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Increased Activation of the Mammalian Target of Rapamycin Pathway in Liver and Skeletal Muscle of Obese Rats: Possible Involvement in Obesity-Linked Insulin Resistance

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The mammalian target of rapamycin (mTOR) pathway integrates insulin and nutrient signaling in numerous cell types. Recent studies also suggest that this pathway negatively modulates insulin signaling to phosphatidylinositol 3-kinase/Akt in adipose and muscle cells. However, it is still unclear whether activation of the mTOR pathway is increased in obesity and if it could be involved in the promotion of insulin resistance. In this paper we show that basal (fasting state) activation of mTOR and its downstream target S6K1 is markedly elevated in liver and skeletal muscle of obese rats fed a high fat diet compared with chow-fed, lean controls. Time-course studies also revealed that mTOR and S6K1 activation by insulin was accelerated in tissues of obese rats, in association with increased inhibitory phosphorylation of insulin receptor substrate-1 (IRS-1) on Ser^{636}/Ser^{639} and impaired Akt activation. The relationship between mTOR/S6K1 overactivation and impaired insulin signaling to Akt was also examined in hepatic cells *in vitro*. Insulin caused a time-dependent activation of mTOR and S6K1 in HepG2 cells. This was associated with increased IRS-1 phosphorylation on Ser^{636}/Ser^{639}. Inhibition of mTOR/S6K1 by rapamycin blunted insulin-induced Ser^{636}/Ser^{639} phosphorylation of IRS-1, leading to a rapid (~5 min) and persistent increase in IRS-1-associated phosphatidylinositol 3-kinase activity and Akt phosphorylation. These results show that activation of the mTOR pathway is increased in liver and muscle of high fat-fed obese rats. *In vitro* studies with rapamycin suggest that mTOR/S6K1 overactivation contributes to elevated serine phosphorylation of IRS-1, leading to impaired insulin signaling to Akt in liver and muscle of this dietary model of obesity. (*Endocrinology* 146: 1473–1481, 2005)

THE MAMMALIAN TARGET of rapamycin (mTOR) is a nutrient sensor and a crucial checkpoint control for integrating growth factor signaling and cellular metabolism. The mTOR protein belongs to the family of phosphatidylinositol kinase-like kinases, which is a unique family of large proteins with Ser/Thr kinase activities (1–3). mTOR activates translation initiation in response to both hormonal and nutrient signals by phosphorylating at least two translational modulators, p70 ribosomal S6 kinase 1 (S6K1) and an inhibitor of translation initiation, eIF-4E-binding protein 1 (4–7). Both mTOR and S6K1 possess Ser/Thr kinase activities that are potently activated by insulin. Activation of mTOR and S6K1 by insulin is relayed through the insulin receptor/insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K)/Akt pathway (8, 9). Ser^{2448} in mTOR has been identified as a site phosphorylated by Akt *in vivo*, and this phosphorylation is indeed dependent upon PI3K (10). Moreover, it has recently been suggested that the heterodimer formed by the tumor suppressors TSC1 (hamartin) and TSC2 (tuberin) may be implicated in mTOR activation (11). TSC2 appears to be a direct Akt substrate and thus may represent a primary effector of Akt-mediated signaling to the mTOR/S6K1 pathway (11).

We (12) and others (13–16) previously showed that chronic activation of the mTOR/S6K1 pathway by insulin, amino acids, or TNF-α promotes insulin resistance in fat and muscle cells through increased IRS-1 serine phosphorylation and degradation, leading to impaired PI3K stimulation. In fact, activation of the mTOR/S6K1 pathway by insulin and amino acids speeds up the temporal deactivation of PI3K activity even before IRS-1 degradation is detectable in L6 myocytes (12). It was thus proposed that the mTOR/S6K1 pathway represents a physiological feedback mechanism that negatively modulates the ability of insulin to transmit signal to PI3K via IRS-1. Whether overactivation of the mTOR/S6K1 pathway may contribute to defective activation of PI3K and Akt in insulin target tissues of obese insulin-resistant animals remains to be investigated. Sun and colleagues (17, 18) recently reported increased serine kinase activity in liver and muscle of obese insulin-resistant rodents, leading to increased inhibitory serine phosphorylation of IRS-1, a recognized molecular mechanism of insulin resistance for glucose metabolism (19). The identity of the serine kinase(s) responsible for elevated serine phosphorylation of IRS-1 remains to be determined, but the kinase activity was found to principally target serine(s) located in the middle region (amino acid 526–859 region) of the IRS-1 molecule (17, 18).

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Abbreviations: HFHS, High fat, high sucrose; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene difluoride.

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In the present study we tested whether mTOR/S6K1 overactivation may be involved in the pathogenesis of hepatic insulin resistance in diet-induced obesity. Our results indicate that mTOR and S6K1 activation are increased in liver and skeletal muscle of high fat-fed, obese rats, implicating these Ser/Thr kinases as potential mediators of insulin resistance in this model. We show, using the mTOR inhibitor rapamycin, that insulin activation of mTOR and S6K1 inhibits insulin signaling to the PI3K/Akt pathway in hepatic cells, possibly from increased inhibitory phosphorylation of IRS-1 on Ser^{636}/Ser^{639}.

Materials and Methods

Materials

All cell culture solutions and supplements were purchased from Invitrogen Life Technologies, Inc. (Burlington, Canada), except for fetal bovine serum, which was purchased from Wisent (St. Bruno, Canada). Reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories, Inc. (Mississauga, Canada). Enhanced chemiluminescence was from NEN Life Science Products (Boston, MA). [γ-^{32}P]ATP, myo-[2-^{3}H]inositol, protein A- and G-Sepharose, and antimouse or antirabbit immunoglobulin G conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, Canada). Polyclonal antibodies against IRS-1 [raised against 20 C-terminal amino acids (C-20)], S6K1 (C-18), and Akt (C-20), S6K1 substrate, were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiphospho-specific antibodies against Akt (Ser^{473} and Thr^{308}), IRS-1 (Ser^{636}/Ser^{639}), and S6K1 (Thr^{421}/Ser^{424}) were from New England Biolabs (Beverly, MA). Antibodies against IRS-1, IRS-2, p85/PI3K, and Akt substrate (Crosstide) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Human insulin was obtained from Eli Lilly & Co. (Toronto, Canada). Rapamycin was purchased from Biomol (Plymouth Meeting, PA). L-α-Phosphatidylinositol was obtained from Avanti Polar Lipids (Alabaster, AL). Oxalate-treated thin layer chromatography silica gel H plates were obtained from Analtech (Newark, DE). All other chemicals were of the highest analytical grade.

Cell culture and treatment

Human HepG2 hepatoma cells (gift from Dr. Luc Bélanger, Hotel Dieu Hospital, Laval University, Québec, Canada) were grown and maintained in monolayer culture in DMEM containing 10% (vol/vol) fetal bovine serum and 1% (vol/vol) antibiotic/antimycotic solution (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B) in an atmosphere of 5% CO_2 at 37 C. Cells were deprived of serum 6 h before experimental treatments with or without rapamycin (25 nM) for 1 h and insulin (100 nM) for different times, as indicated in figure legends.

