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Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation

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Abstract

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays an important role in cell growth and metabolism. mTOR has been postulated as a nutrient sensor, but its role in the regulation of fatty acid and glucose metabolism is poorly understood. For the first time, we show that mTOR inhibition in skeletal muscle cells has pronounced effects on intermediary metabolism. Rapamycin, a uniquely specific mTOR inhibitor with clinical applications, increased fatty acid oxidation by 60% accompanied by increased activities of carnitine palmitoyltransferases I and II, the former believed to be the primary intracellular regulatory enzyme of the fatty acid oxidation pathway. Furthermore, glucose transport capacity, glycogen synthesis, and glycolysis were reduced by approximately 40% under the same conditions. In addition, in the presence of rapamycin, hyperinsulinemic conditions (100 nmol/L insulin, 24 hours) were unable to suppress fatty acid oxidation in L6 myotubes. Rapamycin treatment also decreased baseline phosphorylation of mTOR residues S2448 and S2481 by 30% and almost completely abolished p70 S6 kinase phosphorylation. These results show that rapamycin causes a metabolic shift from glucose utilization to fatty acid oxidation in model muscle cells in the presence of nutrient abundance and underline the importance of mTOR as a key regulator in glucose and lipid metabolism.

1. Introduction

The mammalian target of rapamycin (mTOR) performs an important function in the regulation of cell growth and proliferation. Physiologically, mTOR is regulated by mitogens (including insulin), glucagon, nutrient status, and amino acids, particularly leucine [1-6], and, consequently, mTOR has been termed a "nutrient sensor." The ribosomal p70 S6 kinase (p70S6K) and the translational repressor 4EBP1 are 2 major targets of mTOR, involved in mediation of downstream signaling events [7]. However, the role of mTOR in the regulation of intermediary metabolism is poorly understood.

Rapamycin is a uniquely specific mTOR kinase inhibitor, having no known targets in addition to mTOR [8]. It is an immunosuppressive drug extensively used after kidney, liver, and heart transplant to prevent acute graft rejection [9]. In addition, mTOR inhibitors have been shown to be effective anticancer agents for some types of cancers [10]. However, prolonged use of rapamycin in humans is associated with severe hypertriglyceridemia, excessive weight gain, and insulin resistance [11-14].

In summary, despite the evidence that suggests a role for mTOR in controlling intermediary metabolism, this possibility has not been fully explored; particularly in terms of how rapamycin-mediated inhibition of mTOR affects cellular fuel utilization. To address the contribution of mTOR to the control of intermediary metabolism we studied the effects of rapamycin on lipid and glucose metabolism in a model of skeletal muscle cells (L6 myotubes). Our results indicate that rapamycin exerts a direct effect on metabolism in muscle cells, promoting β-oxidation at the expense of glucose utilization. This is accompanied by a decrease in the capacity of insulin to regulate these processes. The data underscore the importance of mTOR in the regulation of intermediary metabolism and point out that inhibition of the mTOR pathway induces an inappropriate "fasting" metabolic phenotype even in the presence of nutrient abundance.

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2. Materials and methods

2.1. Materials

Cell culture reagents were from Mediatech (Herdon, VA). [9,10-^{3}H]Palmitic acid, [^{3}H]H_2O, and 2-[^{3}H]deoxyglucose (2-DOG) were from Perkin-Elmer Life Sciences (Shelton, CT). L-[*N-Methyl*-^{14}C]carnitine was from American Radiolabeled Chemicals (St Louis, MO). D-[5-^{3}H]Glucose and D-[U-^{14}C]glucose were from Amersham Biosciences (Piscataway, NJ). Antibodies were from Cell Signaling Technology (Beverly, MA) and Sigma (St Louis, MO). Rapamycin (sirolimus) was from Sigma. Antibodies against mTOR, mTOR-(pS2481), mTOR-(pS2448), p70S6K, p70S6K-(pT389), and p70S6K-(pT421/424), were from Cell Signaling Technology, and antiactin was from Sigma. The second antibody used for detection in all cases was antirabbit IgG, horseradish peroxidase–linked antibody, from Cell Signaling Technology.

2.2. Cell culture

Stock L6 cells were grown in α-minimum Eagle's medium containing 10% (vol/vol) fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin (growth medium). For differentiation into myotubes, cells were plated in 6-well plates at a density of 4000 cells/cm^2. After 24 hours the medium was changed to α-minimum Eagle's medium containing 2% (vol/vol) fetal bovine serum and antibiotics as above (differentiation medium) that was replaced after 2 and 4 days of culture. After 6 days, myotube differentiation was complete, and experimental procedures were initiated.

