# mTOR Inhibition by Rapamycin Prevents β-Cell Adaptation to Hyperglycemia and Exacerbates the Metabolic State in Type 2 Diabetes

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**OBJECTIVE**—Mammalian target of rapamycin (mTOR) and its downstream target S6 kinase 1 (S6K1) mediate nutrient-induced insulin resistance by downregulating insulin receptor substrate proteins with subsequent reduced Akt phosphorylation. Therefore, mTOR/S6K1 inhibition could become a therapeutic strategy in insulin-resistant states, including type 2 diabetes. We tested this hypothesis in the *Psammomys obesus* (*P. obesus*) model of nutrition-dependent type 2 diabetes, using the mTOR inhibitor rapamycin.

**RESEARCH DESIGN AND METHODS**—Normoglycemic and diabetic *P. obesus* were treated with 0.2 mg · $kg^{-1}$ · $day^{-1}$ i.p. rapamycin or vehicle, and the effects on insulin signaling in muscle, liver and islets, and on different metabolic parameters were analyzed.

**RESULTS**—Unexpectedly, rapamycin worsened hyperglycemia in diabetic *P. obesus* without affecting glycemia in normoglycemic controls. There was a 10-fold increase of serum insulin in diabetic *P. obesus* compared with controls; rapamycin completely abolished this increase. This was accompanied by weight loss and a robust increase of serum lipids and ketone bodies. Rapamycin decreased muscle insulin sensitivity paralleled by increased glycogen synthase kinase 3β activity. In diabetic animals, rapamycin reduced β-cell mass by 50% through increased apoptosis. Rapamycin increased the stress-responsive c-Jun $NH_2$-terminal kinase pathway in muscle and islets, which could account for its effect on insulin resistance and β-cell apoptosis. Moreover, glucose-stimulated insulin secretion and biosynthesis were impaired in islets treated with rapamycin.

**CONCLUSIONS**—Rapamycin induces fulminant diabetes by increasing insulin resistance and reducing β-cell function and mass. These findings emphasize the essential role of mTOR/S6K1 in orchestrating β-cell adaptation to hyperglycemia in type 2 diabetes. It is likely that treatments based on mTOR inhibition will cause exacerbation of diabetes. ***Diabetes* 57:945–957, 2008**

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FFA, free fatty acid; IRS, insulin receptor substrate; JNK, c-Jun $NH_2$-terminal kinase; KRBH, Krebs-Ringer bicarbonate HEPES buffer; mTOR, mammalian target of rapamycin; S6K1, S6 kinase 1; SAPK, stress-activated protein kinase; TORC1, rapamycin-sensitive complex with the regulatory-associated protein of mTOR (raptor); TORC2, rapamycin-resistant complex with rapamycin-insensitive companion of mTOR (rictor); TUNEL, transferase-mediated dUTP nick-end labeling.

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Type 2 diabetes occurs in obese subjects when pancreatic β-cells fail to increase insulin secretion in response to insulin resistance and nutrient overload (1). Recent data (2–4) suggest that the nutrient sensor mammalian target of rapamycin (mTOR) mediates the nutrient effects on insulin resistance. mTOR integrates signals from growth factors, hormones, nutrients, and cellular energy levels to regulate protein translation and cell growth, proliferation, and survival (3). mTOR exists in two distinct complexes, a rapamycin-sensitive complex, with the regulatory-associated protein of mTOR (raptor) mTOR (raptor) (TORC1), and a rapamycin-resistant complex with rapamycin-insensitive companion of mTOR (rictor) (TORC2) (5–8). TORC1 induces phosphorylation of ribosomal S6 kinase 1 (S6K1) and of the translation inhibitor factor eukaryotic translation initiation factor 4E binding protein (4EBP1), which regulate cellular responses via downstream effectors, such as eukaryotic translation initiation factor 4B (eIF4B), eukaryotic elongation factor 2 kinase (eEF2k), and S6 (9,10).

mTOR stimulation by insulin and growth factors is relayed through the canonical phosphatidylinositol 3-kinase–Akt pathway (11). However, there is ample evidence that mTOR/S6K1 signaling generates a negative-feedback loop that reduces insulin sensitivity (4). TORC1/S6K1 activation augments insulin receptor substrate (IRS) 1 phosphorylation at Ser307(302) and Ser636/639, leading to increased IRS1 degradation (12,13). In vitro studies (14–16) have shown that treatment with the TORC1 inhibitor rapamycin or S6K1 RNAi knockdown increased insulin-induced Akt phosphorylation. Moreover, S6K1 knockout mice and knockin mice resistant to S6 phosphorylation present increased insulin sensitivity (16,17). Several studies showed elevated S6K1 with reduced Akt activity in obese insulin-resistant rodents, suggesting that overactivation of mTOR/S6K1 is responsible for impaired insulin action (16,18). This was substantiated in humans by demonstrating that mTOR/S6K1 activation by amino acid infusion caused insulin resistance, prevented by rapamycin (19,20).

mTOR/S6K1 regulates IRS2 in a similar manner (15). IRS2 is the most abundant and functionally important IRS family member in the β-cell (21). In vitro and in vivo studies (22–24) have shown that increased IRS2 expres-

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sion promotes β-cell growth, proliferation, and survival, whereas its reduction causes β-cell apoptosis (25). Recently, it was shown that chronic mTOR activation by glucose or IGF-1 in INS-1 β-cells led to increased serine phosphorylation of IRS2, targeting it for proteosomal degradation. This resulted in increased β-cell apoptosis, which was prevented by rapamycin (24).

These studies (3,4) underlie the hypothesis that TORC1 could become a therapeutic target in insulin-resistant states. We tested this hypothesis in the *Psammomys obesus* (*P. obesus*) model of diet-induced type 2 diabetes. Changing from its natural low-energy diet to a calorie-rich laboratory diet, *P. obesus* develops moderate obesity with increased insulin resistance and postprandial hyperglycemia (26). The effects of TORC1 inhibition on the development and progression of type 2 diabetes were studied in normoglycemic and diabetic *P. obesus*.

## RESEARCH DESIGN AND METHODS

Diabetes-prone, 2.5- to 3.5-month-old male *P. obesus* (Hebrew University Colony; Harlan, Jerusalem, Israel) were fed the LE normoglycemia-maintaining diet (2.38 kcal/g; Koffolk, Petach-Tikva, Israel). Diabetes, defined by random blood glucose levels >8.3 mmol/l, was induced by feeding a high-energy diet (3.4 kcal/g, cat. no. 2018; Teklad Global Diets, Boston, MA) (26,27). Animal use was approved by the institutional animal care and use committee of the Hebrew University and the Hadassah Medical Organization.

*P. obesus* were treated by a daily intraperitoneal injection of 0.2 mg/kg rapamycin (Sigma, Rehovot, Israel) or the vehicle (0.4% DMSO in saline). Animals had free access to food and water. Body weight, food intake, and tail blood glucose (Accutrend Sensor; Roche Diagnostics, Mannheim, Germany) were monitored periodically. A subgroup of normoglycemic animals underwent an intraperitoneal glucose tolerance test after a 4-h fast by injecting 1.5 g/kg glucose i.p.