Animals

All experiments performed in this study were approved by the Laval University animal care and handling committee. Male Wistar rats (Charles River, Montréal, Canada), weighing 200–250 g at the beginning of the study, were housed individually in plastic cages in animal quarters maintained at 22 C with a 12-h dark, 12-h light schedule. Animals were fed either a low fat chow diet (Charles River rodent chow 5075, Ralston Purina, St. Louis, MO) or a purified high fat diet for 4 wk. The high fat diet was prepared in our laboratory as previously described (20). The composition of the high fat diet consisted of, as a percentage of total energy, 32.5% lard, 32.5% corn oil, 20% sucrose, and 15% protein, whereas the chow diet contained, as a percentage of total energy, 57.3% carbohydrate, 18.1% protein, and 4.5% fat. The high fat diet was supplemented with 1.4% vitamin mixture (Harlan Teklad, Madison, WI), 6.7% AIN-76 mineral mix, 0.2% choline bitartrate, and 0.004% butylated hydroxytoluene (ICN Nutritional Biochemicals, Cleveland, OH).

Whole body insulin sensitivity was determined in conscious overnight-fasted rats by performing an hyperinsulinemic-euglycemic clamp (insulin infused at 4 mU insulin/kg·min), as previously described (20). Other groups of chow and high fat-fed rats under ketamine/xylazine (87.5:12.5 mg/kg, ip) anesthesia were killed 5, 10, 15, 20, or 35 min after ip administration of insulin (0.075 U/g) or PBS as a control. In preliminary studies we determined that plasma insulin concentrations remain in the upper physiological range (>5 nM) throughout the 35-min period. Livers and extensor digitorum longus muscles were quickly excised and immediately frozen in liquid nitrogen.

Immunoprecipitation and immunoblotting

Protein extraction was performed in triplicate from livers, muscles, or HepG2 cells; extracts were prepared using buffer A (20 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and insoluble material was removed by centrifugation at 12,000 $\times$ *g*. Protein content was determined by the Lowry method using the DC reagent (Bio-Rad Laboratories, Inc., Richmond, CA). For direct Western blotting, protein extracts were resolved by SDS-PAGE. Where noted, proteins were immunoprecipitated (500 μg) after incubation with specific antibody (1–2 μg) or irrelevant immunoglobulin G for 18 h at 4 C. Immunocomplexes were collected, washed with buffer A three times, resuspended in Laemmli sample buffer, resolved in SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% nonfat dry milk in 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20; incubated with primary antibody; and then reincubated with a secondary horseradish-conjugated antibody. Immunoreactive bands were visualized by enhanced chemiluminescence. Quantification was achieved by laser densitometry of the bands.

PI3K assay

PI3K activity was measured in IRS-1 and IRS-2 immunoprecipitates as previously described (12). In brief, after experimental treatment, medium was removed, and cells were rinsed twice in ice-cold PBS and lysed in 20 mM Tris (pH 7.4), 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 2 mM Na_3VO_4, and 10 mM NaF. Five hundred micrograms of lysates were immunoprecipitated with 2 μg anti-IRS-1 or anti-IRS-2 coupled to protein A-Sepharose overnight at 4 C. After washing, immune complexes were resuspended in 70 μl kinase buffer [8 mM Tris (pH 7.5), 80 mM NaCl, 0.8 mM EDTA, 15 mM $MgCl_2$, 180 μM ATP, and 5 μCi [γ-^{32}P]ATP] and 10 μl sonicated phosphatidylinositol mixture [20 μg L-α-phosphatidylinositol, 10 mM Tris (pH 7.5), and 1 mM EGTA] for 15 min at 30 C. Reaction was stopped by the addition of 20 μl 8 M HCl, mixed with 160 μl $CHCl_3/CH_3OH$ (1:1), and centrifuged. The lower organic phase was spotted on an oxalate-treated silica gel thin layer chromatography plate and developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.6:2). The plate was dried and visualized by autoradiography with intensifying screen at −80 C.

Statistical analysis

Data are presented as the mean ± SE. The effects of the diet and rapamycin were analyzed by ANOVA. The effect of diet was analyzed by a 2 $\times$ 5 (standard diet/high fat, high sucrose (HFHS) diet and 0/5/10/20/35 min of insulin) factorial ANOVA. Likewise, the effect of rapamycin treatment was analyzed by a 2 $\times$ 4 (control/rapamycin and 0/5/15/30 min of insulin) factorial ANOVA. When justified by a significant treatment interaction, differences between individual group means were analyzed by Fisher's protected least squares difference test. Significance was accepted at $P < 0.05$.

Results

Increased activation of the mTOR pathway in liver and skeletal muscle of obese insulin-resistant animals

The mTOR pathway is thought to be an important regulator of insulin action on glucose metabolism. However, it is still unknown whether this pathway is altered in liver of obese animals, a main site of insulin resistance in obesity. We used the HFHS-fed rat, a well-established dietary model of

obesity to test this hypothesis. As expected, feeding a diet high in fat and sucrose for 4 wk caused obesity (16.6 ± 2.6 *vs.* 7.1 ± 1.0 g perirenal and epididymal fat for HFHS and chow-fed rats; $P < 0.01$) and marked whole body insulin resistance, as revealed by reduced glucose infusion rate during an hyperinsulinemic-euglycemic clamp (11.7 ± 1.6 *vs.* 30.9 ± 2.5 mg/kg·min for HFHS and chow-fed rats; $P < 0.01$). HFHS rats also exhibited fasting hyperinsulinemia (421 ± 65 *vs.* 122 ± 28 pM for HFHS and chow-fed rats; $P < 0.01$), another typical hallmark of insulin resistance.

HFHS-fed obese rats and their lean chow-fed controls were studied in the basal fasting state or after injection of insulin at the times indicated in Figs. 1-3. Protein extracts from livers of these animals were prepared and analyzed by Western blotting for the expression and phosphorylation levels of mTOR, S6K1, IRS-1, and Akt. mTOR and S6K1 phosphorylation on Ser^{2448} and Thr^{421}/Ser^{424}, respectively, was barely detectable in noninsulin-stimulated, chow-fed rats. Insulin injection rapidly increased the phosphorylation of both enzymes ($t_{1/2}$, ~10 min), reaching a 5-fold (mTOR) and approximately 20-fold (S6K1) phosphorylation at 35 min of insulin stimulation (Fig. 1, A–C). We next examined mTOR and S6K1 activation in liver of HFHS-fed obese insulin-resistant rats. Basal (noninsulin-stimulated) phosphorylation of both mTOR and S6K1 was markedly increased (4- to 5-fold) in liver of obese rats. Insulin-induced mTOR and S6K1 phosphorylation was also increased in the obese rats, but different patterns of activation were observed. Whereas mTOR phosphorylation in obese rat liver remained elevated compared with that in lean rats for up to 35 min of insulin stimulation, S6K1 phosphorylation was transiently increased at 5–10 min and then rapidly declined to basal levels within 35 min.

