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The mammalian target of rapamycin regulates lipid metabolism in primary cultures of rat hepatocytes

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Abstract

The mammalian target of rapamycin (mTOR) is a conserved serine-threonine kinase that regulates cell growth and metabolism in response to nutrient signals. However, the specific involvement of mTOR in regulation of energy metabolism is poorly understood. To determine if signaling via mTOR might be directly involved in regulation of fatty acid metabolism in hepatocytes, we performed studies with rapamycin, a specific inhibitor of mTOR. Rapamycin-mediated inhibition of mTOR (18-48 hours) increased oxidation of exogenous fatty acids (46%-100%, respectively). In addition, esterification of exogenous fatty acids and de novo lipid synthesis were reduced (40%-60%, respectively). Consistent with inhibition of lipogenic pathways, rapamycin decreased expression of genes encoding acetyl–coenzyme A carboxylase I and mitochondrial glycerol phosphate acyltransferase. Non–insulin-dependent glucose transport and glycogen synthesis were decreased by 20% to 30%, whereas glucose utilization was unaffected by rapamycin. The data suggest that the hyperlipidemia observed with the drug in vivo is likely not the result of enhanced hepatic synthesis, but rather of delayed peripheral clearance. However, these results are consistent with the idea that mTOR may play a significant role, not only in "energy sensing," but also in regulation of energy production through profound effects on hepatic fatty acid metabolism.

1. Introduction

The mammalian target of rapamycin (mTOR) pathway plays an essential role in the control of cell growth and proliferation, integrating hormonal and nutritional signals in eukaryotic cells. Physiologically, mTOR is stimulated by mitogens (including insulin) and nutrient abundance as signaled by amino acids, particularly leucine [1]. As a consequence, mTOR has been termed a *nutrient sensor*. Its role in the regulation of cell growth has been extensively studied and is well defined [2]. However, the contribution of mTOR to the regulation of intermediary metabolism is poorly understood.

Rapamycin (sirolimus), a highly specific inhibitor of mTOR [3], has wide clinical application as an immunosuppressant in transplant recipients [4], as an inhibitor of tumor growth [5], and in prevention of occlusion of coronary arteries after stent placement [6]. The mammalian target of rapamycin elicits its effect by binding to the cytosolic immunophilin FKBP12 (FK506 binding protein, 12 kd), and it is the rapamycin/FKBP complex that binds to and inhibits mTOR [7].

In the clinic, prolonged use of rapamycin is associated with serious adverse effects including hyperlipidemia [8-10], weight gain [11,12], and insulin resistance [13,14], suggesting that, physiologically, mTOR signaling may play a significant role in lipid homeostasis. Because the liver holds a central position in the regulation of whole-body lipid metabolism, we chose to investigate if blockade of mTOR signaling in hepatocytes could elicit alterations in major pathways of fatty acid metabolism in hepatocytes.

Our studies determined the direct effects of rapamycin-mediated inhibition of mTOR on several major pathways of hepatic fatty acid metabolism, including rates of β-oxidation, de novo fatty acid synthesis, and fatty acid esterification to triglyceride in primary cultures of rat hepatocytes. We show for the first time that rapamycin does indeed have profound direct effects on hepatocyte fatty acid metabolism, promoting β-oxidation while decreasing flux into anabolic storage pathways. These changes are accompanied by a decreased dependence on glucose metabolism. Our observations are

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consistent with the notion that mTOR signaling is involved in nutrient sensing, such that inhibition of the pathway induces a "fasting" hepatic metabolic phenotype, even in the presence of nutrient abundance, and emphasize the importance of mTOR as a key regulator of hepatic metabolism.

2. Materials and methods

2.1. Materials

Cell culture reagents were from Mediatech, Herdon, VA, (fetal bovine serum) and BioWhittaker, Walkersville, MD, (Dulbecco modified Eagle medium). [9,10-^{3}H]-Palmitic acid, [^{3}H]-H_2O, [1,2-^{14}C]-acetate, and 2-[^{3}H]-deoxyglucose were from PerkinElmer Life Sciences (Shelton, CT). [8-^{3}H]-Octanoic acid, [9,10-^{3}H]-oleic acid, and L-[*N*-methyl-^{14}C]-carnitine were 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).