At termination of the studies, animals anesthetized with Ketalar (Parke-Davis, Gwent, U.K.) were exsanguinated by cardiac puncture. The gastrocnemius muscle, liver, and epididymal fat pads were rapidly removed, frozen in liquid nitrogen, and stored at −80°C for subsequent analysis. The head of the pancreas was used for determination of insulin content by radioimmunoassay (28) and the tail part immersion-fixed in 10% formalin for morphometric analysis. Serum was stored at −20°C for analysis of insulin, triglycerides (GPO-Trinder assay; Sigma), nonesterified fatty acids (Half-Micro test; Roche Diagnostics), and β-hydroxybutyrate according to Hansen and Freier (29).

**Insulin sensitivity.** Insulin sensitivity was studied after a 48-h rapamycin treatment and a 4-h fast. An insulin tolerance test was performed with 2.5 and 5 units/kg insulin i.p. (Humulin R; Eli Lilly, Fegersheim, France) in normoglycemic and diabetic *P. obesus*, respectively. Tail blood glucose was measured before and after insulin injection. Insulin signaling was studied by Western blot in tissues harvested from 4-h fasted animals 10 min after intraperitoneal insulin injection (5 units/kg).

**Islet isolation and culture for in vitro studies.** Islets were isolated by collagenase digestion (Collagenase P; Roche Diagnostics), as described (30). Islets were either incubated in suspension for 48 h or cultured on extracellular matrix–coated plates for 96 h at 3.3 or 16.7 mmol/l glucose and 50 nmol/l rapamycin or its vehicle. Islet extracts were used for Western blot analysis. The effects of rapamycin on insulin secretion, proinsulin biosynthesis, and total protein synthesis were studied after the 96-h treatment.

**Insulin secretion and proinsulin biosynthesis.** Groups of 25 islets were cultured on extracellular matrix—coated plates with the different treatments, as indicated. After 96 h, the islets were preincubated for 30 min at 37°C followed by 1-h incubation in 1 ml of modified Krebs-Ringer bicarbonate buffer containing 20 mmol/l HEPES (KRBH) and 0.25% BSA (KRBH-BSA), supplemented with glucose and rapamycin according to the experimental protocols. Medium was collected at the end of the 1-h incubation, centrifuged, and frozen at −20°C, pending insulin assay.

Proinsulin biosynthesis was studied by labeling islets in the center of the plate in 25 μl fresh KRBH-BSA buffer containing glucose and rapamycin and 25 μCi L-[2,3,4,5-$^{3}$H]leucine (120 Ci/mmol; ARC, St. Louis, MO). After a 15-min pulse at 37°C, leucine incorporation was terminated by ice-cold glucose-free KRBH-BSA buffer. Islets were then scrapped and centrifuged and the pellet subjected to immunoprecipitation with anti-insulin serum (Sigma) (31). Total protein biosynthesis was determined by trichloroacetic acid precipitation (32).

**Immunohistochemical studies.** Immunohistochemistry was performed on consecutive sections (7 μm) of paraffin-embedded pancreatic tissue (33). Cell proliferation was assessed by Ki-67 immunostaining using mouse anti-human Ki-67 antibody (1:100; Dako, Glostrup Denmark) and guinea pig anti-porcine insulin (1:500; ICN, Irvine, CA). Ki-67 immunoreactivity was detected by the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector, Burlingame, CA) developed with the DAB/Ni peroxidase substrate kit (Vector); peroxidase-conjugated rabbit anti-guinea pig IgG (1:100, Dako) developed with the DAB peroxidase substrate kit (Vector) was used for insulin. Slides were counterstained with hematoxylin. The proliferation index (percent of Ki-67–positive β-cells) was determined by counting ∼1,500 β-cells per pancreas.

Apoptosis was assessed by the transferase-mediated dUTP nick-end labeling (TUNEL) reaction (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA). Detection was by the DAB peroxidase substrate kit (Vector). Insulin immunoreactivity was determined using an alkaline phosphatase-conjugated goat anti-guinea pig IgG (1:100; Jackson ImmunoResearch, Suffolk, U.K.) developed with an alkaline phosphatase substrate kit I (Vector Red; Vector). The β-cell apoptotic index (percent of TUNEL-positive β-cells) was evaluated by analyzing ∼1,500 β-cells per pancreas.

Insulin-stained sections were used for morphometric analysis. Islet cells whose staining ranged from very light to dark brown were considered insulin positive; thus, even highly degranulated cells were included in the calculation of β-cell mass. Quantitative evaluation was performed using the Histolab 6.0.5 software (Microvision Instruments, Evry, France). β-Cell mass was calculated by multiplying the relative β-cell area by the pancreas weight (33).

**Immunoprecipitation and Western blot analysis.** IRS1 tyrosine phosphorylation was studied by immunoprecipitation followed by phosphotyrosine immunoblotting. Expression and site-specific phosphorylation of IRS1 and other proteins were studied by Western blot. X-ray film densitometry was used for quantification (ImageMaster VDS-CL; Amersham Pharmacia Biotech, Cardiff, U.K.).

**Antibodies.** Polyclonal rabbit antibodies (Cell Signaling Technology, Beverly, MA) against the following proteins were used: p70S6 kinase, phospho-p70S6 kinase (Thr389), Akt, phospho-Akt (Ser473), S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), IRS1, phospho-IRS1 (Ser636/639), phospho-IRS1 (Ser302), IRS2, phosphor stress-activated protein kinase (SAPK)/c-Jun, $NH_2$-terminal kinase (JNK) (Thr183/Tyr185), SAPK/JNK, and phospho-GSK3β (Ser9). Mouse antibody against GSK3α and -β was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for IRS1 immunoprecipitation and anti-phosphotyrosine antibody were from Upstate (Lake Placid, NY). Peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA).

**Data presentation and statistical analysis.** Data are shown as means ± SE. Samples were compared by the nonparametric Mann-Whitney *U* test. Multiple groups were compared using ANOVA followed by a Newman-Keuls test. A *P* value <0.05 was considered significant.

## RESULTS

**Metabolic effects of rapamycin in normoglycemic and diabetic *P. obesus*.** Nonfasted blood glucose and body weight in normoglycemic *P. obesus* on the low-energy diet did not change significantly when treated with rapamycin for 16 days (Fig. 1*A* and *B*). Rapamycin did not affect food intake, epididymal fat pad mass, pancreatic insulin content, serum triglycerides, free fatty acids (FFAs), and ketone bodies in normoglycemic animals (Fig. 1*C*, *E–G* and online appendix Table 1 [available at http://dx.doi.org/10.2337/db07-0922]). Serum insulin was elevated in the rapamycin group (870.1 ± 99.5 vs. 511.5 ± 86.6 pmol/l in control animals; $P = 0.015$) (Fig. 1*D*). Intraperitoneal glucose tolerance tests showed that rapamycin-treated animals achieved slightly higher blood glucose levels than controls, although the difference was not significant (Fig. 1*H*).