2.3. Fatty acid oxidation in L6 cells

Fatty acid oxidation was measured as described previously [15]. Cells were incubated for 24 or 48 hours in the presence or absence of 15 nmol/L rapamycin (4 independent experiments). For experiments relating to fatty acid oxidation under long-term insulin-stimulation conditions, cells were preincubated in the presence or absence of 15 nmol/L rapamycin and/or 100 nmol/L insulin for 24 hours (6 independent experiments). In both cases, for the last 18 hours the culture medium was replaced with differentiation medium containing [9,10-^{3}H] palmitic acid (1.25% [wt/vol] essentially fatty-acid-free bovine serum albumin, 0.2 mmol/L palmitate [0.5 μCi/mL]) in the presence or absence of rapamycin and other additions as in the prior incubation. Fatty acid oxidation was determined in triplicate wells for each condition by collecting 200 μL of medium from each well and determining the tritiated water content by the vapor-phase equilibration method of Hughes et al [16]. After aspiration of the remaining medium, cells were washed 3 times with 2 mL of ice-cold phosphate-buffered saline (PBS) and collected in 1 mL of 1 N NaOH for measurement of protein content by the bicinchoninic acid method (Pierce, Rockford, IL).

2.4. Carnitine palmitoyltransferase assay

Activities of carnitine palmitoyltransferase (CPT) I and CPT II were determined by a radiochemical assay in the direction of acylcarnitine formation as described previously [15]. Briefly, cells were harvested in a buffer containing 150 mmol/L KCl and 5 mmol/L TRIS-HCl (pH 7.2) and broken with a glass homogenizer. The cell homogenate was used for assay of CPT I. For assay of CPT II, a portion of the homogenate was incubated for 5 minutes at 4°C with 1% (wt/vol) octylglucoside, which inactivates CPT I and releases CPT II from the mitochondrial matrix in active form. Six independent experiments were carried out in duplicate.

2.5. Glucose metabolism in L6 myotubes

Glucose transport was measured after 24 or 48 hours' incubation in the absence or presence of 15 nmol/L rapamycin. To determine the effect of rapamycin on glucose transport in the context of hyperinsulinemic conditions, cells were preincubated in the presence or absence of 15 nmol/L rapamycin and 100 nmol/L insulin for 24 hours (long-term insulin). After 24 or 48 hours, the medium was changed to serum-free medium with the same additions and culture was continued for a further 4 hours. Cells were then incubated for 30 minutes in fresh medium in the absence or presence of 100 nmol/L insulin (short-term insulin). Uptake of 2-DOG was determined in triplicate wells for each condition (4 independent experiments) over 5 minutes in the presence of 10 μmol/L (0.5 μCi/mL) 2-DOG [15]. Protein content was measured as above.

Glycogen synthesis was determined as previously described [15]. Briefly, cells were treated as described above for 24 hours, followed by 2 hours in serum-free medium with the same additions and a further 60 minutes in fresh medium containing 2 μCi/mL D-[U-^{14}C]glucose in the presence or absence of 100 nmol/L insulin. Triplicate wells were used for each condition (4 independent experiments). After incubation with the isotope, the medium was removed and the cells were washed 4 times with 2 mL of ice-cold PBS. To solubilize the cells, 500 μL/well of 1 N NaOH was added to each well and the plates were incubated for 1 hour at 50°C. A 300-μL portion was then collected and 10 μL of 50 mg/mL unlabeled glycogen was added as carrier. Glycogen was precipitated by the addition of 750 μL ethanol (prechilled to −20°C) and incubation at −20°C for 30 minutes. Samples were centrifuged at 18 000*g* for 10 minutes at 4°C, the pellets were redissolved in 250 μL of 1 N NaOH by heating at 60°C for 30 minutes, and 200 μL was taken for liquid scintillation counting. Protein content was measured as above.

Glucose utilization (equivalent to glycolysis) was determined as previously described [16]. Cells were treated as described above for 24 hours, followed by 2 hours in differentiation medium containing 6.5 μCi/mL [5-^{3}H]glucose with the same additions. Triplicate wells were used for each condition (4 independent experiments). The medium

was collected and tritiated water was determined by the vapor-phase equilibration method (as for fatty acid oxidation experiments). Cells were washed 3 times with 2 mL of ice-cold PBS and collected in 1 mL of 1 N NaOH for measurement of protein content as above.

2.6. Animal studies and fatty acid oxidation in soleus muscle

Male Sprague-Dawley rats were purchased from Taconic Farms (Hudson, NY). Animals were housed in sterile cages with a 12-hour light/dark cycle and fed with standard rodent chow and water ad libitum. To determine the effects of rapamycin on fatty acid oxidation in muscle in vivo, male Sprague-Dawley rats (n = 5) were injected subcutaneously with rapamycin (2 mg/kg body weight) on alternate days for 10 days to mimic typical therapeutic concentrations (8.7 ± 2.5 ng/mL) [11,13,14]. Control animals (n = 5) received a corresponding volume of vehicle (2% [vol/vol] Tween 80/ 1% [wt/vol] carboxymethylcellulose). Body weight was monitored during the experiment and no significant changes between control and rapamycin-treated rats were observed (before treatment, 233 ± 30 and 237 ± 32 g; after treatment, 269 ± 26 and 257 ± 28 g for control and treated