Activation of the mTOR pathway by insulin has been shown to increase phosphorylation of IRS-1 on Ser^{636}/Ser^{639} (21). This site is located in the close vicinity of a tyrosine-phosphorylated motifs of IRS-1 and is thought to reduce its interaction with PI3K (22, 23). We thus assessed whether the alterations in mTOR and S6K1 activation were associated with increased IRS-1 phosphorylation on Ser^{636}/Ser^{639} in liver of HFHS-fed obese rats. As depicted in Fig. 1, A and D, IRS-1 Ser^{636}/Ser^{639} phosphorylation was low in liver of chow-fed, fasting rats. Insulin stimulation increased IRS-1 Ser^{636}/Ser^{639} phosphorylation, peaking after 20–35 min of hormonal treatment, a time at which activation of both mTOR and S6K1 was prominent (Fig. 1, B and C). Basal IRS-1 Ser^{636}/Ser^{639} phosphorylation was increased by about 2.5-fold in liver of obese rats compared with lean, chow-fed controls. Interestingly, insulin-induced IRS-1 Ser^{636}/Ser^{639} phosphorylation was also increased, but only after 5 and 10 min of hormonal stimulation, suggesting that IRS-1 Ser^{636}/Ser^{639} phosphorylation is accelerated in insulin-stimulated liver of obese, insulin-resistant rats. IRS-1 protein expression was not reduced by obesity or by this acute insulin treatment

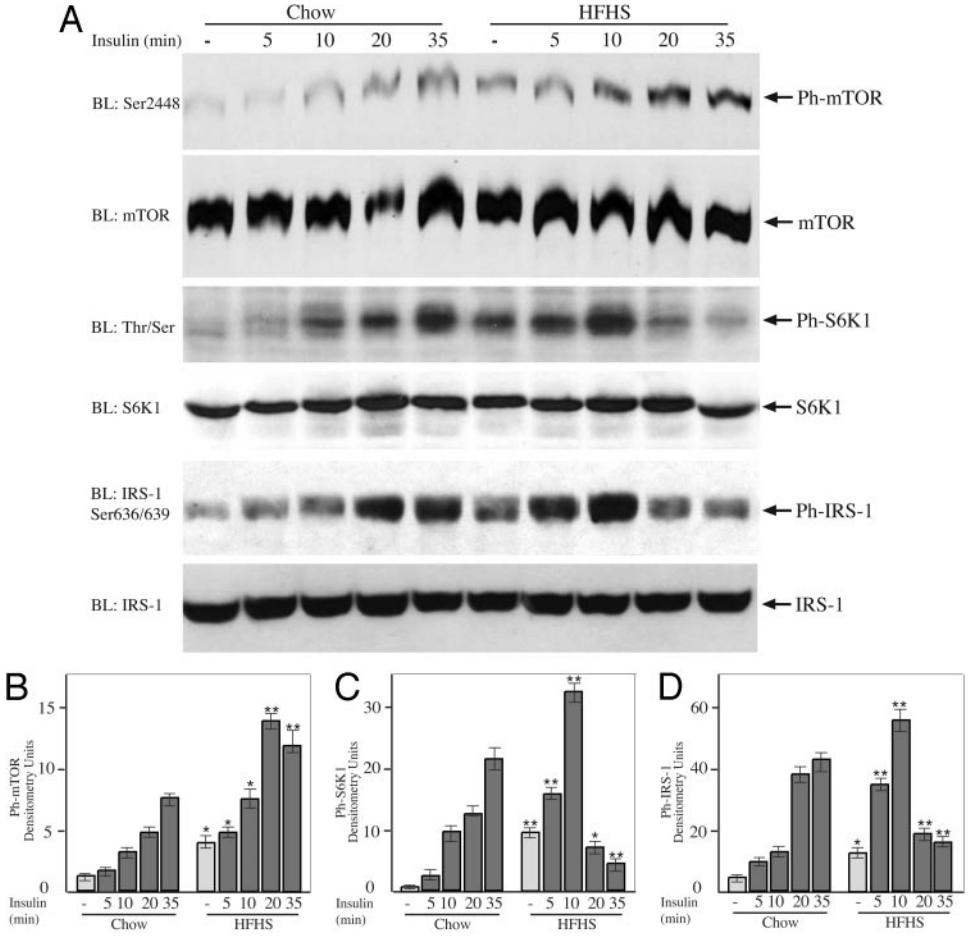

FIG. 1. Increased activation of the mTOR pathway is associated with elevated Ser^{636}/Ser^{639} IRS-1 phosphorylation in liver of obese rats. Male Wistar rats were fed either a low fat chow diet (Chow) or a HFHS for 4 wk. Livers were collected from fasting rats, as well as 5, 10, 20, or 35 min after ip administration of insulin or PBS (−). A, Homogenates were prepared, and equal amounts of protein were subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with antibodies to mTOR, mTOR-Ser^{2448}, S6K1, S6K1-Thr^{421}/Ser^{424}, IRS-1, or IRS-Ser^{636}/Ser^{639}. *Arrows* indicate the expected migration for these proteins. The figure shows representative Western blots that were replicated a total of three times in liver of each group of rats. B–D, The *graphs* depict densitometric analysis of the phosphorylation of mTOR-Ser^{2448}, S6K1-Thr^{421}/Ser^{424}, or Ser^{636}/Ser^{639} IRS-1 in three independent experiments performed in livers of lean or obese rats. Basal (PBS treatment; ▨) and insulin-induced (■) phosphorylation of mTOR, S6K1, and IRS-1 are shown. Results are expressed in this and subsequent figures as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ (*vs.* chow-fed rats at same time point).