In contrast to these modest effects, rapamycin markedly worsened the metabolic state of diabetic animals (Fig. 1*A*). Blood glucose gradually increased in rapamycin-treated diabetic *P. obesus*, whereas glycemia remained unchanged in diabetic controls (24.2 ± 1.4 vs. 16.9 ± 3.1 mmol/l glucose at 2 weeks; $P < 0.001$). Rapamycin reduced the body weight of diabetic animals. At 2 weeks, rapamycin-

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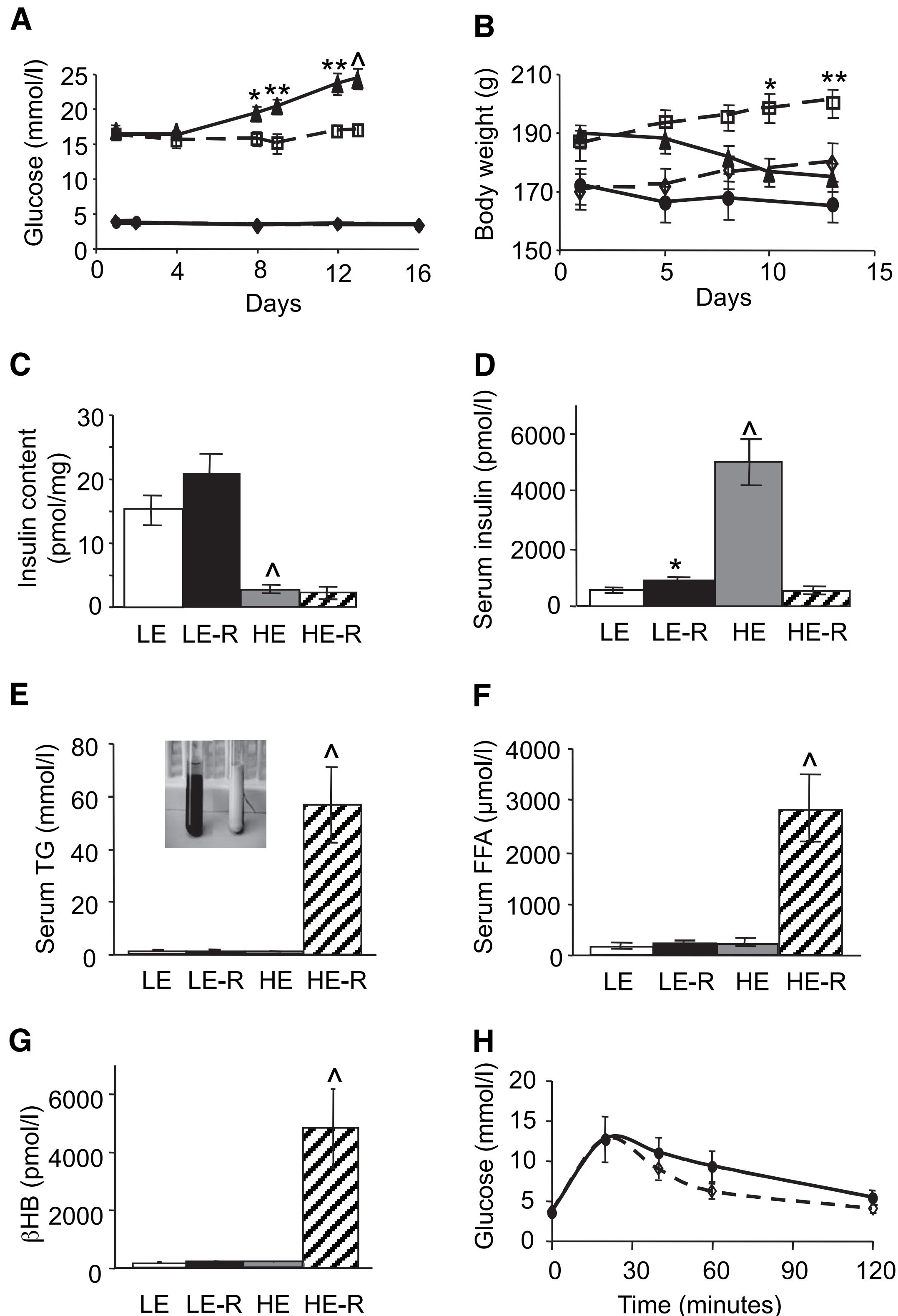


**FIG. 1. Metabolic effects of rapamycin treatment in normoglycemic and diabetic *P. obesus*. Hyperglycemia was induced by feeding *P. obesus* a high-energy diet (HE) for 5–7 days. Normoglycemic animals were given low-energy diet (LE) throughout the study. Normoglycemic and diabetic *P. obesus* were treated by intraperitoneal injection of 0.2 mg/kg rapamycin (R) or vehicle for 16 and 14 days, respectively. Nonfasting blood glucose (*A*) and body weight (*B*) were determined on the indicated days before morning injection. ● and solid line, rapamycin-treated normoglycemic animals; ◇ and dashed line, normoglycemic controls; ▲ and solid line, rapamycin-treated diabetic animals; □ and dashed line, diabetic controls. Pancreatic insulin content (*C*), serum insulin (*D*), triglycerides (*E*) (with *inset* showing lipemic serum of a diabetic animal treated with rapamycin [right] and serum of diabetic control [left], nonesterified fatty acids (*F*), and β-hydroxybutyrate (*G*) were determined on the day mice were killed. Intraperitoneal glucose tolerance test (1.5 g/kg body wt) was performed in rapamycin-treated normoglycemic *P. obesus* and their controls at 2 weeks (*H*). Results are shown as means ± SE of 10–21 animals in each group. *$P < 0.05$; **$P < 0.01$; ˆ$P < 0.001$; compared with rapamycin-treated animals on the same diet at the same time point (*A* and *B*); LE-R and HE compared with LE (*C* and *D*) and compared with all other groups (*E–G*).**