2.2. Cell culture

Hepatocytes from male Sprague-Dawley rats (200-250 g) were isolated by collagenase perfusion of the liver, as described previously [15]. Viability, as assessed by trypan blue exclusion, was routinely in excess of 90%. Six-well collagen-coated plates were loaded with 10^6 live cells per well with 2 mL of Dulbecco modified Eagle medium containing 10% (vol/vol) fetal calf serum. After 1 hour, during which time only the live cells became attached to the plate, the medium was aspirated (along with unattached dead cells) and replaced with fresh medium. Cells were maintained for up to 48 hours (with at least one change of medium at 24 hours). Animal procedures were in accordance with the humane care criteria of the National Academy of Sciences and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.3. Fatty acid oxidation

Fatty acids (palmitate, oleate, or octanoate) were preconjugated with essentially fatty acid–free bovine serum albumin (BSA) to generate stock solutions of 25% (wt/vol) BSA, 4 mmol/L fatty acid in serum free medium [16]. [^{3}H]-Palmitate, [^{3}H]-oleate, or [^{3}H]-octanoate was added; and the stock solution was diluted into the final culture medium to give concentrations of 1.25% BSA, 0.2 mmol/L (0.5 μCi/mL) fatty acid.

Cells were incubated for 18 or 48 hours in the presence or absence of immunosuppressant agents (cyclosporin A [CsA], rapamycin, or FK506), and concentrations were based on pharmacological plasma levels [17-19]. In 18-hour experiments, the medium also contained labeled fatty acids throughout. In 48-hour experiments, the medium was replaced after an initial (30 hours) incubation with the drug, such that fatty acids were introduced and drug levels were maintained for the last 18 hours. The medium was then collected, and tritiated water was determined by the vapor-phase equilibration method of Hughes et al [20]. Cells were washed 3 times with 2 mL of ice-cold phophate-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. [^{3}H]-Fatty acid incorporation into total lipids

After similar incubations with labeled fatty acids to those used for measurements of β-oxidation, cells were washed 3 times with 2 mL of ice-cold PBS and harvested in 90 μL of PBS; and after a total lipid extraction (according to Bligh and Dyer [21]), the radioactive content was determined. Protein content was measured as above.

2.5. [^{14}C]-Acetate incorporation into total lipids

De novo lipid synthesis was determined using [^{14}C]-acetate according to Kuhajda et al [22]. Cells were incubated with vehicle or rapamycin as above, plus [^{14}C]-acetate (0.05 μCi/mL) during the last 18 hours. The cells were then washed and harvested, and radioactive content and protein were measured as above.

2.6. Mitochondrial carnitine palmitoyltransferase I assay

Activities of mitochondrial carnitine palmitoyltransferases (CPTs) I and II were determined by a radiochemical assay in the direction of acylcarnitine formation as described previously [23]. For assay of CPT I, located on the outer aspect of the mitochondria, hepatocytes were broken using a glass homogenizer. Assay of this material determines only CPT I because the mitochondria remain largely intact. For measurement of CPT II, a portion of this material was made 1% (wt/vol) with regard to the detergent octylglucoside that inactivates CPT I but releases CPT II from the mitochondrial matrix in active form [24]. Proteins were determined as above.

2.7. Glucose metabolism

Parameters of hepatocyte glucose metabolism were measured after 18-hour incubation in the absence or presence of 30 nmol/L rapamycin. Glucose utilization was determined as the production of tritiated water (as for fatty acid oxidation experiments) after incubation of hepatocytes for 2 hours in the presence of [5-^{3}H]-glucose (6.5 μCi/mL). Uptake of 2-[^{3}H]-deoxyglucose (DOG; 10 μmol/L, 0.5 μCi/mL) was determined over 5 minutes after 30 minutes of preincubation with or without 100 nmol/L insulin during each period [25]. Incorporation of D-[^{14}C]-glucose (2 μCi/mL) into glycogen was measured in the absence or presence of insulin over 1 hour [25].