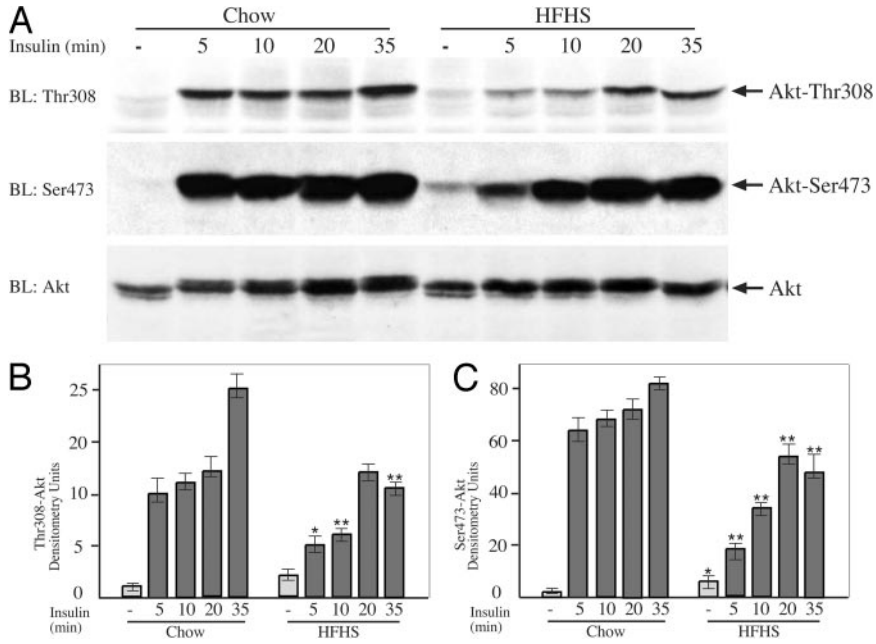

FIG. 2. Impaired Akt activation in liver of HFHS-fed, obese rats. Animal treatments were the same as described in Fig. 1. A, Homogenates were prepared, and equal amounts of protein were subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with antibodies against Akt, Akt-Thr^{308}, or Akt-Ser^{473}. *Arrows* indicate the expected migration of Akt. Shown are representative Western blots from three independent experiments. B and C, The *graphs* depict densitometric analysis of the phosphorylation of Akt on Thr^{308} and Ser^{473} in liver of lean or obese rats. Basal (▨) and insulin-induced (■) phosphorylation of Akt are shown. *, $P < 0.05$; **, $P < 0.01$ (*vs.* chow-fed rats at same time point).

in liver of chow- or HFHS-fed rats. The time course of insulin-mediated IRS-1 Ser^{636}/Ser^{639} phosphorylation was almost identical to that of S6K1 activation in liver of obese rats.

To evaluate the impact of increased activation of the mTOR pathway on insulin signaling to glucose metabolism, we next measured activation of Akt, a key effector of insulin-induced inhibition of glucose production (24) and of stimulation of glycogen synthesis (25). Basal Akt activation was slightly increased in liver of obese animals, and this reached the level of significance for the phosphorylation of Akt on Ser^{473}. Insulin rapidly and markedly increased Akt phosphorylation on both Thr^{308} and Ser^{473} residues in liver of chow-fed rats (Fig. 2). In contrast, insulin's ability to increase Akt phosphorylation was blunted in liver of obese rats, particularly at the 5- and 10-min points. Akt phosphorylation increased after 20–35 min of insulin injection in obese rats,

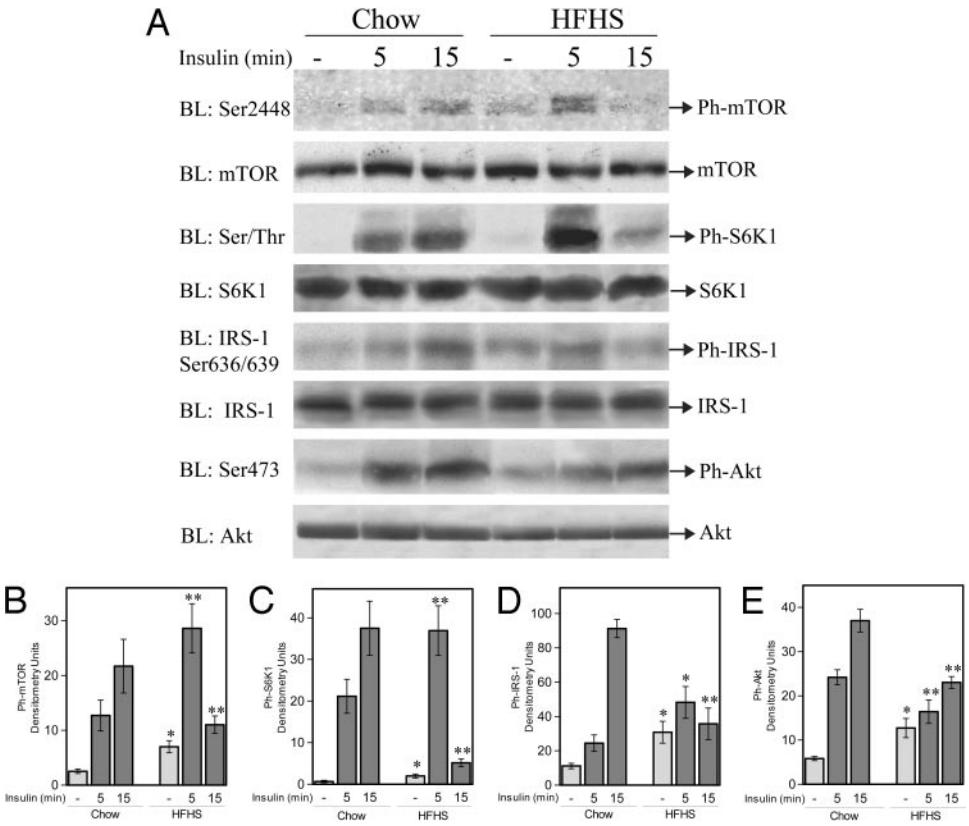

FIG. 3. Increased activation of the mTOR pathway is associated with elevated Ser^{636}/Ser^{639} IRS-1 phosphorylation and impaired Akt stimulation by insulin in skeletal muscle of obese rats. Male Wistar rats were fed either a low-fat chow diet (Chow) or a HFHS for 4 wk. Muscles were collected from fasting rats as well as 5 or 15 min after ip administration of insulin or PBS (−). A, Homogenates were prepared, and equal amounts of protein were subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with antibodies to mTOR, mTOR-Ser^{2448}, S6K1, S6K1-Thr^{421}/Ser^{424}, IRS-1, IRS-Ser^{636}/Ser^{639}, Akt, or Akt-Ser^{473}. *Arrows* indicate the expected migration for these proteins. The figure shows representative Western blots from four to six independent determinations. B–D, The *graphs* depict quantification of densitometric analysis from four to six lean or obese rats. Basal (PBS treatment; ▨) and insulin-induced (■) phosphorylation of mTOR, S6K1, IRS-1, and Akt are shown. *, $P < 0.05$; **, $P < 0.01$ (*vs.* chow-fed rats at same time point).

but it remained lower than that of liver from insulin-injected, chow-fed rats. It is interesting to note that insulin-induced Akt activation was more severely reduced at the same time (*i.e.* 5–10 min) that S6K1 activation and IRS-1 Ser^{636}/Ser^{639} phosphorylation were found to be markedly elevated in liver of obese rats.

We next tested whether activation of the mTOR/S6K1 pathway was also increased in skeletal muscle of HFHS-fed obese rats. As depicted in Fig. 3A and quantified in Fig. 3, B and C, basal (fasting state) phosphorylation of both mTOR and S6K1 was significantly increased (~2- to 3-fold; $P < 0.05$) in extensor digitorum longus muscle of obese rats. Moreover, phosphorylation of mTOR and S6K1 was significantly enhanced in muscle of obese rats injected for only 5 min with insulin, compared with chow-fed animals. However, the insulin-dependent activation of both enzymes declined to basal levels within 15 min in HFHS-fed obese, but not chow-fed, rats. The inhibitory phosphorylation of IRS-1 on Ser^{636}/Ser^{639} was increased in both the fasting state and 5 min after insulin injection in obese animals, but decreased to the basal level within 15 min of insulin stimulation (Fig. 3, A and D). Akt phosphorylation on Ser^{473} was also increased in obese fasted rats, but its activation by insulin was impaired both after 5 and 15 min of insulin stimulation (Fig. 3, A and E).