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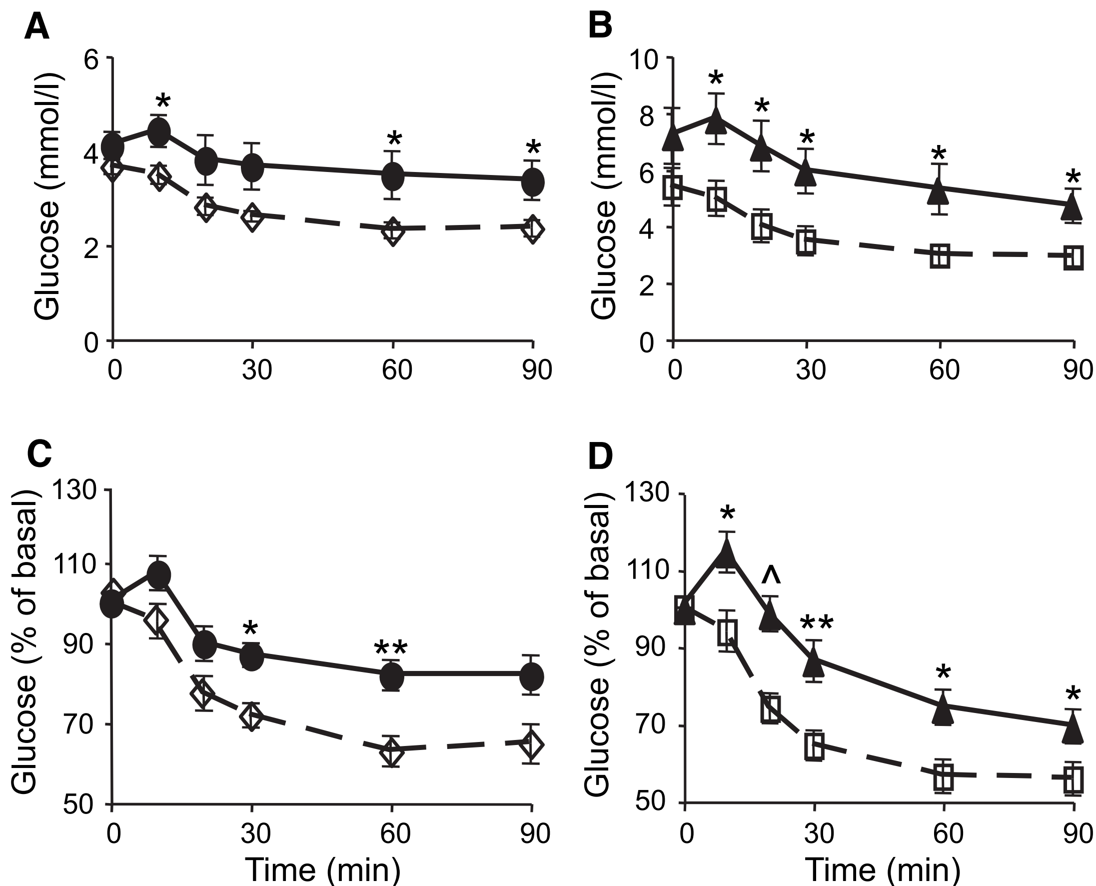


**FIG. 2. Effects of rapamycin on insulin sensitivity. Normoglycemic and diabetic *P. obesus* were treated by daily intraperitoneal injection of 0.2 mg/kg rapamycin (solid line) or vehicle (dashed line) for 48 h. An insulin tolerance test was performed after a 4-h fast: 2.5 and 5 units/kg of regular insulin was injected intraperitoneally to normoglycemic (*A* and *C*) and diabetic (*B* and *D*) *P. obesus*. Glucose concentrations were measured at the indicated time points. Symbols and lines are as described in the Fig. 1 legend. Results shown in absolute glucose concentrations (*A* and *B*) and as percent of basal glucose concentrations (time 0) (*C* and *D*) are expressed as means ± SE of 10–15 animals in each group. *$P < 0.05$; **$P < 0.01$ and ˆ$P < 0.001$; rapamycin-treated animals vs. controls at the same time points.**

treated animals weighed 174.4 ± 4.7 vs. 200.3 ± 4.4 g in controls ($P < 0.001$). This was not explained by changed food intake (online appendix Table 1).

Pancreatic insulin was depleted by ~90% in diabetic *P. obesus* with or without rapamycin (Fig. 1*C*). Hyperglycemia was accompanied by a 10-fold increase of serum insulin in diabetic *P. obesus*, which was completely abolished by rapamycin (Fig. 1*D*). The hypoinsulinemia in rapamycin-treated diabetic *P. obesus* was accompanied by a 55-, 12-, and 23-fold increase in serum triglycerides, FFAs, and ketone bodies, respectively ($P < 0.001$) (Fig. 1*E–G*). The hyperlipidemia was accompanied by hepatic steatosis (online appendix Figure S1).

**Rapamycin effect on insulin sensitivity.** The higher serum insulin level in the rapamycin-treated normoglycemic *P. obesus* suggests worsening of the insulin resistance inherent to this species. This was assessed by an insulin tolerance test in *P. obesus* treated with rapamycin for 48 h. Rapamycin-treated animals were more insulin resistant than controls (Fig. 2*A* and *C*); blood glucose in controls was reduced by 37% in response to insulin versus only 18% in the rapamycin group ($P < 0.05$) (Fig. 2*C*).

Diabetic *P. obesus* are more insulin resistant than normoglycemic animals (34); therefore, a higher dose of insulin (5 units/kg) was used to study the effect of the 48-h rapamycin treatment on insulin sensitivity. At this early time point, there was no increase in serum FFAs (not shown), which could directly affect insulin sensitivity. Similar to its effect in normoglycemic *P. obesus*, rapamycin increased insulin resistance also in diabetic animals (Fig. 2*B* and *D*); blood glucose of untreated diabetic animals was reduced by 45% after insulin injection versus 31% in the rapamycin group ($P < 0.05$) (Fig. 2*D*).

**Regulation of mTOR/S6K1 in diabetic *P. obesus*.** Based on the hypothesis that increased TORC1 activity is the main cause of insulin resistance in type 2 diabetes, we expected an inverse relationship between Akt and S6K1 phosphorylation. We therefore studied the phosphorylation of these kinases in fed and fasted diabetic animals. To further assess the impact of the hyperinsulinemia characterizing fed diabetic animals on kinase activity, NPH insulin was injected to fasted diabetic animals and Akt and S6K1 phosphorylation were measured after 4 h.

Steady-state Akt and S6K1 phosphorylations were higher in muscles of fed diabetic animals (Fig. 3 and online appendix Figure S2). These changes were reversed by a 4-h fast, in parallel to the decrease of serum insulin. Hyperinsulinemia induced by NPH insulin reinstituted both Akt and S6K1 phosphorylation. The direct relationship between serum insulin, Akt, and S6K1 phosphorylation in the diabetic state suggests that the higher steady-state S6K1 phosphorylation in diabetic animals could result from the activation of the insulin receptor/Akt/TORC1 pathway by insulin.

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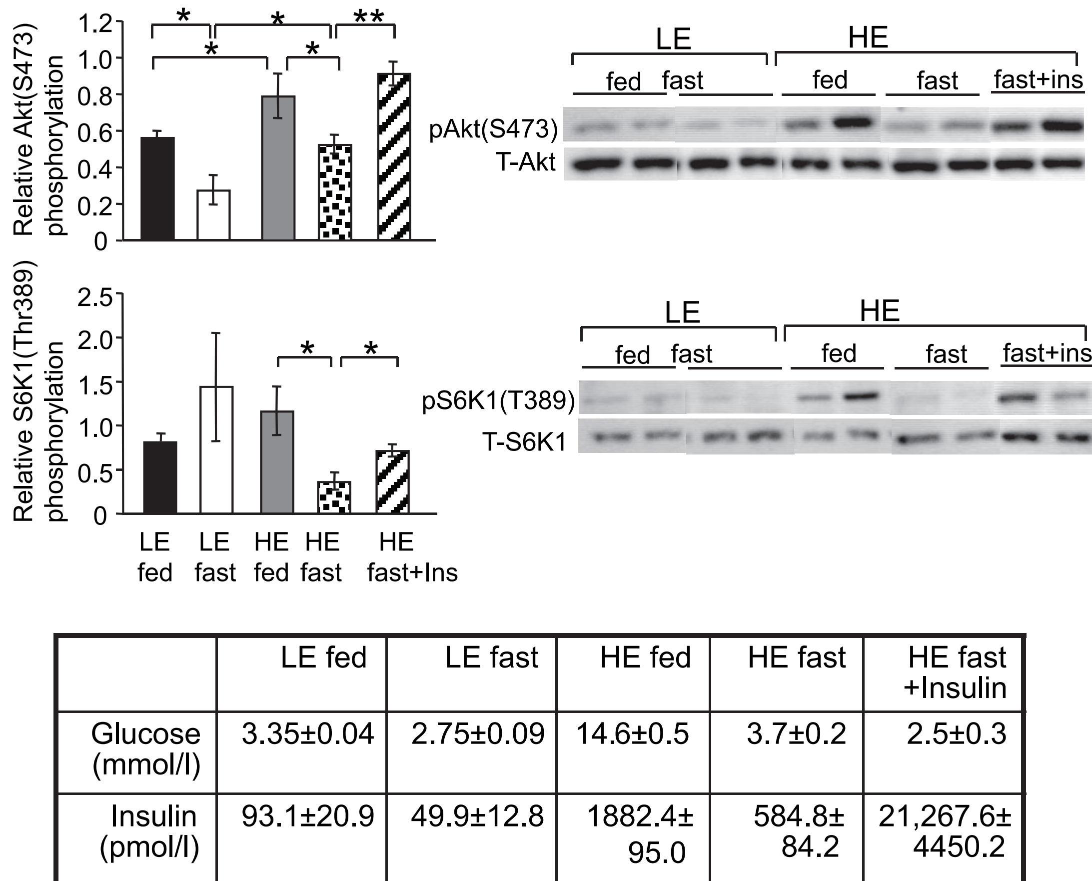