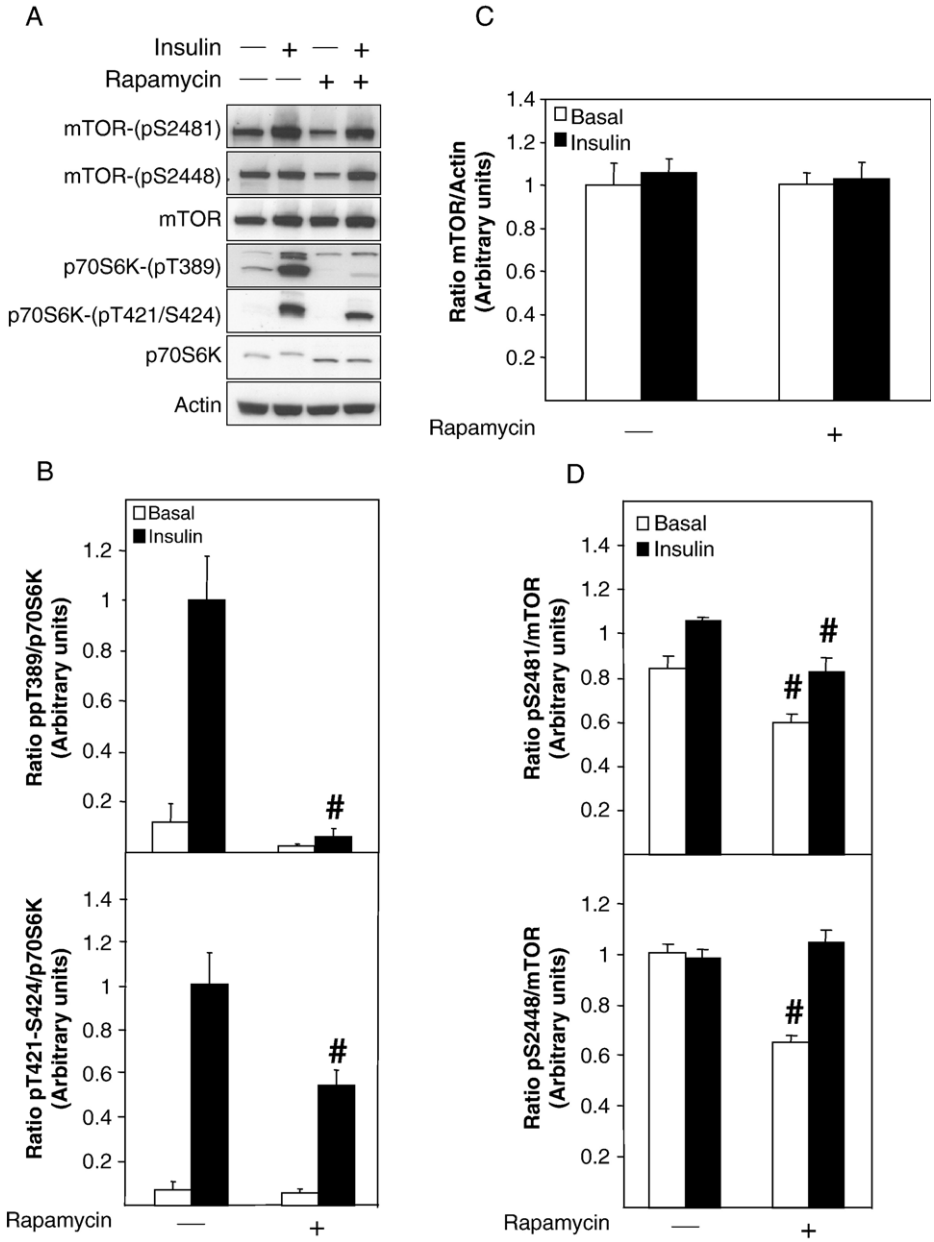


Fig. 1. Western blot analysis of signaling pathways in differentiated L6 myotubes treated with rapamycin (long-term treatment). Cells were incubated for 24 hours in the absence or presence of 15 nmol/L rapamycin, with or without 100 nmol/L insulin for the final 30 minutes (short-term insulin) before preparation of cell lysates and immunoblot analysis. A, Typical immunoblots using phosphorylation-specific or total kinase-directed antibodies (and antiactin antibody as control). B, C, and D, Densitometric quantification of bands. Mean relative values ± SE for 4 independent experiments. Significant differences are indicated as follows: #Versus no rapamycin.