Insulin-induced mTOR/S6K1 activation causes IRS-1 Ser^{636}/Ser^{639} phosphorylation and inhibition of the PI3K/Akt pathway in hepatocytes

The data obtained in HFHS- and chow-fed rats indicate that the mTOR pathway is overactivated in association with increased IRS-1 Ser^{636}/Ser^{639} phosphorylation and impaired activation of Akt in liver and muscle of obese rats. Overactivation of the mTOR pathway may be explained by hyperinsulinemia, but several other humoral and local factors (*e.g.* TNF-α) may be implicated. Moreover, these *in vivo* data do not establish the causal relationship between mTOR/S6K1 activation and impaired insulin signaling to Akt. To precisely address these issues, we next performed studies in cultured hepatocytes (HepG2 cells) exposed to insulin and in the presence of rapamycin, a specific inhibitor of the mTOR pathway.

HepG2 cells were preincubated for 1 h with the mTOR inhibitor rapamycin (25 nM) or vehicle (0.01% dimethylsulfoxide) before insulin stimulation. Cells were stimulated with 100 nM insulin for 5, 15, and 30 min or were left untreated. As shown in Fig. 4, A and C, mTOR phosphorylation on Ser^{2448} was increased by insulin, reaching maximal activation (~4-fold) after 30 min of stimulation. A very similar pattern was observed for S6K1 phosphorylation on Ser^{424}/Thr^{421},

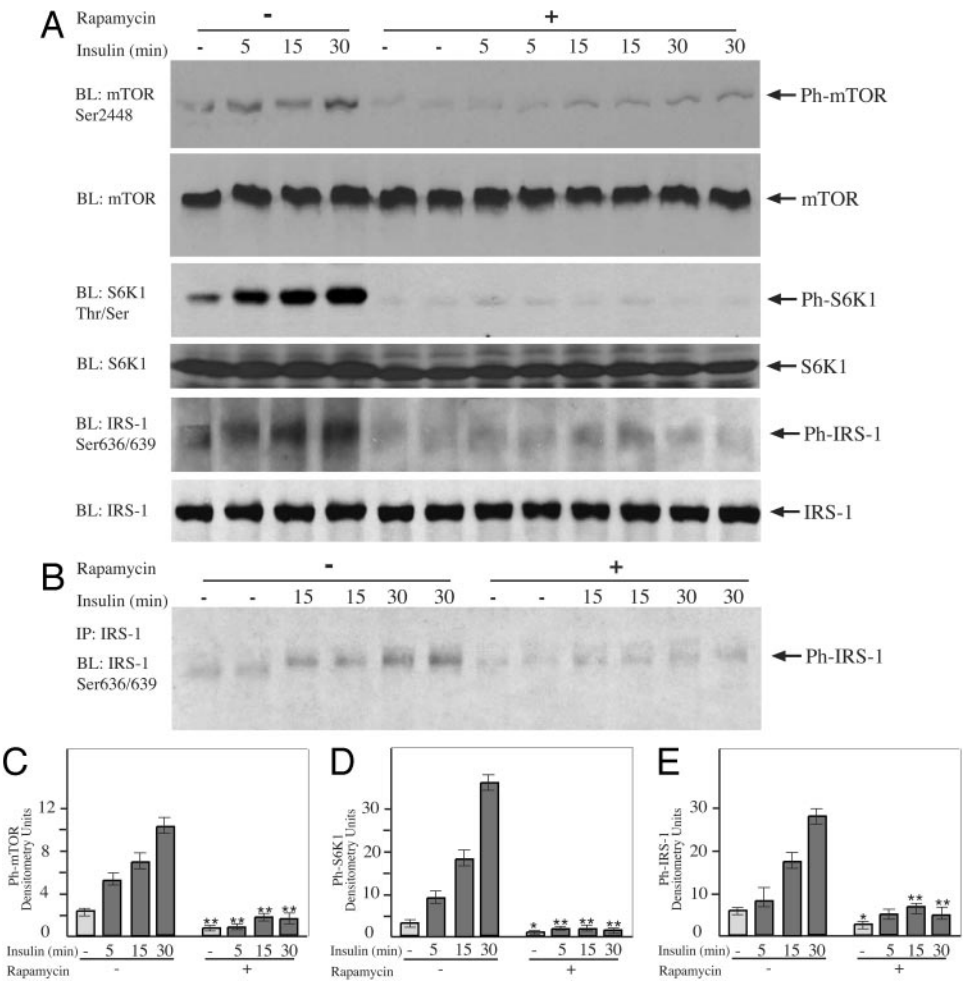

FIG. 4. Effects of insulin and rapamycin on mTOR, S6K1, and Ser^{636}/Ser^{639} IRS-1 phosphorylation in HepG2 cells *in vitro*. Lysates were prepared from HepG2 cells treated, or not, with 25 nM rapamycin for 1 h and stimulated with or without 100 nM insulin for 5, 15, and 30 min. A, Equal amounts of protein were subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with antibodies to mTOR, mTOR-Ser^{2448}, S6K1, S6K1-Thr^{421}/Ser^{424}, IRS-1, or IRS-Ser^{636}/Ser^{639}. *Arrows* indicate the expected migration of these proteins. The figure shows representative Western blots that were replicated a total of three times in three independent experiments in HepG2 cells. B, Equal amounts of proteins (300 μg) were immunoprecipitated with IRS-1 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with either IRS-Ser^{636}/Ser^{639} or IRS-1 antibody (data not shown). *Arrows* indicate the migration of IRS-1 phosphorylated on Ser^{636}/Ser^{639}. C–E, The *graphs* depict densitometric analysis of the phosphorylation of mTOR, S6K1, or IRS-1 in three independent experiments performed in HepG2 cells. Basal and insulin-induced levels of phosphorylation of mTOR, S6K1, and IRS-1 are shown as *light-colored bars* and *darker bars*, respectively. *, $P < 0.05$; **, $P < 0.01$ (*vs.* no rapamycin treatment at same time point).

which was increased by ~9-fold after 30 min of insulin stimulation (Fig. 4, A and D). Insulin also promoted IRS-1 phosphorylation on Ser^{636}/Ser^{639} in HepG2 cells, as detected by direct immunoblotting of cell lysates (Fig. 4A) or by probing IRS-1 immunoprecipitates (Fig. 4B). The time dependence of insulin-induced IRS-1 phosphorylation on Ser^{636}/Ser^{639} was very similar to that observed for activation of mTOR and S6K1 by the hormone (Fig. 4, compare C–E). As expected, the ability of insulin to activate mTOR and S6K1 was markedly reduced in cells preincubated with rapamycin (Fig. 4, A, C, and D), and this also blunted IRS-1 Ser^{636}/Ser^{639} phosphorylation (Fig. 4E), indicating that activation of the mTOR pathway is responsible for phosphorylation of this site in insulin-treated hepatocytes.