| | LE fed | LE fast | HE fed | HE fast | HE fast +Insulin |
|---|---|---|---|---|---|
| Glucose (mmol/l) | 3.35±0.04 | 2.75±0.09 | 14.6±0.5 | 3.7±0.2 | 2.5±0.3 |
| Insulin (pmol/l) | 93.1±20.9 | 49.9±12.8 | 1882.4± 95.0 | 584.8± 84.2 | 21,267.6± 4450.2 |

**FIG. 3. Effects of diet and hyperinsulinemia on Akt (Ser473) and S6K1 (Thr389) phosphorylation. Diabetes was induced by feeding *P. obesus* the high-energy diet (HE) diet for 5–7 days. The analysis was performed in fed and 4-h fasted normoglycemic and diabetic *P. obesus*. The effect of hyperinsulinemia was studied by subcutaneous injection of 4 units NPH insulin/kg to fasted animals. Gastrocnemius muscle was isolated 4 h after insulin injection. Representative gels are shown in the *right panels* and quantification of the ratio of phosphorylated to total band intensity are shown in the *left panel*. The corresponding blood glucose and serum insulin concentrations when the animals were killed are shown in the *lower panel*. Results are expressed as means ± SE of 4–5 animals in each group. *$P < 0.05$; **$P < 0.01$ for the difference between the indicated groups.**

**Mechanisms of rapamycin-induced insulin resistance.** We first studied the effect of acute insulin stimulation on Akt phosphorylation in muscle of *P. obesus*. Akt phosphorylation was increased in muscle of normoglycemic and diabetic animals 10 min after insulin injection (Fig. 4*A*). In the fasting state, basal Akt phosphorylation was higher in diabetic than normoglycemic animals, which is associated with decreased stimulation by insulin (1.2- vs. 2.1-fold increase in diabetic and normoglycemic animals, respectively) and compatible with the higher insulin resistance of diabetic animals.

Rapamycin effects on insulin signaling were studied in the fasting state 10 min after insulin injection. IRS1 tyrosine phosphorylation was similar in muscle of rapamycin-treated *P. obesus* and controls (Fig. 4*B*). Moreover, rapamycin did not affect phosphorylation at IRS1 serines 636/639 and 302 (Fig. 4*B*), suggesting that rapamycin-induced insulin resistance is not mediated via changes in IRS1 phosphorylation.

As expected, rapamycin completely abolished S6K1 phosphorylation (Fig. 5*A*). However, Akt phosphorylation in muscle (Fig. 5*B* and online appendix Figure S3) and liver (online appendix Figure S4) was decreased despite S6K1 inhibition. Steady-state Akt phosphorylation in fed diabetic animals was dramatically reduced following the 2-week rapamycin treatment (online appendix Figure S2). Thus, rapamycin induced insulin resistance by decreasing Akt phosphorylation.

Increased GSK3 activity alters glucose and fat metabolism and causes insulin resistance (35,36). Akt phosphorylates GSK3β at the conserved serine 9 site, resulting in its inactivation (35). Rapamycin markedly decreased GSK3β phosphorylation in muscle of normoglycemic and diabetic *P. obesus*, indicating increased GSK3β activity (Fig. 5*C*).

Activation of the JNK pathway leads to insulin resistance (37). It has been previously reported that rapamycin activates JNK in tumor cells (38). Therefore, we studied the effect of rapamycin on JNK phosphorylation in muscle of *P. obesus* and observed increased JNK 2/3 and, to a lesser extent, JNK1 phosphorylation in both normoglycemic and diabetic animals (Fig. 5*D*). In summary, rapamycin reduced Akt phosphorylation independent of IRS1 tyrosine phosphorylation and increased GSK3β and JNK activities, which may account for rapamycin-induced insulin resistance.

**Rapamycin effects on β-cell function in vitro.** Exposure of *P. obesus* islets to 16.7 mmol/l glucose for 96 h resulted in a fivefold increase of insulin secretion, accompanied by a 55% decrease of islet insulin content (Fig. 6*A* and *B*). Thus, chronic exposure of islets to high glucose mimicked the hyperinsulinemia and the depletion of pancreatic insulin content characterizing the in vivo diabetic state. Rapamycin inhibited insulin secretion by 33% in the presence of 16.7 mmol/l glucose without affecting insulin secretion at 3.3 mmol/l glucose or insulin content (Fig. 6*A* and *B*). In addition, rapamycin reduced glucose-stimulated