rats, respectively). The nonsignificant trend toward a decrease in weight gain seen in the rapamycin-treated animals may result from a mild short-term reaction to the drug. As previously reported [14], this regimen for rapamycin treatment did not alter plasma glucose (122.5 ± 5.5 and 117.8 ± 6.2 mg/dL on day 8, for control and treated rats, respectively, measured by using a portable glucose meter, Medisense, Bedford, MA). To determine the fatty acid oxidation capacity, soleus muscles (~120 mg) were removed and incubated in an atmosphere of 95% oxygen/5% carbon dioxide in a total volume of 1 mL of Dulbecco's modified Eagle's medium containing bovine serum albumin and labeled palmitate (as for fatty acid oxidation measurements in L6 cells) for 90 minutes. at 37°C. The medium was collected and tritiated water determined as described above [16]. Animal procedures were in accordance with the humane care criteria of the National Academy of Sciences and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.7. Immunoblot Analysis

Cells were incubated for 24 hours in the absence or presence of 15 nmol/L rapamycin. The culture medium was then replaced with fresh serum-free medium with the same additions as above for another 4 hours. In the last 30 minutes, 100 nmol/L insulin (short-term insulin stimulation) was added before preparation of cell lysates and immunoblot analysis. Cell lysates were prepared by addition of 150 μL of lysis buffer (20 mmol/L TRIS-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% [vol/vol] Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride) to the cells. After 10 minutes on ice, the lysis buffer was collected, and triplicate wells were pooled. Samples were sonicated followed by centrifugation at 18 000g for 10 minutes at 4°C. Pellets were discarded, and the solubilized proteins (~20 μg/sample) were resolved by 7.5% SDS-PAGE and electrotransferred onto polyvinylidene difluoride filters for immunoblotting by conventional means (using 1:1000 dilution for the first antibodies and 1:3000 for the second antibody). After probing with phosphorylation-specific antibodies, the membranes were stripped and reprobed with antibodies against total kinase proteins and actin. Signals were detected by chemiluminescence (ECL Plus detection system, Amersham Biosciences) and exposure to x-ray film to produce bands within the linear range. Four independent experiments were used for quantification with NIH Image software.

2.8. Statistical analysis

Data are expressed as mean ± S.E. Statistical significance was determined by paired Student t test using the statistics module of Microsoft Excel or 1-way analysis of variance followed by all pairwise multiple comparison procedures (Student-Newman-Keuls method). Statistical significance was considered to be present at P less than .05.

3. Results

3.1. Effects of rapamycin on signaling pathways in L6 myotubes

To confirm the effective blockade of mTOR signaling by rapamycin in L6 myotubes, we determined the effect of the drug on phosphorylation of p70S6K, an established downstream target of mTOR. Fig. 1A shows immunoblot analysis of extracts from cells treated in the presence or absence of 15 nmol/L rapamycin for 24 hours and/or insulin