We have previously reported that inhibition of mTOR by rapamycin in L6 muscle cells maintains PI3K maximally activated for up to 30 min (12). To determine whether a similar phenomenon could be observed in liver cells, we next measured IRS-1- and IRS-2-associated PI3K activity in insulin-treated HepG2 cells. Insulin induced a transient increase in IRS-1-associated PI3K activity, reaching maximal activation at 5 min and then rapidly declining to basal levels after

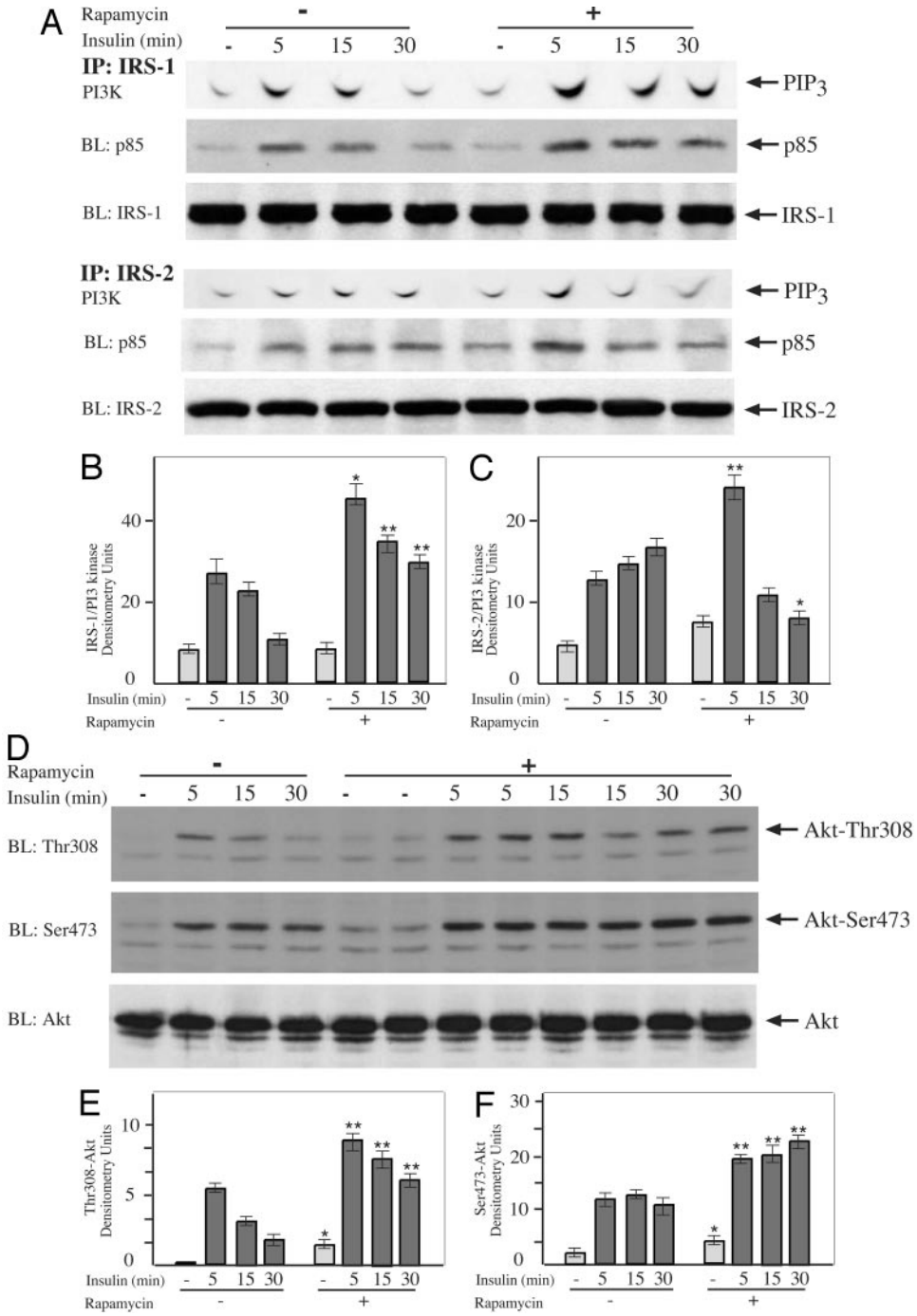

FIG. 5. Effects of insulin and rapamycin on IRS-1- and IRS-2-associated PI3K activity and Akt phosphorylation in HepG2 cells. Lysates were prepared from HepG2 cells treated, or not, with 25 nM rapamycin for 1 h and stimulated with or without 100 nM insulin for 5, 15, and 30 min. A, Equal amounts of protein (300 μg) were immunoprecipitated with IRS-1 or IRS-2 antibody. Immunoprecipitates were analyzed for PI3K activity. Representative autoradiograms are shown at the *top* of the figure. The *graphs* depict densitometric analysis of IRS-1 or IRS-2-associated PI3K activity from basal (▨) or insulin-stimulated (■) cells. All results shown in this figure are representative of three independent experiments. B, Equal amounts of protein were subjected to SDS-PAGE, transferred to a PVDF membrane, and then immunoblotted with antibodies against Akt, Akt-Thr^{308}, or Akt-Ser^{473}. *Arrows* indicate the expected migration of phosphorylated and total Akt. The figure shows representative Western blots that were replicated a total of three times in three independent experiments in HepG2 cells. C and D, The *graphs* depict densitometric analysis of Thr^{308} or Ser^{473} Akt phosphorylation in three independent experiments in HepG2 cells. Basal (▨) and insulin-mediated (■) phosphorylation of Akt are shown. *, $P < 0.05$; **, $P < 0.01$ (*vs.* no rapamycin treatment at same time point).

30 min (Fig. 5, A and B). Rapamycin pretreatment increased insulin-stimulated IRS-1-associated P13K and retarded its time-dependent deactivation. IRS-2-associated PI3K activity was also increased by insulin, but remained elevated throughout the 30-min period (Fig. 5, A and C). Rapamycin treatment also enhanced IRS-2-associated PI3K at 5 min, but not at 15 and 30 min. This suggests that inhibition of the mTOR pathway speeds up IRS-2-associated PI3K, but that this effect is transient.

We next assessed the activation of the PI3K downstream target Akt in rapamycin-treated cells, as revealed by its phosphorylation on Thr^{308} and Ser^{473} residues. Insulin induced phosphorylation of both sites in HepG2 cells (Fig. 5D). Whereas insulin only transiently increased Thr^{308} phosphorylation (Fig. 5E), the hormone effect was maintained for up to 30 min on Ser^{473} (Fig. 5F). Rapamycin pretreatment increased insulin-stimulated Akt phosphorylation on both sites and slowed down the temporal dephosphorylation of Thr^{308}. Thus, inhibition of the mTOR pathway by rapamycin increased insulin signaling to Akt in HepG2 cells.