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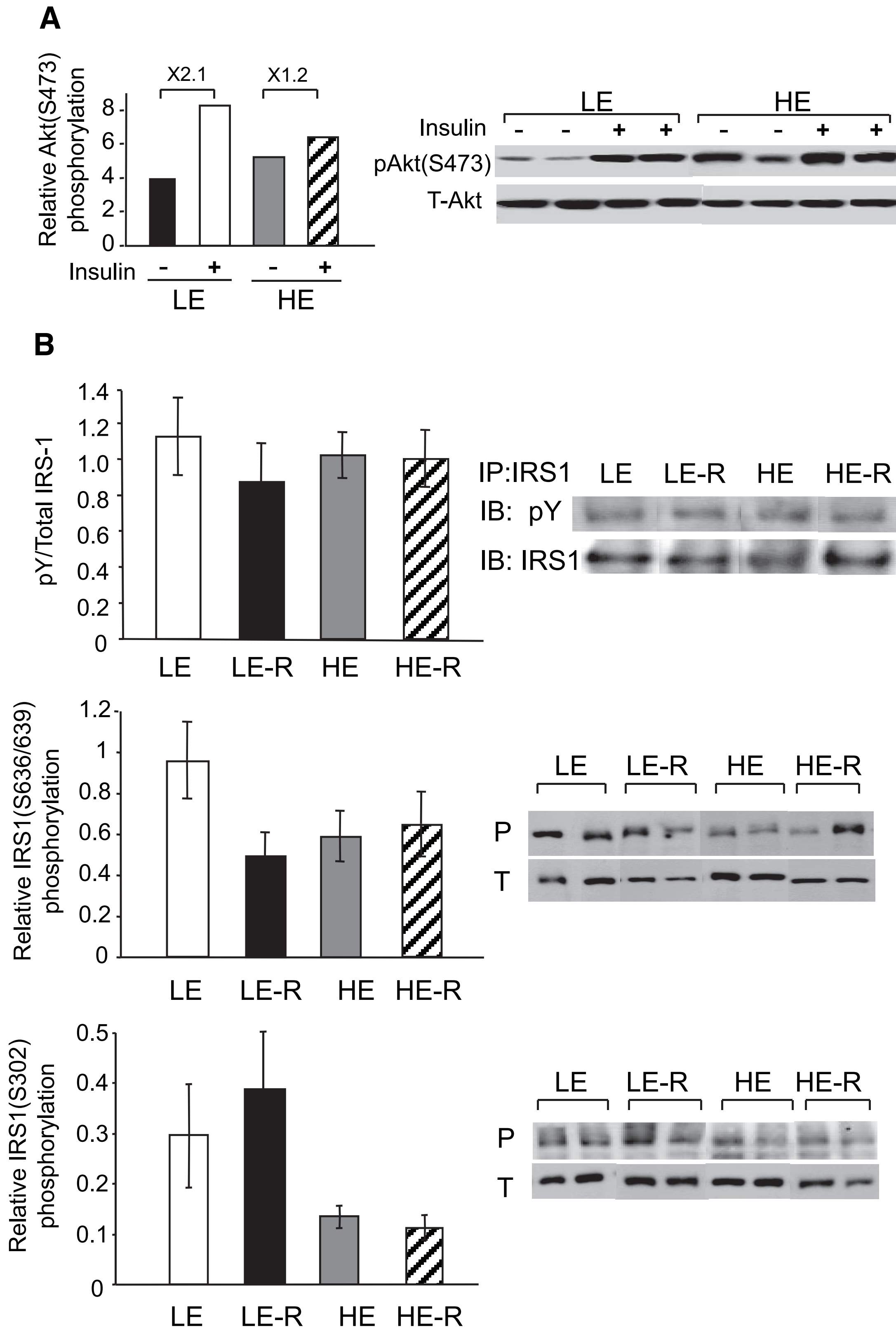


**FIG. 4. Effects of rapamycin on IRS1 phosphorylation.** ***A*****: The stimulatory effect of acute insulin stimulation on Akt (Ser473) phosphorylation in normoglycemic (low-energy diet [LE]) and diabetic (high-energy diet [HE]) animals. Diabetes was induced by feeding** ***P. obesus*** **HE diet for 5–7 days. After a 4-h fast, the animals were injected intraperitoneally with 5 units/kg insulin followed 10 min later by isolation of the gastrocnemius muscle.** ***B*****: Effects of rapamycin on IRS1 tyrosine phosphorylation and IRS1 Ser636/639 and Ser302 phosphorylation. Normoglycemic and diabetic** ***P. obesus*** **were treated daily with rapamycin (R) or vehicle for 48 h. After a 4-h fast, the animals were injected intraperitoneally with 5 units/kg regular insulin followed 10 min later by isolation of the gastrocnemius muscle. IRS1 was immunoprecipitated followed by immunoblotting using IRS1 or phosphotyrosine antibodies. IRS1 Ser636/639 and Ser302 phosphorylation were analyzed by Western blot.**

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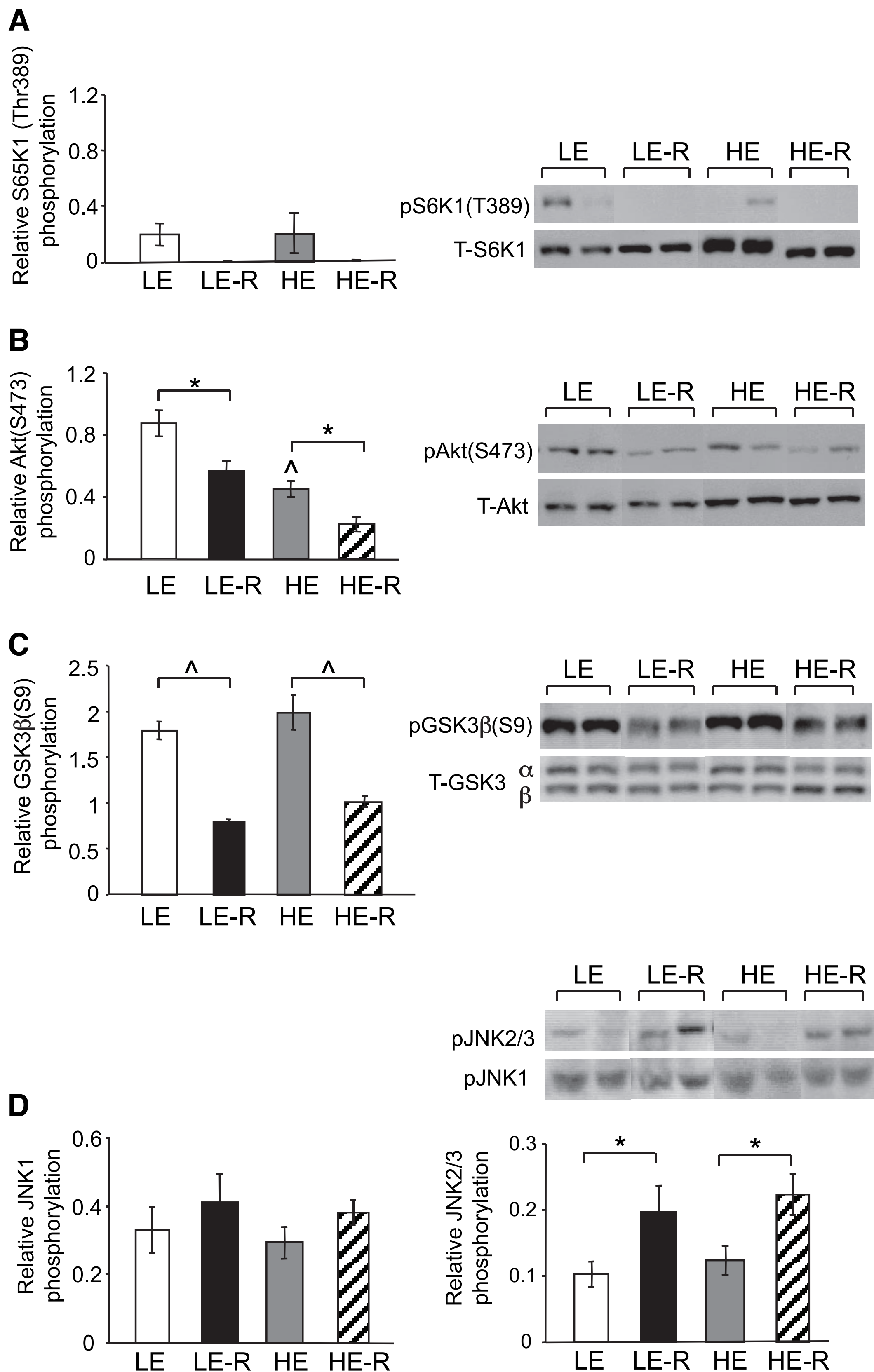