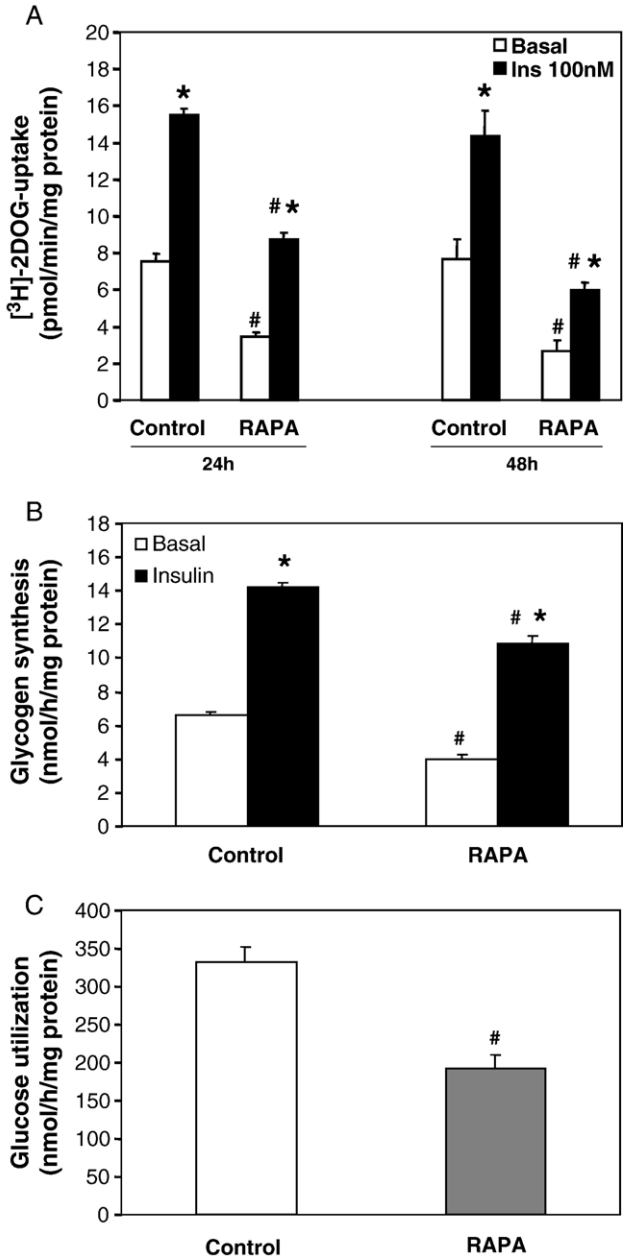


Fig. 2. Glucose metabolism in differentiated L6 myotubes treated with rapamycin. L6 cells were treated 24 to 48 hours with 15 nmol/L rapamycin (RAPA) before measurement of glucose uptake (A), glycogen synthesis (B), or glucose utilization (C) as described in Materials and methods. Mean ± SE for 4 independent experiments. Significant differences are indicated as follows: *Versus basal; #versus no rapamycin.

(short-term insulin stimulation), using 2 antibodies recognizing phosphorylation at T389 and T421/S424 in p70S6K. Insulin treatment increased phosphorylation in both cases, but insulin-stimulated levels were decreased in the presence of rapamycin (Fig. 1A and B).

Two phosphorylation sites critical for mTOR activity have been described: S2481 (reported as an autophosphorylation site in vivo and in vitro) and S2448, potentially phosphorylated by PKB in vivo [17]. Rapamycin did not influence total mTOR protein levels under basal or insulin-stimulated conditions (Fig. 1C). However, rapamycin treatment resulted in significant decreases in basal levels of mTOR phosphorylation on both residues S2481 and S2448 as shown in a representative immunoblot (Fig. 1A) and after quantification of multiple experiments (Fig. 1D). In the absence of rapamycin, short-term insulin stimulation increased mTOR phosphorylation at S2481, whereas phosphorylation at S2448 was unaffected. In the presence of rapamycin, brief exposure to insulin increased phosphorylation at both sites, restoring S2448 phosphorylation to control levels (Fig 1D). These results demonstrate effective blockade of mTOR activity by rapamycin in this system.

3.2. Rapamycin decreases glucose utilization in L6 myotubes

Differentiated L6 myotubes were incubated in the presence or absence of 15 nmol/L rapamycin, corresponding to a typical pharmacologic plasma concentration [11,13,14], for 24 or 48 hours before determination of several parameters of glucose metabolism. In cells not pretreated with rapamycin, short-term insulin treatment stimulated 2-DOG uptake by approximately 2-fold (Fig. 2A), as previously reported [15]. In rapamycin-treated cells, however, basal and short-term insulin-stimulated 2-DOG uptake were significantly decreased by approximately 40% to 50% at 24 or 48 hours (Fig .2A), such that the *proportional* effect of insulin on this process was maintained. Insulin also doubled the rate of glycogen synthesis by the cells (Fig 2B). However, as with 2-DOG uptake, above, both baseline and insulin-stimulated rates were decreased by approximately 40% in cells treated with rapamycin for 24 hours (Fig. 2B). Glucose utilization (measured in the absence of insulin only) was similarly decreased by approximately 40% after exposure to rapamycin (Fig. 2C).

Chronic rapamycin treatment of rodents and humans in vivo causes hyperinsulinemia, whereas plasma glucose levels remain normal, indicating an insulin-resistant state [13,14]. In view of this hyperinsulinemia that accompanies rapamycin use in vivo, we investigated the effect of rapamycin on the L6 cells' glucose metabolism in the context of long-duration high levels of insulin. L6 myotubes were incubated in the absence or presence of 100 nmol/L insulin and/or 15 nmol/L rapamycin for 24 hours (long-term insulin) before measurement of 2-DOG uptake over 5 minutes in the absence or presence of insulin (short-term insulin). Long-term (24 hours) exposure to insulin resulted in a 3-fold increase in basal 2-DOG uptake in control cells (Fig. 3). In control cells, or in cells with long-term exposure to high levels of insulin, the inclusion of insulin during the 5-minute DOG uptake assay (short-term insulin treatment) further increased 2-DOG uptake by 40% to 75%.

Rapamycin treatment decreased basal glucose transport (as also seen in Fig. 2A), but this effect was prevented in the simultaneous long-term presence of insulin. Thus, under the simultaneous influences of long-term rapamycin treatment and long-duration elevated insulin levels, the rate of DOG uptake was similar to that in control cells.