Discussion

The present study demonstrates that the mTOR pathway is overactivated in liver and muscle of obese HFHS rats. Indeed, both mTOR and S6K1 are hyperphosphorylated in tissues of obese rats, even when assessed in the fasting state. The elevated phosphorylation of Ser^{2448} in mTOR in tissues of fasted obese rats may be explained by hyperinsulinemia, because it is well known that insulin induces phosphorylation of this site (8, 10, 26). Ser^{2448} is a direct target of Akt, and the slight increase in basal Akt phosphorylation in liver and muscle of obese rats may thus contribute to elevated mTOR-Ser^{2448} phosphorylation. Interestingly, mTOR can also be activated by TNF-α (15), which is elevated in HFHS-fed obese rats (20). Finally, Ser^{2448} in mTOR can also be phosphorylated by amino acids (8, 27) through a mechanism independent from Akt activation, and this could also contribute to mTOR phosphorylation, because circulating amino acid levels are increased in obesity (28).

Time-course experiments also revealed that mTOR and S6K1 are both rapidly phosphorylated in liver and muscle after insulin injection of obese animals, compared with lean chow-fed controls. In liver, S6K1 activation by insulin was only increased at early time points (5–10 min) despite the fact that mTOR-Ser^{2448} hyperphosphorylation was maintained for up to 35 min in obese animals. This apparent discrepancy between activation of mTOR and its downstream target S6K1 at the late time points of insulin injection may be explained by compensatory activation of Ser/Thr phosphatases that selectively target S6K1. Alternatively, it may indicate differential modulation of mTOR and S6K1 activity in obesity. Indeed, Ser^{2448} in mTOR can also be phosphorylated by glucagon and cAMP, but this is associated with inhibition of insulin-induced S6K1 activity in rat hepatocytes (26). Thus, glucagon and/or other cAMP-enhancing pathways may explain the persistent increase in mTOR-Ser^{2448} phosphorylation despite the time-dependent loss of S6K1 phosphorylation in liver of obese rats.

Our studies also strongly suggest that mTOR and/or S6K1 contribute to the pathogenesis of insulin resistance in liver and muscle tissues. Increased phosphorylation of IRS-1 on serine residues is considered a molecular hallmark of insulin resistance in insulin target tissues (19). *In vitro* studies suggest that both mTOR and S6K1 can phosphorylate multiple serines in IRS-1 (12, 14–16, 29–32). More recently, Gual *et al.* (21) confirmed that Ser^{636}/Ser^{639} is a target of the mTOR pathway *in vivo*, because rapamycin treatment fully blocked phosphorylation of this site in muscle and fat of normal mice. However, the contribution of the mTOR pathway to phosphorylation of Ser^{636}/Ser^{639} in IRS-1 remains to be established in obese insulin-resistant animals. We found that in liver of obese rats, elevated IRS-1 phosphorylation on Ser^{636}/Ser^{639} and impaired Akt activation by insulin were evident at early time points, suggesting that S6K1, and not mTOR directly, may be more linked with hepatic insulin resistance in obesity. In muscle, however, the time course of overactivation of mTOR and S6K1 were similar and paralleled the elevated IRS-1 Ser^{636}/Ser^{639} phosphorylation in HFHS-fed obese rats. The marked decline in S6K1 activity in muscle of obese rats 15 min after insulin injection is in agreement with a previous study showing a lack of insulin activation of S6K1 in skeletal muscle of gold thioglucose-treated, obese mice compared with lean mice (33). Indeed, S6K1 activity was measured after 15 min of insulin stimulation in the latter study, a time at which overactivation of S6K1 is no longer apparent in the obese rat model. Importantly, a role for S6K1 as a primary inducer of inhibitory serine phosphorylation of IRS-1 and insulin resistance in obesity is also supported by the recent observation that S6K1 knockout mice are more resistant to diet-induced obesity and more insulin sensitive than wild-type mice (34).

Our data for liver of obese rats suggest a role for the overactivation of the mTOR pathway in the development of insulin resistance in liver of obese rats. However, the connection between the mTOR pathway and the phosphorylation of Ser^{636}/Ser^{639} in IRS-1 in obese animals is correlative, because multiple kinases may increase phosphorylation of this site (see below). In contrast, such a connection is supported by our *in vitro* studies in HepG2 cells, showing that inhibition of mTOR/S6K1 by rapamycin reverses insulin-mediated IRS-1 phosphorylation on Ser^{636}/Ser^{639} while increasing Akt activation by insulin. Rapamycin is a very selective inhibitor of the mTOR pathway, with no inhibitory activity toward other known kinases even at concentrations in the high nanomolar range (35). These *in vitro* studies also suggest that hyperinsulinemia contributes to mTOR/S6K1 overactivation in the fasting state and probably in the postprandial state, because glucose-induced insulin secretion is increased by up to 2-fold in HFHS-fed, obese rats compared with chow-fed controls (Dombrowski, L., and A. Marette, unpublished observations). These studies in liver cells are in line with recent studies by Gual *et al.* (21), showing that rapamycin blunted phosphorylation of IRS-1 on Ser^{632} (Ser^{636} in humans) by prolonged insulin treatment of 3T3-L1 adipocytes as well as fat and muscle tissues of normal mice *in vivo*.

Although evidence is accumulating about the potential role of the mTOR/S6K1 pathway in promoting inhibitory serine phosphorylation of IRS-1 and insulin resistance in

obesity, other pathways are also likely to contribute to this pathogenic mechanism. Thus, insulin has been recently shown to acutely promote phosphorylation of Ser^{612} and Ser^{632}/Ser^{636} through a MAPK-dependent pathway in 3T3-L1 adipocytes (21). Moreover, cultured muscle cells from type 2 diabetic subjects have increased MAPK (extracellular signal regulated kinase-1 and -2) activity and the MAPK inhibitor PD98059 blunted IRS-1 phosphorylation on Ser^{636} in these cells (29). Interestingly, both Ser^{612} and Ser^{632}/Ser^{636} phosphorylation by insulin were found to be acutely dependent on MAPK (within minutes), but were mostly linked to mTOR activation upon prolonged insulin treatment (~1 h) (21) in normal lean mice. Thus, our finding of an accelerated activation of mTOR and S6K1 phosphorylation by insulin in obese animals suggest that this pathway may be more rapidly promoting Ser^{636}/Ser^{639} phosphorylation in obese animals. Other kinases link to inflammatory pathways are also thought to be responsible for IRS-1 serine phosphorylation in obesity. Thus, Ser^{307} in IRS-1 has been reported to be targeted by the c-Jun N-terminal kinase (JNK) and inhibitor κB kinase (36–38), which are both increased in obesity (38, 39). Interestingly, an unidentified kinase was recently shown to phosphorylate Ser^{789} in IRS-1 in liver of obese rats (18). It will be interesting to see whether this kinase, which is neither JNK nor extracellular signal-regulated kinase, turns out to be mTOR or S6K1, which we show here to be overactivated in liver of obese rats.