2.8. Northern blot analysis

Total RNA was extracted from hepatocytes using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and separated by electrophoresis on

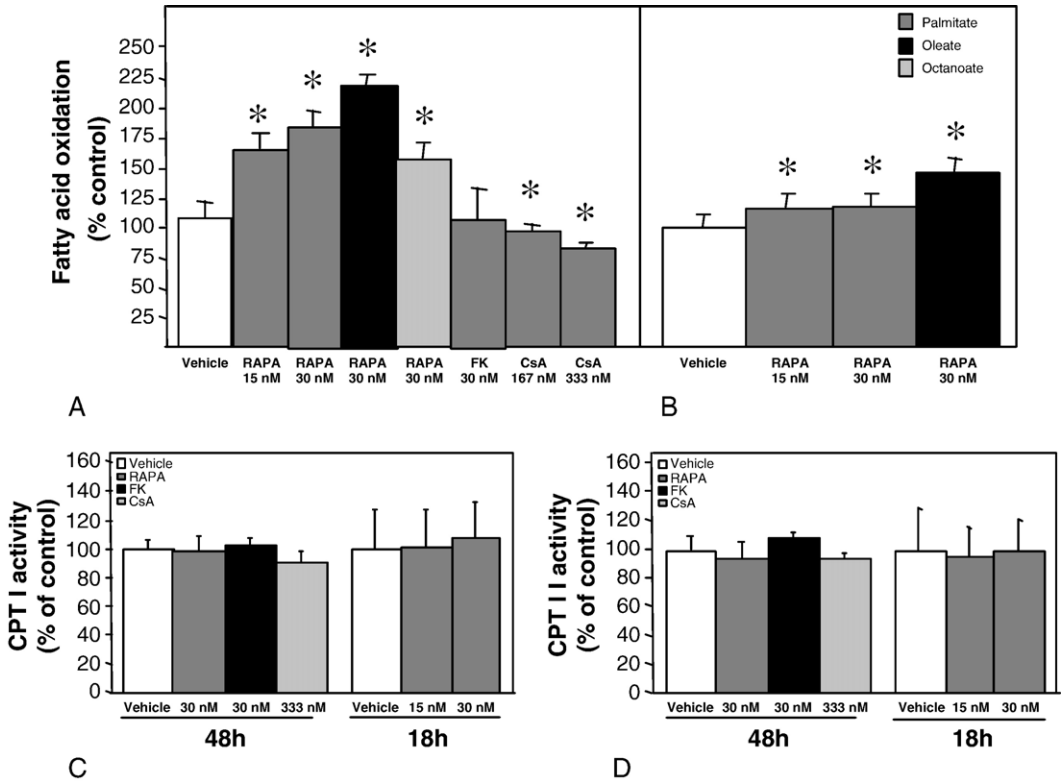

Fig. 1. β-Oxidation of exogenous fatty acids and CPT activities in hepatocytes treated with immunosuppressants. Primary rat hepatocytes were treated for 48 hours (A) or 18 hours (B) with rapamycin, CsA, or FK506 at the indicated concentrations. Tritiated fatty acids were included during the final 18 hours for measurement of fatty acid oxidation rates. C, CPT I activities. D, CPT II activities. Results are expressed as percentage of values in untreated cells. Mean ± SE for 4 to 6 independent experiments. Significance (*) is indicated relative to the corresponding control values. RAPA indicates rapamycin; FK, FK506.

1% agarose gels. The DNA templates for synthesis of radioactive probes were generated by polymerase chain reaction (PCR) using rat hepatocyte complementary DNA (Advantage RT for PCR Kit; Clontech, Palo Alto, CA) and oligonucleotide primers based on published sequences (rat malonyl–coenzyme A [CoA] decarboxylase [MCD], rat acetyl-CoA carboxylase [ACC] 1 [the predominant form in hepatocytes], rat adenosine monophosphate [AMP]–activated protein kinase [AMPK] α-2 catalytic subunit, and rat β-actin). The products were ligated into phage M13mp18, and [^{32}P]-labeled single-stranded DNA probes were generated and used to detect individual messages as described [26]. The identities of the cloned PCR products were confirmed by DNA sequencing.

2.9. Immunoblot analysis

Immunoblots were conducted on cell lysates using commercially available antibodies as previously described [25].

2.10. Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined by paired Student *t* test using the statistics module of Microsoft Excel (Microsoft Excel X for Mac; Microsoft, Redmond, WA) 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 $P < .05$.

3. Results and discussion

3.1. Effects of rapamycin on pathways of lipid metabolism in hepatocytes

To determine the effects of rapamycin on hepatic lipid metabolism, we used a previously established primary rat hepatocyte model [15]. This system has multiple advantages. First, by using a single cell type, it is possible to test the direct effect of the drugs on hepatocytes in the absence of potential secondary effects of other cell types, hormones, or plasma metabolites. Second, the cells are nonproliferating, which removes the potential complication of the effect of rapamycin/mTOR inhibition on that process. Third, measurement of the important metabolic fluxes under study can be performed with relative precision in the culture system.

