fat mass and an increase in intramyocellular triglyceride (IMTG) content, along with increases in several lipid metabolites in muscle. Unbiased proteomics revealed an increase in the expression of the lipid droplet binding protein Perilipin 3 (PLIN3) in muscle from Ric mKO mice. This was associated with increased AMPK activity in Ric mKO muscle. Reducing AMPK kinase activity decreased muscle PLIN3 expression and IMTG content. AMPK agonism, in turn, increased PLIN3 expression in a FoxO1 dependent manner. PLIN3 overexpression was sufficient to increase triglyceride content in muscle cells.
Conclusions: We identified a novel link between mTORC2 and PLIN3, which regulates lipid storage in muscle. While mTORC2 is a negative regulator, we further identified AMPK as a positive regulator of PLIN3, which impacts whole-body substrate utilization and nutrient partitioning.
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Keywords PLIN3; RICTOR; mTOR; Metabolism; Akt

1. INTRODUCTION

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is found in two distinct mTOR complexes, mTOR complex 1 (mTORC1) and mTORC2. RAPTOR (regulatory-associated protein of mTOR) is the defining subunit of mTORC1, which is a vital regulator of cell growth. The subunits RICTOR (rapamycin-insensitive companion of mTOR) and SIN1 (SAPK-interacting protein 1) are unique to mTORC2. Growth factors activate mTORC2 [31,45], and its substrates include Akt, SGK1, and PKCα. As a kinase for Akt Ser473 phosphorylation [34], mTORC2 is positioned in the canonical insulin signaling pathway responsible for regulating glucose uptake. Therefore, it is not surprising that in adipose tissue [11,25] and in liver [14,26,43] mTORC2 is required for maintaining glucose homeostasis. Similarly, evidence from cultured muscle cells [42] and incubated mouse skeletal muscles [24] implicated mTORC2 as a regulator of muscle glucose metabolism. In agreement, muscle specific RICTOR knockout (Ric mKO) mice are glucose intolerant [24]. Moreover, we recently demonstrated that mTORC2 is necessary for normal insulin-stimulated muscle glucose uptake *in vivo* [22] and others have suggested that mTORC2 is required for adrenergic stimulation of glucose uptake in skeletal muscle [35]. Despite its position upstream of Akt within the insulin signaling network, questions remain about how mTORC2 signaling impacts glucose metabolism specifically in muscle, because Akt signaling to substrates appears to be normal in muscle lacking mTORC2 activity [3,22,35]. For example, in response to a physiological insulin stimulation *in vivo*, phosphorylation of AS160, a well described Akt substrate and key regulator of glucose uptake, was not inhibited, but hyper-phosphorylated in muscle lacking RICTOR, despite a robust reduction in Akt Ser473 phosphorylation [22] in Ric mKO muscle.

Since muscle is an important tissue for whole-body glucose homeostasis, especially in humans [12,20], understanding mTORC2-mediated regulation of energy metabolism may reveal new therapeutic strategies for treating diseases that are characterized by dysregulated glucose metabolism (e.g. type 2 diabetes). We have recently shown that acute inhibition of both mTOR complexes increases whole-body lipid utilization in mice, while mTORC1 inhibition had no effect

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[22]. We therefore hypothesized that, in addition to its role in glucose metabolism, mTORC2 regulates lipid metabolism. In agreement with our hypothesis, we found evidence for mTORC2 regulation of muscle lipid metabolism, specifically lipid storage, which appears to be linked to specific changes in the mTORC2-controlled proteome.

2. MATERIAL AND METHODS

2.1. Animals

Female muscle-specific RICTOR knockout (Ric mKO: RICTOR$^{floxed/floxed}$, HSA-Cre$^{-/+}$) and WT (Ric WT: RICTOR$^{floxed/floxed}$, HSA-Cre$^{-/-}$) littermates aged 9–16 weeks were used [3]. Female C57Bl/6 mice overexpressing a muscle-specific, kinase-dead AMPKα_2 construct (AMPK KD) and corresponding WT littermates [30], aged 12–21 weeks were used. Muscle-specific FoxO1 knockout muscle samples (FoxO1 mKO) on a C57BL/6 background were a gift from Dr. Febbraio, Baker Heart and Diabetes Institute, Melbourne, Australia. Mice were group-housed when possible, kept on a 12:12-h light–dark cycle, and had free access to standard rodent chow diet (Altromin no. 1324; Brogaarden, Denmark) and water. All experiments were approved by the Danish Animal Experimental Inspectorate.