**FIG. 5. Effect of rapamycin on S6K1, Akt, GSK3β, and JNK phosphorylation in muscle. Normoglycemic and diabetic *P. obesus* were treated daily with rapamycin (R) or vehicle for 48 h. After a 4-h fast, the animals were injected intraperitoneally with 5 units/kg regular insulin followed 10 min later by isolation of the gastrocnemius muscle. Phosphorylated and total S6K1 (Thr379), Akt (Ser473), GSK3β (Ser9), and SAPK/JNK (Thr183/Tyr185) were analyzed by Western blot. Representative gels and quantification of the ratio between phosphorylated and total band intensity are shown. Results are expressed as means ± SE of six animals in each group. *$P$ < 0.05 and ˆ$P$ < 0.001 for the difference between the indicated groups or between HE and LE groups in *B*.**

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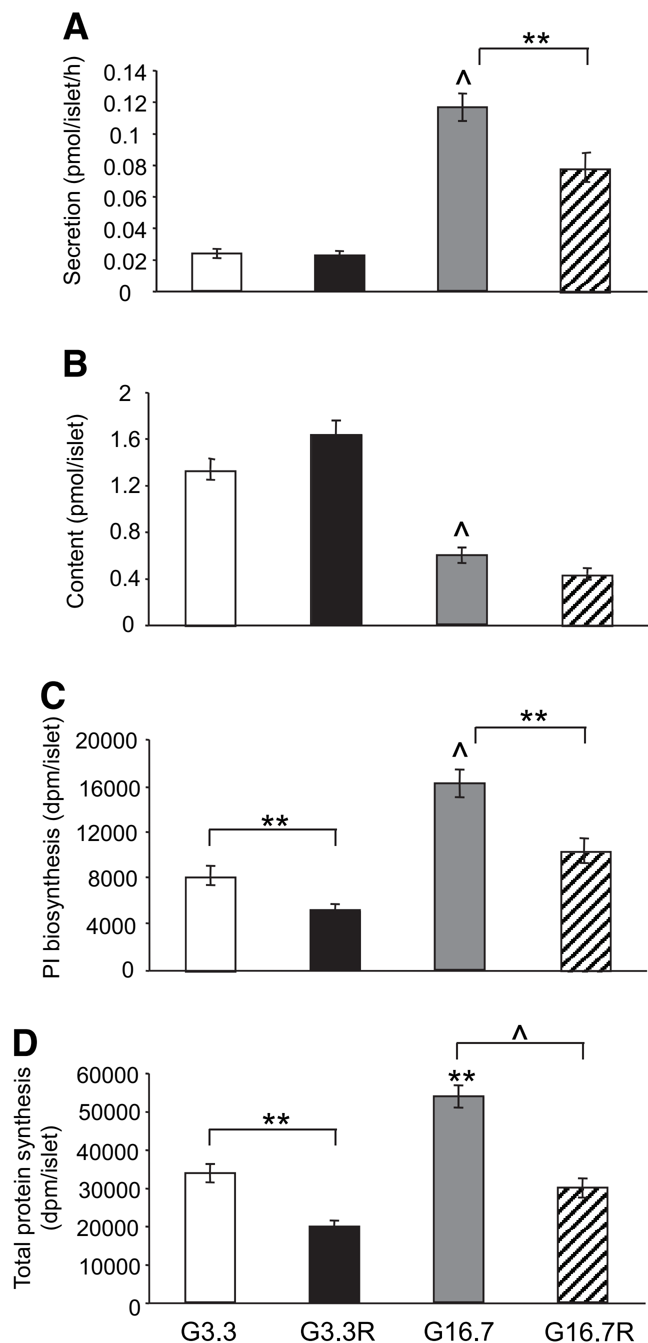


**FIG. 6. Effect of rapamycin on insulin secretion (*A*), islet insulin content (*B*), proinsulin (*C*), and total protein (*D*) biosynthesis. Islets were isolated from normoglycemic *P. obesus* and cultured on extracellular matrix–coated plates at 3.3 (G3.3) and 16.7 (G16.7) mmol/l glucose with and without 50 nmol/l rapamycin (R) for 96 h. Insulin secretion was analyzed by static incubations for 1 h in buffer containing the same glucose and rapamycin concentrations. Proinsulin and total protein biosynthesis were analyzed after a 15-min pulse with $^3$H-leucine, as described in RESEARCH DESIGN AND METHODS. Results are means ± SE of six individual experiments, each performed on islets pooled from three animals. **$P < 0.01$; ^$P < 0.001$; for the differences between the indicated groups or between G16.7 and G3.3.**

proinsulin biosynthesis by 35%, paralleling its effect on total protein biosynthesis (Fig. 6*C* and *D*). Taken together, our data show that rapamycin is deleterious to β-cell function.

**Rapamycin effects on insulin signaling in pancreatic islets.** mTOR activation by glucose was shown to inhibit IRS2, an important regulator of β-cell function and mass (24); we therefore studied IRS2 expression in islets of diabetic versus normoglycemic *P. obesus* with and without rapamycin. IRS2 expression was twofold higher in islets derived from diabetic animals. Rapamycin increased IRS2 expression in islets from normoglycemic *P. obesus* but failed to further augment IRS2 expression in diabetic animals (Fig. 7*A*).

The lengthy islet isolation process prevented monitoring of in vivo phosphorylation events. Therefore, we studied the effects of high glucose and rapamycin in vitro (Fig. 7*B*). Islets derived from normoglycemic *P. obesus* were cultured at 3.3 and 16.7 mmol/l glucose with and without rapamycin, respectively, for 48 h. Exposure of islets to 16.7 mmol/l glucose did not affect S6 phosphorylation; rapamycin reduced S6 phosphorylation in the presence of either 3.3 or 16.7 mmol/l glucose. This was associated with a twofold increase of IRS2 expression at 3.3 mmol/l glucose. Islets exposed to 16.7 mmol/l glucose showed a trend for higher IRS2 expression, which was further augmented by rapamycin. The higher IRS2 expression was associated with increased Akt phosphorylation. In summary, rapamycin increased IRS2 expression both in vivo and in vitro mainly under a physiological glucose concentration. This was accompanied by activation of Akt.

**Rapamycin effects on pancreatic morphology and β-cell mass.** Changes in IRS2 expression and Akt phosphorylation in response to hyperglycemia and to rapamycin could affect β-cell mass. We therefore studied β-cell proliferation, apoptosis, and mass in rapamycin-treated normoglycemic and diabetic *P. obesus* (Fig. 8 and online appendix Figure S5). Rapamycin treatment had no effect on islet morphology in normoglycemic *P. obesus*. A marked heterogeneity of islet size with large distorted islets was observed in diabetic *P. obesus*, whereas islets from rapamycin-treated diabetic animals were smaller and more uniform in size. β-Cell degranulation and vacuolization were observed in islets of diabetic *P. obesus* with and without rapamycin.