Initial experiments were designed to determine how rapamycin influenced the rate of oxidation of exogenous fatty acids by hepatocytes. As shown in Fig. 1A, 15 or 30 nmol/L rapamycin increased oxidation of the saturated long-chain fatty acid palmitate by 55% or 72%, respectively. Rapamycin (30 nmol/L) increased oxidation of the monounsaturated long-chain fatty acid oleate and the saturated

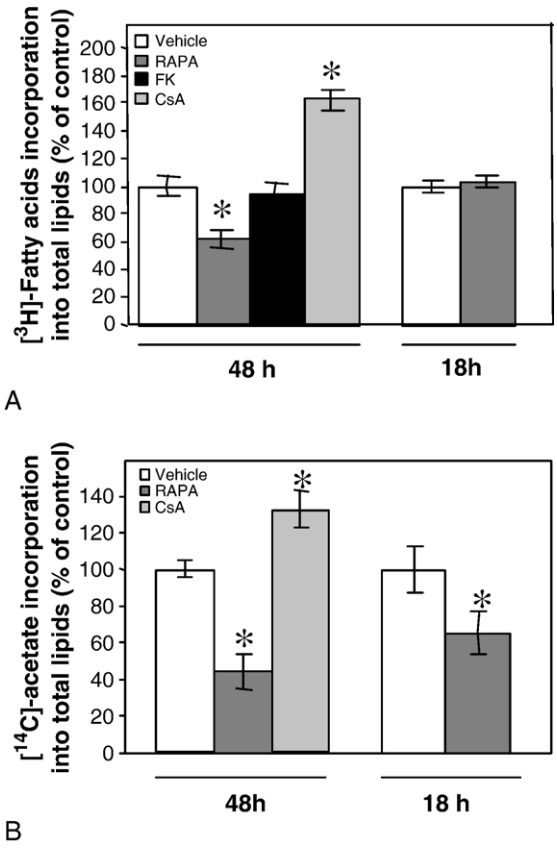

Fig. 2. Lipid metabolism in hepatocytes treated with immunosuppressants. A, Incorporation of exogenous fatty acids into total lipids in hepatocytes treated with immunosuppressants. Cells were treated for 48 or 18 hours with 30 nmol/L rapamycin, 333 nmol/L CsA, or 30 nmol/L FK506. Tritiated fatty acids were included during the final 18 hours before determination of incorporation into total cellular lipid. Mean ± SE for 3 to 7 independent experiments. Results are expressed as percentage of vales in untreated cells. B, De novo lipid synthesis in hepatocytes treated with immunosuppressants. Cells were treated for 48 or 18 hours with 30 nmol/L rapamycin, 333 nmol/L CsA, or 30 nmol/L FK506 before measurement of [^{14}C]-acetate incorporation into total lipids as described in "Materials and methods." Mean ± SE for 3 to 7 independent experiments. Results are expressed as percentage of values in untreated cells. Significance (*) is indicated relative to the corresponding control values.

medium-chain octanoate by 100% and 46%, respectively. When rapamycin and the labeled fatty acids were included together for only 18 hours (Fig. 1B), rates of palmitate and oleate oxidation were also significantly increased, but to a lesser degree (15% and 47%, respectively). FK506 (tacrolimus) is another immunosuppressant agent that also binds to the eponymous immunophilin FKBP12; but in this case, the complex has no significant affinity for mTOR, but rather inhibits calcineurin signaling [27]. In contrast to the effect of rapamycin, 48-hour incubation with FK506 had no effect on oxidation of exogenous palmitate (Fig. 1A). A third immunosuppressant, cyclosporine, which acts through binding to cellular cyclophilin, decreased palmitate oxidation during the 48-hour experiments. These data demonstrate a highly specific dose-dependent increase in flux through β-oxidation in response to rapamycin. The time course of the effect is consistent with a long-term mechanism, such as transcription or translation, in accordance with the known pathways of mTOR action.