2.2. qRT-PCR

Ric mKO mice: ~20 mg quadriceps was homogenized in TRIzol (Life Technologies). Total RNA was isolated using 1-Bromo 3-Chloropropane for phase separation and isopropanol for total RNA precipitation according to manufacturer's instructions. RNA quantity and purity was determined by UV measurements using a Nanodrop ND-1000. cDNA was synthesized from all samples at the same time using the same batch of enzyme mix from 1 µg total RNA using reverse transcriptase and oligo-dT primers (Omniscript RT kit, Qiagen). The primer sequences are listed in the Supplementary data online. LightCycler 480 SYBR Green 1 Master Mix and LightCycler 480 (both Roche Applied Science) were used to quantify cDNA. The PCR cycle conditions were 94 °C for 10 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Assay efficiencies were checked using serial dilution of mouse cDNA, and only primers that fell in the 85–115% range were used. Gene expression levels were normalized to the house-keeping gene, EEF2 or HRPT, (no Ct difference between Ric WT and mKO) and made relative to the Ric WT condition using the ΔΔCt method. *AMPK KD mice*: Total RNA was isolated from ~20 mg AMPK KD and wildtype quadriceps muscle with a modified guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi [10], described previously by Pilegaard et al. [33], except that the tissue was homogenized for 3 min at 30 Hz in a TissueLyzerII (Qiagen, CA, USA). RNA concentration and purity was tested by Nanodrop (Nanodrop 1000, Thermo Scientific, MA, USA). 4 µg RNA was transcribed to cDNA by Superscript II RNase H^{-} system and Oligo dT (Invitrogen, USA) [33]. mRNA content was determined in triplicates by real-time PCR (ABI PRISM 7900 Sequence Detection Systems; Applied Biosystems) using the fluorogenic 5′ nuclease assay with TaqMan probes and universal mastermix with UNG using the following cycle profile: 50 °C for 2 min + 95 °C for 10 min + (95 °C for 15 s + 60 °C for 1 min) × 40. PLIN3 and β-actin probes and primers were pre-developed assay reagents from Applied Biosystems, USA (PLIN3 # mm04208646_g1 and β-actin # 4352341E). The total amount of PLIN3 and β-actin mRNA was determined by gene-specific standard curves made from a serial dilution of a pooled sample made from all samples. PLIN3 mRNA was normalized to total amount of β-actin mRNA, and there was no difference in β-actin mRNA between AMPK KD and wildtype. *L6 cells and C2C12 cells*: Total RNA was extracted from using TRIzol reagent (Invitrogen). cDNA synthesis was performed using PrimeScript 1st strand cDNA Synthesis Kit (Clontech, Takara Bio Company). PCR reactions were carried out using FastStart Universal SYBR Green Master (Roche) on the LightCycler 480 II (Roche). β-actin (Actb) or HRPT were used as internal controls and gene expression levels were normalized to Actb or HRPT and made relative to the control condition using the ΔΔCt method. Primers used in L6 cells: Plin3 F: 5′-CCTGATTGCCACATCTCCA-3′; Plin3 R: 5′-GCCCAACCTGACAAAGTAGC-3′; Actb F: 5′-CTGGCTCCTAGCACCATGA-3′; Actb R: 5′-ACTCCTGCTTGCTGATCCAC-3′; and FoxO1 F: 5′-GGA-TAAGGGCGACAGCAACA-3′; FoxO1 R: 5′-TTCCCACTCTTGCCTCCCT-3′. Primers used in C2C12 cells: HRPT: F: 5′-AAGCTTGCTGGTGAAAAGGA-3′; HRPT R: 5′-TTGCGCTCATCTTAGGCTTT-3′; Plin3 F: 5′-GCCCAA-GAGATGGTGTCTAGC-3′; Plin3 R: 5′-CCGGTCACTACGGACTTTGT-3′. Their efficiency was tested.

2.3. Nuclear fractionation

As adapted from Senf et al. [37] with minor changes. ~30 mg of quadriceps muscle from fed Ric mKO and AMPK KD mice were homogenized. The nuclear pellet was rotated for 2 h with 0.42 M NaCl. In pilot experiments efficient separation of the nuclear fraction from the cytosolic fraction was verified with β-actin (cytosolic marker) and lamin A/C (nuclear marker).

2.4. TG determinations

Muscle (quadriceps) and liver were freeze-dried and dissected free of all visible fat, blood and connective tissue and IMTG and liver TG content was assayed as previously described [21]. TG determinations in C2C12 myotubes were carried out as described in [36].

2.5. PLIN3 overexpression experiments

C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing antibiotics and 10% FCS. To differentiate cells into myotubes, cells were maintained in DMEM (containing antibiotics) now supplemented with 2% HS (differentiation medium) for 5–6 days. PLIN3 overexpression was accomplished by Lipofectamine-2000 mediated transfection with a pCMV-Myc-C PLIN3 ORF clone. Control cells were transfected with a pCAG-GFP empty vector clone. To verify transfection myc-tag and GFP levels were assessed by standard WB technique and PLIN3 expression was assayed by qPCR 24 h post transfection. Other transfected myotubes were fixed with 4% paraformaldehyde and Dapi stained after 24 h. Staining intensity fluorescence was measured at 340/460 nM. Other transfected myotubes were incubated for 6 h or 18 h with either 750 µmol/L palmitic acid made up in differentiation medium containing 2% BSA (free of fatty acids) or for 18 h in differentiation medium only containing 2% BSA.

2.6. Western blot analysis

Tissues were homogenized with stainless steel pellets that shook 2 × 45 s at 30 Hz using a Tissuelyser II (Qiagen, USA) in ice-cold homogenization buffer (10% Glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8.0), 1 mM, EGTA (pH 8.0), 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 2 mM Na3VO4, 3 mM Benzamidine, 5 mM Nicotinamide). Homogenates were rotated end-over-end for 1 h at 4 °C, and cleared by centrifugation at 13000 ×g for 20 min at 4 °C. Lysate protein content was determined by bicinchoninic acid method and lysates were diluted to the same protein concentration (1–2 µg/µl). Total protein and phosphorylation levels of indicated proteins were determined by standard immunoblotting, loading equal amounts of protein (5–40 ug).

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