3.3. Effects of rapamycin on fatty acid oxidation in muscle cells

To determine the effect of rapamycin on the rate of fatty acid oxidation by muscle cells, differentiated L6 myotubes were again incubated with 15 nmol/L rapamycin for 24 or 48 hours, but with the addition of tritiated palmitate during the final 18 hours. As shown in Fig. 4A, rapamycin significantly increased the oxidation of the fatty acid palmitate by 30% and 60% in 24- and 48-hour experiments, respectively. This was accompanied by a statistically significant increase in the activity of CPT I (Fig. 4B), the first and most tightly regulated component of the CPT system that allows mitochondrial entry of fatty acyl groups destined for β-oxidation [18]. Although not considered a regulatory step, the matrix enzyme CPT II, the final component of the CPT system, was similarly increased. To confirm whether rapamycin could induce a similar effect on fatty acid oxidation in vivo, Sprague-Dawley rats were either vehicle treated or rapamycin treated (2 mg/kg body weight) for 10 days, and the rate of palmitate oxidation in soleus muscle was analyzed ex vivo. As shown in Fig. 4C, palmitate oxidation was significantly increased (~25%) by

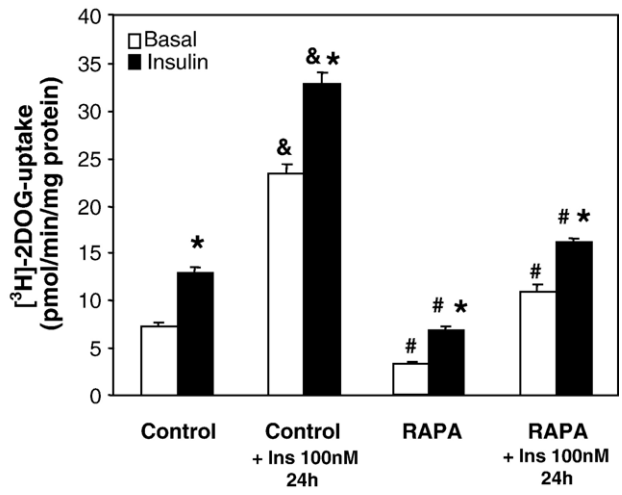
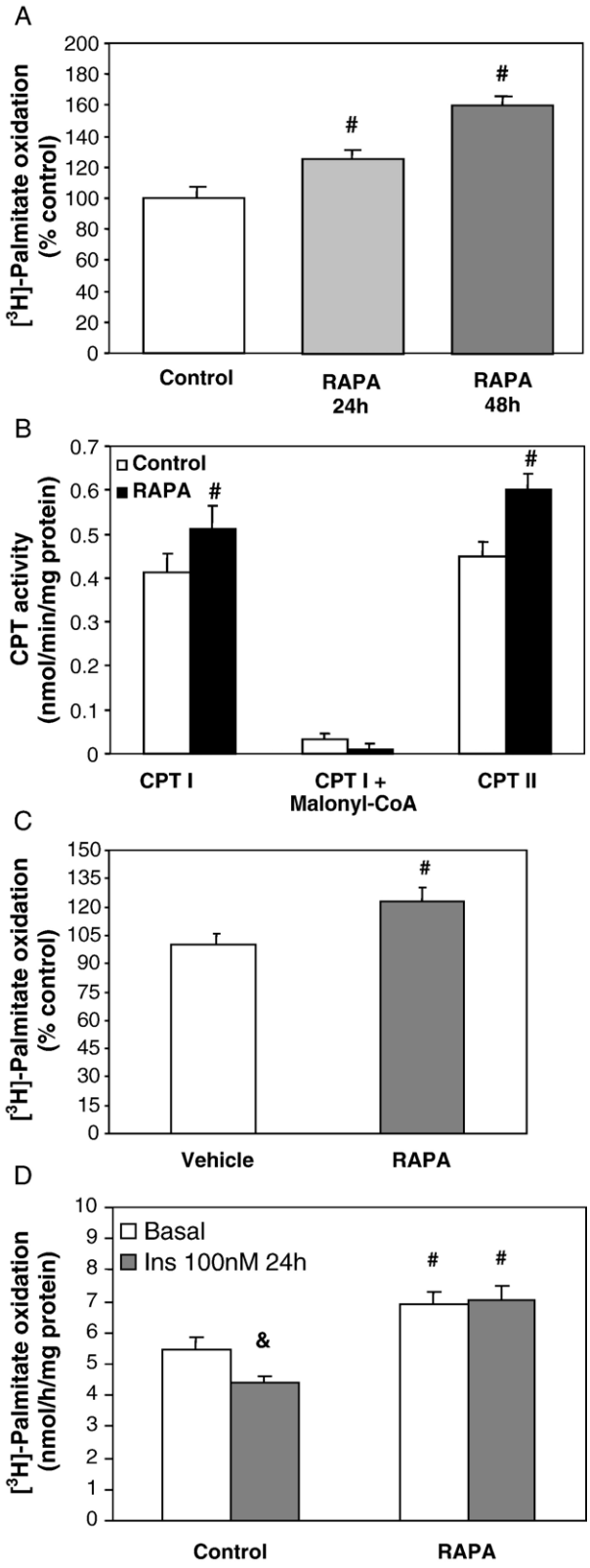


Fig. 3. Effects of rapamycin on 2-DOG uptake in the context of long-term and short-term insulin treatment. L6 cells were incubated in the presence or absence of 15 nmol/L rapamycin (RAPA) and/or 100 nmol/L insulin (long-term insulin) for 24 hours before determination of 2-DOG uptake over 5 minutes in the absence or presence of insulin (short-term insulin). Mean ± SE for 4 independent experiments. Significant differences are indicated as follows: *Versus basal; #versus no rapamycin; &versus RAPA.

rapamycin treatment. Therefore, both in vitro and in vivo rapamycin treatment/mTOR inactivation increased fatty acid oxidation in muscle cells.