The primary regulated step in mitochondrial oxidation of long-chain fatty acids by hepatocytes under most physiological circumstances is CPT I [28,29]. This mitochondrial outer membrane protein catalyzes the transfer of long-chain acyl groups from CoA to carnitine. Whereas the outer mitochondrial membrane is permeable to acyl-CoA species, the inner membrane presents a barrier. However, acylcarnitine generated from the CPT I reaction can cross the inner membrane to the mitochondrial matrix by means of a specific carnitine/acylcarnitine carrier, where a distinct gene product, CPT II, reverses the CPT I reaction, regenerating acyl-CoA, which can then enter the β-oxidation pathway. Mitochondrial carnitine palmitoyltransferase I is potently inhibited by malonyl-CoA, the first committed intermediate in the opposing pathway of de novo fatty acid biosynthesis. Hence, when the de novo synthesis pathway is active, the elevated levels of malonyl-CoA inhibit CPT I and prevent the mitochondrial entry and futile oxidation of the newly synthesized fatty acids. Fig. 1C and D shows that none of the immunosuppressants affected the activities of CPT I or CPT II. It is possible that the increased flux through β-oxidation induced by rapamycin involves a decreased production of the physiological CPT I inhibitor malonyl-CoA. However, because oxidation of octanoate, which enters the mitochondrion independently of CPT I, was also accelerated, changes in the level of malonyl-CoA alone are unlikely to provide an explanation for the increased flux through β-oxidation.

At first inspection, the effect of rapamycin—to promote fatty oxidation—may be surprising because clinical use of the drug is associated with lipid accumulation. However, Um et al [30] reported that mice deficient in S6K1 (a downstream target of mTOR) also have increased fatty acid oxidation capacity in vivo, in association with elevated messenger RNA levels for genes involved in fatty acid oxidation (CPT I), and mitochondrial content (peroxisome proliferator–activated receptor [PPAR] γ coactivator 1α, uncoupling protein 3, and PPAR-β/δ) in skeletal muscle and adipose tissue. We have also shown that rapamycin-mediated inhibition of mTOR increases the fatty acid oxidation rate in skeletal muscle cells both in vivo and in vitro [31]. Furthermore, Luong et al [32] have reported that reducing the function of *Drosophila* TOR results in breakdown of lipid stores and conversion to ketone bodies in that organism. Thus, our observation of a direct effect of rapamycin to enhance fatty acid oxidation in hepatocytes is consistent with other models, suggesting that mTOR plays a physiological role in the regulation of fatty acid metabolism and that in certain circumstances, mTOR inhibition can have pro-oxidative effects.

To determine the effect of rapamycin on the rate of esterification of exogenous fatty acids, experiments were designed similarly to those for measurement of β-oxidation,