In summary, this study shows that the activation of mTOR and S6K1 is increased in liver and skeletal muscle of high fat-fed, obese rats. We demonstrate, using rapamycin *in vitro*, that mTOR/S6K1 activation by insulin increases Ser^{636}/Ser^{639} phosphorylation of IRS-1, leading to impaired PI3K/Akt activation in liver cells. Taken together, these results strongly suggest that mTOR and/or S6K1 are potential mediators of elevated inhibitory phosphorylation of IRS-1 on Ser^{636}/Ser^{639} and tissue insulin resistance in obesity.

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References

1. **Schmelzle T, Hall MN** 2000 TOR, a central controller of cell growth. Cell 103:253–262
2. **Raught B, Gingras AC, Sonenberg N** 2001 The target of rapamycin (TOR) proteins. Proc Natl Acad Sci USA 98:7037–7044
3. **Rohde J, Heitman J, Cardenas ME** 2001 The TOR kinases link nutrient sensing to cell growth. J Biol Chem 276:9583–9586
4. **Brunn GJ, Fadden P, Haystead TAJ, Lawrence Jr JC** 1997 The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH terminus. J Biol Chem 272:32547–32550
5. **Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM** 1998 RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci USA 95:1432–1437
6. **Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, Kasuga M, Nishimoto I, Avruch J** 1997 Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem 272:26457–26463
7. **Isotani S, Hara K, Tokunaga C, Inoue H, Avruch J, Yonezawa K** 1999 Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase α in vitro. J Biol Chem 274:34493–34498
8. **Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR** 1999 Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J 344:427–431
9. **Sekulic A, Hudson CC, Homme JL, Yin P, Otterness DM, Karnitz LM, Abraham RT** 2000 A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res 60:3504–3513
10. **Scott PH, Brunn GJ, Kohn AD, Roth RA, Lawrence Jr JC** 1998 Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. Proc Natl Acad Sci USA 95:7772–7777
11. **Shamji AF, Nghiem P, Schreiber SL** 2003 Integration of growth factor and nutrient signaling: implications for cancer biology. Mol Cell 12:271–280
12. **Tremblay F, Marette A** 2001 Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem 276:38052–38060
13. **Berg CE, Lavan BE, Rondinone CM** 2002 Rapamycin partially prevents insulin resistance induced by chronic insulin treatment. Biochem Biophys Res Commun 293:1021–1027
14. **Haruta T, Uno T, Kawahara J, Takano A, Egawa K, Sharma PM, Olefsky JM, Kobayashi M** 2000 A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol 14:783–794
15. **Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB** 2001 A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci USA 98:4640–4645
16. **Takano A, Usui I, Haruta T, Kawahara J, Uno T, Iwata M, Kobayashi M** 2001 Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol Cell Biol 21:5050–5062
17. **Qiao LY, Goldberg JL, Russell JC, Sun XJ** 1999 Identification of enhanced serine kinase activity in insulin resistance. J Biol Chem 274:10625–10632
18. **Qiao LY, Zhande R, Jetton TL, Zhou G, Sun XJ** 2002 In vivo phosphorylation of insulin receptor substrate 1 at serine 789 by a novel serine kinase in insulin-resistant rodents. J Biol Chem 277:26530–26539
19. **Zick Y** 2003 Role of Ser/Thr kinases in the uncoupling of insulin signaling. Int J Obes Relat Metab Disord 27(Suppl 3):S56–S60
20. **Lavigne C, Tremblay F, Asselin G, Jacques H, Marette A** 2001 Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. Am J Physiol 281:E62–E71
21. **Gual P, Gremeaux T, Gonzalez T, Le Marchand-Brustel Y, Tanti JF** 2003 MAP kinases and mTOR mediate insulin-induced phosphorylation of insulin receptor substrate-1 on serine residues 307, 612 and 632. Diabetologia 46:1532–1542
22. **Esposito DL, Li Y, Cama A, Quon MJ** 2001 Tyr^{612} and Tyr^{632} in human insulin receptor substrate-1 are important for full activation of insulin-stimulated phosphatidylinositol 3-kinase activity and translocation of GLUT4 in adipose cells. Endocrinology 142:2833–2840
23. **Mothe I, Van Obberghen E** 1996 Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662, and 731, modulates insulin action. J Biol Chem 271:11222–11227
24. **Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ** 2001 Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ). Science 292:1728–1731
25. **Peak M, Rochford JJ, Borthwick AC, Yeaman SJ, Agius L** 1998 Signalling pathways involved in the stimulation of glycogen synthesis by insulin in rat hepatocytes. Diabetologia 41:16–25
26. **Mothe-Satney I, Gautier N, Hinault C, Lawrence Jr JC, Van Obberghen E** 2004 In rat hepatocytes glucagon increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids. J Biol Chem 279:42628–42637
27. **Reynolds THt, Bodine SC, Lawrence Jr JC** 2002 Control of Ser^{2448} phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. J Biol Chem 277:17657–17662
28. **Felig P, Wahren J, Hendler R, Brundin T** 1974 Splanchnic glucose and amino acid metabolism in obesity. J Clin Invest 53:582–590
29. **Bouzakri K, Roques M, Gual P, Espinosa S, Guebre-Egziabher F, Riou JP,**

Laville M, Le Marchand-Brustel Y, Tanti JF, Vidal H 2003 Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 52:1319–1325
30. **Carlson CJ, White MF, Rondinone CM** 2004 Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. Biochem Biophys Res Commun 316:533–539
31. **Li J, DeFea K, Roth RA** 1999 Modulation of insulin receptor substrate-1 tyrosine phosphorylation by an Akt/phosphatidylinositol 3-kinase pathway. J Biol Chem 274:9351–9356
32. **Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RF** 2004 The TSC1–2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 166:213–223
33. **Chang PY, Le Marchand-Brustel Y, Cheatham LA, Moller DE** 1995 Insulin stimulation of mitogen-activated protein kinase, $p90^{rsk}$, and p70 S6 kinase in skeletal muscle of normal and insulin-resistant mice. Implications for the regulation of glycogen synthase. J Biol Chem 270:29928–29935
34. **Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G** 2004 Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 431:200–205
35. **Davies SP, Reddy H, Caivano M, Cohen P** 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95–105
36. **Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF** 2002 Phosphorylation of Ser^{307} in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem 277:1531–1537
37. **Gao Z, Zuberi A, Quon MJ, Dong Z, Ye J** 2003 Aspirin inhibits serine phosphorylation of insulin receptor substrate 1 in tumor necrosis factor-treated cells through targeting multiple serine kinases. J Biol Chem 278:24944–24950
38. **Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS** 2002 A central role for JNK in obesity and insulin resistance. Nature 420:333–336
39. **Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE** 2001 Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of inhibitor κB kinase β. Science 293:1673–1677

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