As with glucose metabolism above, we studied the effect of rapamycin on the regulation of fatty acid metabolism in the context of high insulin levels. Palmitate oxidation by L6 myotubes was determined over 24 hours in the absence or presence of rapamycin and/or insulin (long-term insulin). Insulin treatment significantly decreased palmitate oxidation in control cells (Fig. 4D). However, rapamycin treatment not only increased basal palmitate oxidation (as above) but also abolished the ability of insulin to inhibit this pathway (Fig. 4D).

4. Discussion

Skeletal muscle is a major target for insulin-stimulated glucose disposal and suppression of fatty acid oxidation after a meal [19]. The mTOR inhibitor, rapamycin, a drug in widespread clinical use, has been shown to induce insulin resistance and dyslipidemia [11-14]. However, the underlying mechanism(s) remains elusive. In the current study, we investigated the effect of rapamycin on glucose and lipid metabolism in muscle cells.

The mTOR/p70S6K pathway plays an important role in the regulation of insulin-stimulated glucose transport in L6 cells [20]. It has also been suggested that amino-acid-induced overactivation of the mTOR/p70S6K1 pathway may impair insulin action in L6 cells and in human skeletal muscle [20,21]. Acute exposure to amino acids (potent modulators of mTOR activity) reduced insulin-stimulated glucose transport in L6 cells with a concomitant activation of the mTOR/p70S6K pathway [20]. Furthermore, inhibition of the mTOR/p70S6K pathway by rapamycin prevented the negative effect of amino acids on insulin signaling and glucose uptake in L6 cells [20]. In L6 cells as well as in 3T3-L1 cells, the molecular mechanism by which the mTOR/p70S6K pathway regulates insulin signaling and insulin-stimulated glucose transport has been partially delineated [20,22,23]. Subcellular localization, insulin-stimulated Ser/Thr phosphorylation, and proteosomal degradation of IRS-1 are all altered by rapamycin, indicating a role for the mTOR/p70S6K pathway. Thus, insulin and/or

Fig. 4. Effects of rapamycin on β-oxidation rates in L6 myotubes and rat soleus muscle. A, Fatty acid oxidation in L6 cells. L6 cells were incubated 24 or 48 hours in the presence or absence of 15 nmol/L rapamycin, with tritiated palmitate included for the final 18 hours. Mean ± SE for 4 independent experiments in triplicate. B, CPT I and II activities. L6 cells were preincubated in the presence or absence of 15 nmol/L rapamycin for 24 hours before measurement of CPT activities. Mean ± SE for 6 independent experiments in duplicate. C, Fatty acid oxidation in rat soleus muscle. Soleus muscles were obtained from rats that received long-term treatment with rapamycin as described in Materials and methods and palmitate oxidation measured over 90 minutes ex vivo. Mean ± SE for 5 independent experiments. D, Fatty acid oxidation in L6 cells under hyperinsulinemic conditions. L6 cells were treated as above in the presence or absence of 100 nmol/L insulin (long-term insulin) before fatty acid oxidation was measured. Mean ± SE for 6 independent experiments in triplicate. Significant differences are indicated as follows: $^{\#}$Versus no rapamycin; $^{\&}$versus no insulin.

amino acid activation of the mTOR/p70S6K pathway may constitute a feedback mechanism that down-regulates insulin signaling and integrates nutritional status and metabolic signals.

However, none of these studies [20,22,23] examined long-term effects of rapamycin (24-48 hours) on insulin-stimulated glucose transport, but instead were focused on short-term inhibition of the mTOR/p70S6K pathway (~1-hour rapamycin treatment). Because in a clinical context immunosuppression is normally a long-term situation and one associated with insulin resistance in both rodents and humans [13,14], we were interested to study the effects of rapamycin on glucose uptake in skeletal muscle over a longer time frame than had been addressed by previous studies. Our results show that in L6 cells long-term exposure to rapamycin alone causes a reduction in basal glucose uptake, whereas the short-term response to insulin, in relative terms, is unaffected. These novel observations are consistent with those reported by Taha et al [24] where rapamycin was shown to modulate glucose transport in 3T3-L1 cells, preventing long-term insulin-induced increases in GLUT 1 glucose transporter protein synthesis via partial inhibition at the level of both transcription and translation. Thus, we propose that the rapamycin-mediated reduction in basal glucose uptake in L6 cells might involve transcriptional/translational regulation of basal glucose transporters (ie, those not subject to rapid, short-term regulation by insulin). Although mTOR inhibition may have as yet undefined direct effects on other steps in glucose metabolism, this likely explains, at least in part, the observed decreases in glucose utilization and glycogen synthesis.