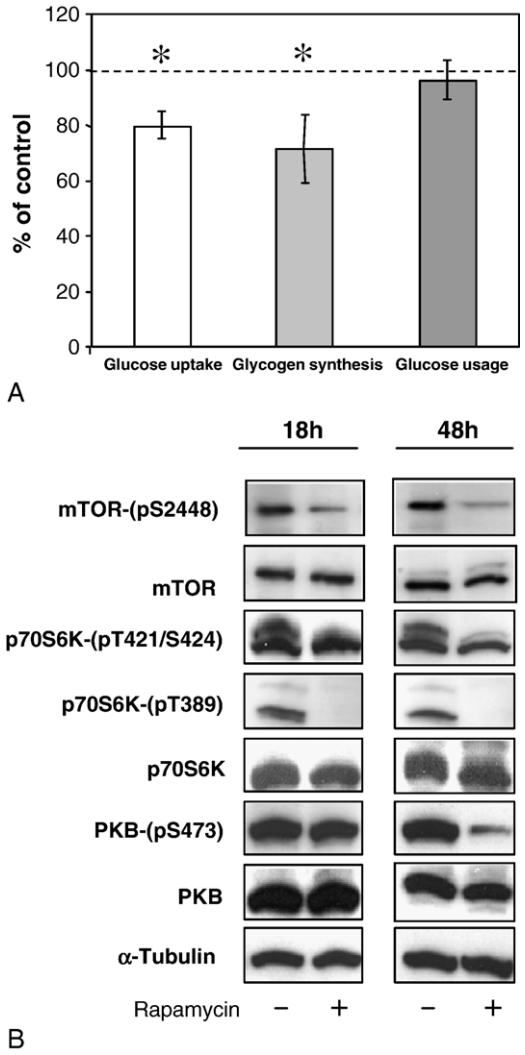

Fig. 3. Glucose metabolism and signaling pathways in hepatocytes treated with rapamycin. A, Glucose metabolism. Cells were treated for 18 hours with 30 nmol/L rapamycin before determination of rates of DOG uptake, glycogen synthesis, and glucose utilization, as described under "Materials and methods." Mean ± SE for 5 to 6 independent experiments. Significance (*) is indicated relative to the corresponding control values. B, Western blot analysis of signaling pathways in hepatocytes treated with rapamycin. Cells were treated for 18 or 48 hours with 30 nmol/L rapamycin before preparation of cell lysates and immunoblot analysis. Typical immunoblots (of 3 independent experiments) for phosphorylation specific or total kinase antibodies and α-tubulin (control), as indicated.

with up to 48 hours of exposure to the drugs in the presence of labeled palmitate for the last 18 hours before determination of label incorporation into cellular lipids (primarily triglyceride). As shown in Fig. 2A, rapamycin decreased esterification by 40%, whereas FK506 had no effect and cyclosporine caused an increase of 62%. Exposure of hepatocytes to rapamycin for only 18 hours in the simultaneous presence of palmitate revealed no effect on esterification rates in the shorter term.

The rate of de novo lipid synthesis (fatty acids and cholesterol) was determined in similar experiments using labeled acetate as substrate. Fig. 2B shows that long-term (48 hours) exposure of the cells to rapamycin decreased de novo synthesis by 60%. In 18-hour experiments, rapamycin also decreased de novo synthesis, but by only 30%. Thus, this effect is also consistent with a chronic regulatory mechanism. Again, cyclosporine had the opposite effect, increasing de novo lipid synthesis by 32%.

Thus, in addition to the drug's effects on the rate of fatty acid oxidation (above), we show that rapamycin-mediated inhibition of mTOR has profound effects on lipogenesis in hepatocytes. Of note in this regard, Cho et al [33] have suggested that rapamycin, at low doses, might efficiently inhibit lipogenesis by suppressing PPAR-γ and fatty acid synthetase gene expression in 3T3-L1 cells.

An apparent paradox to emerge from the present study is that it is procatabolic, antianabolic effects on fatty acid metabolism that we observe with direct action of rapamycin on hepatocytes, whereas it is hyperlipidemia that is observed with prolonged rapamycin use in a clinical setting [8-10]. However, we have previously shown that the β-oxidation–promoting effects of rapamycin on cultured muscle cells are paralleled in vivo [31]; and there is no evidence to suggest that the direct effects of the drug on hepatocytes are not also manifested in whole animals (or humans). Presumably, however, during prolonged use in the complex environment of the in vivo situation, effects of the drug on other tissues must act au contraire and mask the direct influence of mTOR blockade on hepatic fatty acid metabolism. Indeed, it has been shown that reduction in mTOR activity exerts a variety of different metabolic effects in skeletal muscle [30], adipose tissue [33,34], and brain [35]. However, the immediate goal of this study was to define the direct action of the drug on liver cells rather than to explain the dyslipidemia associated with long-term use of rapamycin.

Notably, however, hyperlipidemia is also a well-known adverse effect of CsA [36]. As we show in Figs. 1 and 2, CsA at pharmacological doses reduced fatty acid oxidation capacity and enhanced de novo fatty acid synthesis and esterification in hepatocytes, observations entirely consistent with and likely explaining, at least in part, the hyperlipidemia associated with that drug.

3.2. Effects of rapamycin on pathways of glucose metabolism in hepatocytes

Similarly to experiments with fatty acid metabolism (above), cells were preincubated for 18 hours in the presence or absence of 30 nmol/L rapamycin; and the rate of glucose uptake, glycogen synthesis, and glucose utilization were determined (Materials and methods). As shown in Fig. 3A, uptake of DOG (a measure of glucose transport capacity) and incorporation of glucose into glycogen were decreased by 20% and 30%, respectively, although glucose utilization, as determined by production of tritiated water from [5-^{3}H]-glucose, was unchanged. Because glycogen synthesis in liver is positively regulated by insulin (whereas glucose uptake is not), it is possible that the effects of rapamycin on glucose

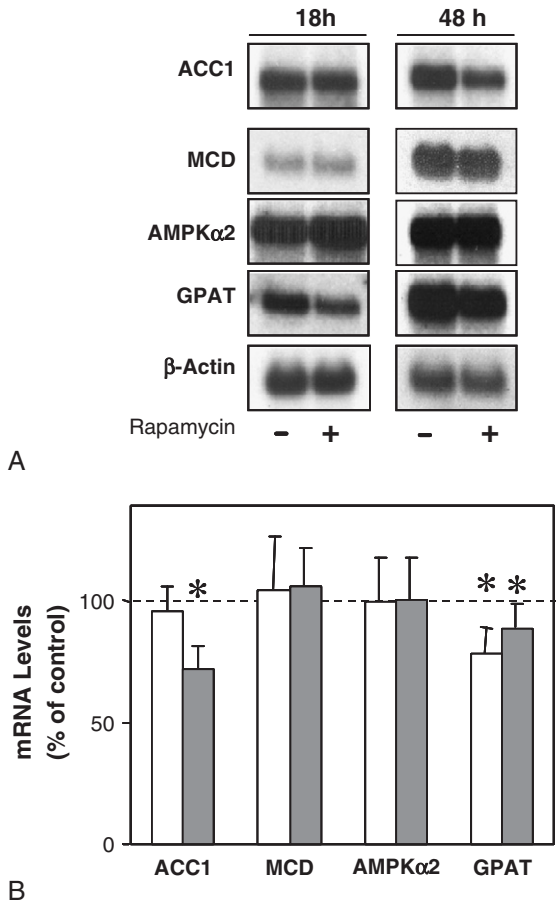

Fig. 4. Northern blot analysis of total RNA from rapamycin-treated hepatocytes. Cells were treated for 18 (white bars) or 48 hours (gray bars) with 30 nmol/L rapamycin or in the absence of the drug before harvesting of cells, preparation of total RNA, and detection of specific messages by Northern blot. A, Representative Northern blots for messenger RNA of ACC1, MCD, AMPK α-2 catalytic subunit, glycerol phosphate acyltransferase, and actin. B, Quantification of the total data set expressed in arbitrary units relative to actin message levels. Mean ± SE for 6 independent experiments. Significance (*) is indicated relative to the corresponding control values.

metabolism could be explained by a decrease in the baseline activity of the insulin signaling pathway (because exogenous insulin was not present in these experiments). Thus, a decrease in glycogen synthesis without a change in glucose utilization would be reflected in a decrease in net glucose uptake (the methodology used to measure the pathways of glucose metabolism in these experiments does not permit comparison of the absolute values of flux through each pathway). Consistent with this hypothesis, prolonged (48 hours) rapamycin treatment significantly reduced the serine 473 phosphorylation on protein kinase B (Akt) (PKB), indicating a decreased activation of that signaling kinase, a critical component of the canonical insulin signaling pathway (Fig. 3B). As expected, phosphorylation of mTOR and p70 S6 kinase (p70S6K) was significantly decreased in rapamycin-treated hepatocytes, confirming the blockage of mTOR signaling in those cells (Fig. 3B), although notably this effect became pronounced in advance of significant changes in PKB phosphorylation.

Reports on the effects of rapamycin on glucose metabolism in vivo are equivocal and may be caused in part by effects on the β-cell and on major glucose-utilizing organs. In humans, an adverse effect of prolonged use of rapamycin is posttransplant diabetes mellitus and insulin resistance [14]. In rats, prolonged use of rapamycin causes hyperglycemia without changes in plasma insulin levels, a phenotype that clearly indicates a relative insulin deficiency [13]; and in mice, rapamycin causes hyperglycemia associated with decreased production of insulin in β-cells, [37]. In contrast, genetic ablation in mice of the p70S6K gene (a downstream target of mTOR) increased whole-body insulin sensitivity in the knockout animals [30], suggesting that the effects of rapamycin on glucose metabolism are mediated in part by a p70S6K-independent mechanism. Further work is warranted to unveil signaling events downstream mTOR and p70S6K that regulate glucose metabolism. Nevertheless, the increased rate of fatty acid oxidation we observed in hepatocytes is in agreement with the enhanced fatty acid use seen in the p70S6K −/− mice and *Drosophila* mutant $dTOR^{7/P}$ [30,32]; and it seems likely that a common mechanism (involving mTOR in conjunction with p70S6K) acts to mediate that particular effect in both models.