In parallel with these changes in glucose metabolism, rapamycin increased fatty acid oxidation in muscle cells and, furthermore, inhibited the ability of insulin to suppress that pathway. The rapamycin-induced increase in fatty acid oxidation in L6 myotubes may, at least in part, be the direct result of increases in the activity of CPT I and CPT II. Interestingly, S6K1-deficient mice (lacking that downstream target of mTOR) exhibit an enhanced fatty acid oxidation capacity in vivo, which correlates with elevated mRNA expression levels of CPT I, peroxisome proliferator–activated receptor γ coactivator 1α, uncoupling protein 3, and peroxisome proliferator–activated receptors β/δ in skeletal muscle [25]. Thus, the S6K1-deficient mouse model and our own data using soleus muscles from rapamycin-treated rats provide 2 lines of evidence that mTOR plays a physiologic role in the regulation of fatty acid metabolism in vivo.

Hence, our data suggest that prolonged rapamycin-mediated inhibition of mTOR increases the rate of fatty acid oxidation in skeletal muscle cells, both in vitro and in vivo, observations that at first glance are at odds with clinical observations. For example, posttransplant hyperlipidemia is one of the most serious side effects of immunosuppressants [26]. Also, weight gain posttransplant affects approximately 50% of patients with immunosuppressive medication and the prevalence of obesity rises from 19%, at the time of transplantation, to 36% 1 year later [27]. There are several possible explanations. First, the prevalence of posttransplant obesity rises slowly, observed only after 1 year, which suggests that it may be a consequence of extended treatment. We did not observe any significant effects of rapamycin on body weight, but our in vivo experiments in rats were conducted over only 10 days. Second, whereas the focus of our current work was on rapamycin's direct actions in skeletal muscle, many other tissues are important in the regulation of fatty acid metabolism such as liver and adipose. In the latter case, it has been shown that rapamycin-mediated inhibition of mTOR blocks adipocyte differentiation by a mechanism that involves 2 key transcription factors in adipogenesis (CCAAT/enhancer binding protein-α and peroxisome proliferator–activated receptor γ) illustrating a direct effect of the immunosuppressant in that organ [28]. However, the effect of rapamycin on fatty acid metabolism in these tissues is unknown. Third, it has been shown that hypothalamic mTOR signaling regulates food intake. In a series of elegant experiments, Cota et al [29] showed that central administration of leucine (intra-third ventricular) increased hypothalamic mTOR signaling and decreased food intake and body weight in rats. In contrast, administration of rapamycin rapidly inhibited hypothalamic p70S6K1 and significantly increased short-term chow intake in presatiated rats. Thus, the effects of rapamycin in vivo are a complex synthesis of what may appear to be contradictory effects on multiple tissues.

However, if our observations in L6 cells are paralleled in vivo (and our data for fatty acid oxidation in isolated soleus muscle suggest that this is indeed the case for that pathway at least) they could explain in large measure the apparent insulin resistance associated with rapamycin treatment in vivo [13,14]. That is to say, despite the retention of the proportional increase in glucose uptake capacity in response to insulin, the absolute capacity is lower in the presence of rapamycin at any level of insulin.

Sometimes described as a nutrient sensor, mTOR might be expected to play a role in intermediary metabolism, and in particular in regulation of the response of a tissue to its nutrient environment and its choice of metabolic fuel. Here we demonstrate that this is indeed the case. Blockade of mTOR in L6 cells leads to increased fatty acid oxidation at the expense of glucose utilization, a behavior characteristic of the fasted metabolic state. Whereas this kind of metabolic "switching" between fatty acids and glucose for energy production is a normal physiologic response (as epitomized by the Randle mechanism proposed decades ago to partially explain short-term changes in muscle fuel utilization in response to glucose and fatty acid availability), the effect of rapamycin is to induce a behavior suitable for the fasted environment regardless of circumstances. Conversely, one may infer that physiologic mTOR signaling likely plays a role in promoting glucose utilization at the expense of fatty acid oxidation under conditions of nutrient abundance.

In summary, our data demonstrate that long-term rapamycin treatment significantly affects muscle cells' fuel

metabolism, promoting β-oxidation while diminishing basal glucose transport and glycogen synthesis. More importantly, in the presence of rapamycin, the net effects of insulin on glucose and fatty acid metabolism are diminished. These observations highlight an important role for the nutrient sensor mTOR in the regulation of fuel metabolism that goes beyond its established role in regulation of cellular proliferation and growth. They also provide novel insight into the metabolic perturbations associated with long-term rapamycin use in a clinical setting.

Acknowledgments

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