3.3. Mechanism of the rapamycin-mediated inhibition of mTOR effect

To gain insight into the mechanism whereby rapamycin was eliciting chronic regulation of pathways of fatty acid metabolism, we performed Northern blot analysis of the messages encoding 4 critical regulatory enzymes (Fig. 4). Long-term (48 hours) exposure of the cells to rapamycin resulted in a significant decrease in the relative expression of the ACC1 message. Because ACC generates malonyl-CoA as the first step in de novo fatty acid synthesis and as an inhibitor of CPT I, a decreased level of the enzyme would be predicted to promote fatty acid oxidation and decrease synthesis. Similarly, rapamycin decreased expression of glycerol phosphate acyltransferase (Fig. 4), the primary regulated step in triglyceride synthesis [38]. These observations are consistent with a mechanism in which rapamycin inhibits flux into these anabolic lipid storage pathways via transcriptional regulation of specific genes. Malonyl-CoA carboxylase, which acts to remove malonyl-CoA, and the catalytic subunit of AMPK, the action of which activates MCD and inhibits ACC, were unaffected at the transcriptional level.

Rapamycin, therefore, acts in a highly specific manner to change the relative expression levels of certain critical genes involved in hepatic lipid metabolism (levels of other transcripts were unaffected, precluding generalized inhibition of cellular transcription as a nonspecific explanation for our observations). These changes would be expected to result in decreased flux through lipid storage pathways. In parallel, fatty acid oxidation is accelerated. This may be due

in part to a drop in cytosolic malonyl-CoA, with consequent deinhibition of CPT I in the cellular milieu and increased flux through the enzyme. This would not be apparent in our measurements of total CPT I activity in ruptured cells, where the cytosolic malonyl-CoA has been essentially removed by dilution. However, as noted above, whereas CPT I regulates oxidation of long-chain fatty acids, medium-chain fatty acids enter the mitochondrial matrix independently of CPT I. That oxidation of rapamycin also increased oxidation of the medium-chain fatty acid octanoate indicates that other steps in the β-oxidation pathway may also be up-regulated. The possibility also exists that blockade of mTOR signaling with rapamycin increases the rate of fatty acid oxidation secondary to increased overall mitochondrial function or number, an idea supported by the observation that deletion of the mTOR downstream effector p70S6K has been reported to increase the expression of PPAR-γ coactivator 1, a critical factor in determining mitochondrial biogenesis [30,39].

These are the first data showing direct effects of rapamycin on flux through pathways of hepatic fatty acid metabolism. However, the data are consistent with a microarray study indicating that rapamycin induced a "fasted" phenotype in lymphocytes, including decreased expression of the fatty acid synthase gene [40]. In addition, rapamycin seemed to decrease expression of glycolytic enzymes in prostate epithelial cells [41], again consistent with the notion that rapamycin treatment favors the use of fatty acids as a source of metabolic fuel, a characteristic fasted behavior.

Although our data indicate that the hepatic metabolic effects of rapamycin are mediated in part through transcriptional regulation of the genes encoding critical metabolic enzymes, we cannot exclude the possibility of additional mechanisms, as indicated by the inhibition of PKB phosphorylation. Some studies suggest that AMPK may have inhibitory effects on mTOR action [39,42]; and it may also be the case, conversely, that mTOR activation acts chronically to oppose the effects of AMPK activation. Interestingly, increased basal (fasting state) activation of mTOR and its downstream target p70S6K has been reported in liver and skeletal muscle of fat-fed obese rats [43]. Furthermore, a fasted hepatic milieu is associated with a decreased sensitivity of liver CPT I to malonyl-CoA in vivo [44]; and it is possible that such a mechanism could contribute to the increased β-oxidation flux in hepatocytes treated with rapamycin.

In summary, our experiments show for the first time that rapamycin has direct specific effects on hepatocyte lipid metabolism, consistent with the role of mTOR as an energy sensor. Rapamycin promotes β-oxidation while decreasing flux into anabolic storage pathways, suggesting that the hyperlipidemia associated with long-term rapamycin use in a clinical setting is likely not the result of enhanced hepatic synthesis, but rather of delayed peripheral clearance. However, these data are consistent with the notion that mTOR signaling is involved not only in energy sensing, but also in regulation of energy production from fatty acids. The mechanism involves specific modulation of the expression of gene products that regulate intermediary metabolism and inhibition of opposing signaling pathways.

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