There was a twofold increase of TUNEL- and Ki-67–positive β-cell nuclei in diabetic *P. obesus*, which did not reach statistical significance (Fig. 8). This was associated with a 50% increase of β-cell mass in the diabetic animals ($P = 0.055$). Rapamycin had no effect on β-cell apoptosis and mass in normoglycemic *P. obesus*. Yet, it increased β-cell apoptosis fivefold and decreased β-cell mass by 50% in diabetic animals compared with controls (Fig. 8). To elucidate the mechanisms underlying rapamycin induction of β-cell apoptosis, we studied its effects on JNK phosphorylation following 48 h in the presence of 3.3 or 16.7 mmol/l glucose (Fig. 8*D*). JNK phosphorylation was not affected by high glucose or by rapamycin at 3.3 mmol/l glucose. However, at 16.7 mmol/l glucose, JNK1 and JNK2/3 were increased 1.8- and 1.6-fold, respectively, by rapamycin ($P < 0.05$).

## DISCUSSION

Previous studies have suggested that inhibition of TORC1/S6K1 may alleviate insulin resistance and improve β-cell survival under conditions of chronic hyperglycemia (13,15,24). However, the chronic effects of rapamycin on glucose metabolism under conditions of nutrition-induced insulin resistance in vivo were not evaluated. Our study shows that inhibition of mTOR by rapamycin dramatically worsened the metabolic syndrome in nutrition-dependent type 2 diabetes. The deleterious effects of rapamycin on

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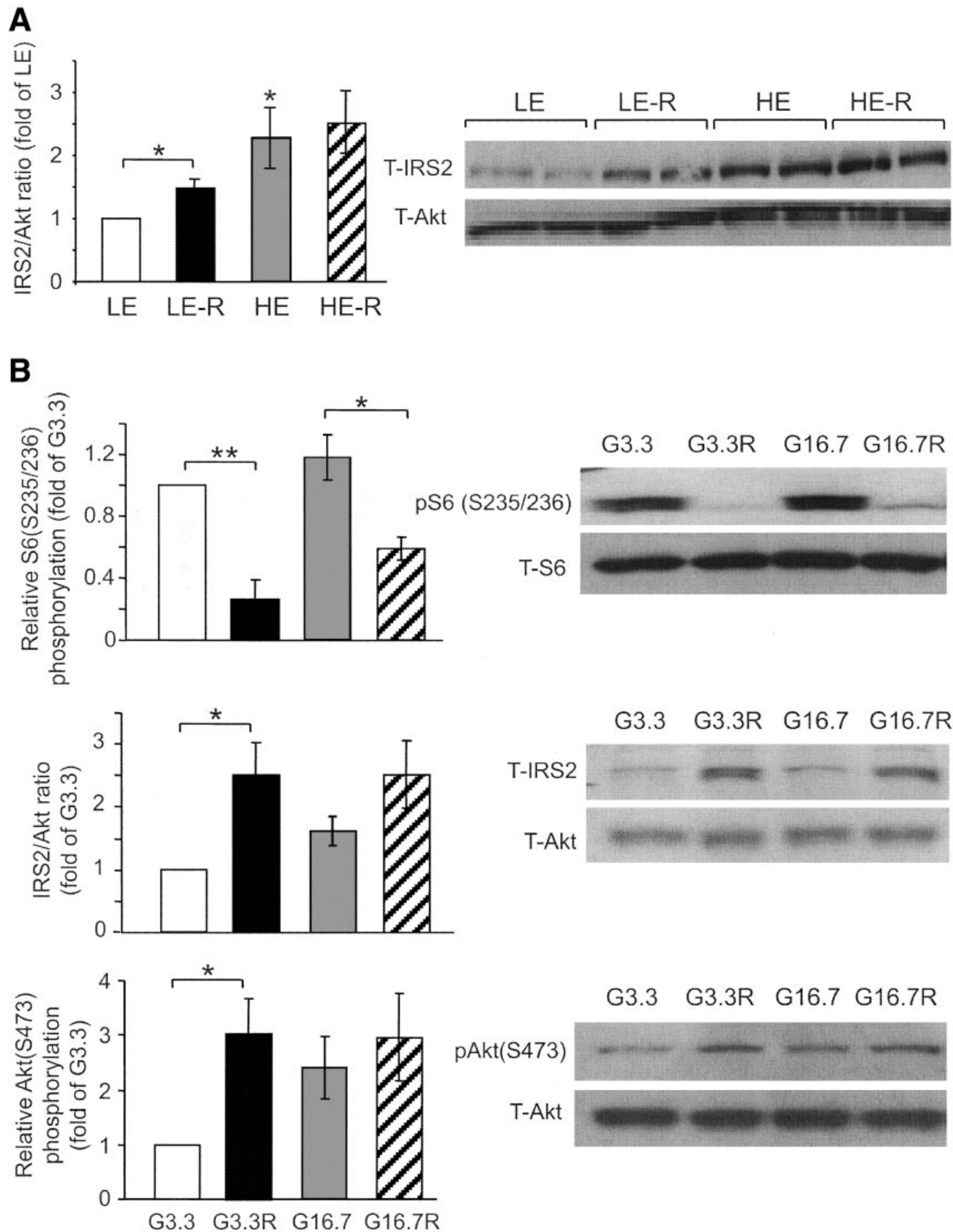


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**FIG. 7. Effects of hyperglycemia and rapamycin on IRS2 expression and insulin signaling in *P. obesus* islets in vivo (*A*) and in vitro (*B*). *A*: Normoglycemic and diabetic *P. obesus* were treated daily with rapamycin (R) or vehicle for 48 h followed by islet isolation, as described in RESEARCH DESIGN AND METHODS. IRS2 expression was analyzed by Western blot and results normalized to total Akt. A representative gel is shown in the *right panel* and quantification of four to five animals in each group is depicted in the *left panel*. The results are expressed as IRS2/Akt and normalized to normoglycemic controls (low-energy diet [LE]). *B*: Islets were isolated from normoglycemic *P. obesus* and incubated at 3.3 and 16.7 mmol/l glucose (denoted as G3.3 and G16.7, respectively) with and without 50 nmol/l rapamycin (R) for 48 h. Western blot analysis for IRS2, total and phosphorylated S6 (Ser235/236), and total and phosphorylated Akt (Ser473) were performed on islet extracts. S6 phosphorylation was normalized to total S6 expression; IRS2 and Akt phosphorylation were normalized to total Akt. Results shown as means ± SE (four to six individual experiments each performed on islets pooled from three animals) are normalized to islets at 3.3 mmol/l glucose. *$P < 0.05$ and **$P < 0.01$ for the differences between the indicated groups and between the HE and LE groups in *A*.**

blood glucose, serum triglycerides, FFAs, and ketone bodies were observed only in diabetic animals, indicating that rapamycin impedes pathways that are important for reducing the metabolic disorders in type 2 diabetes.

Steady-state S6K1 phosphorylation was increased in fed diabetic *P. obesus* muscle, correlating with serum insulin and Akt phosphorylation (Fig. 3*B* and online appendix Figure S2). This suggests that the higher S6K1 activity in diabetic hyperinsulinemic animals results from the activation of the insulin receptor/phosphatidylinositol 3-kinase

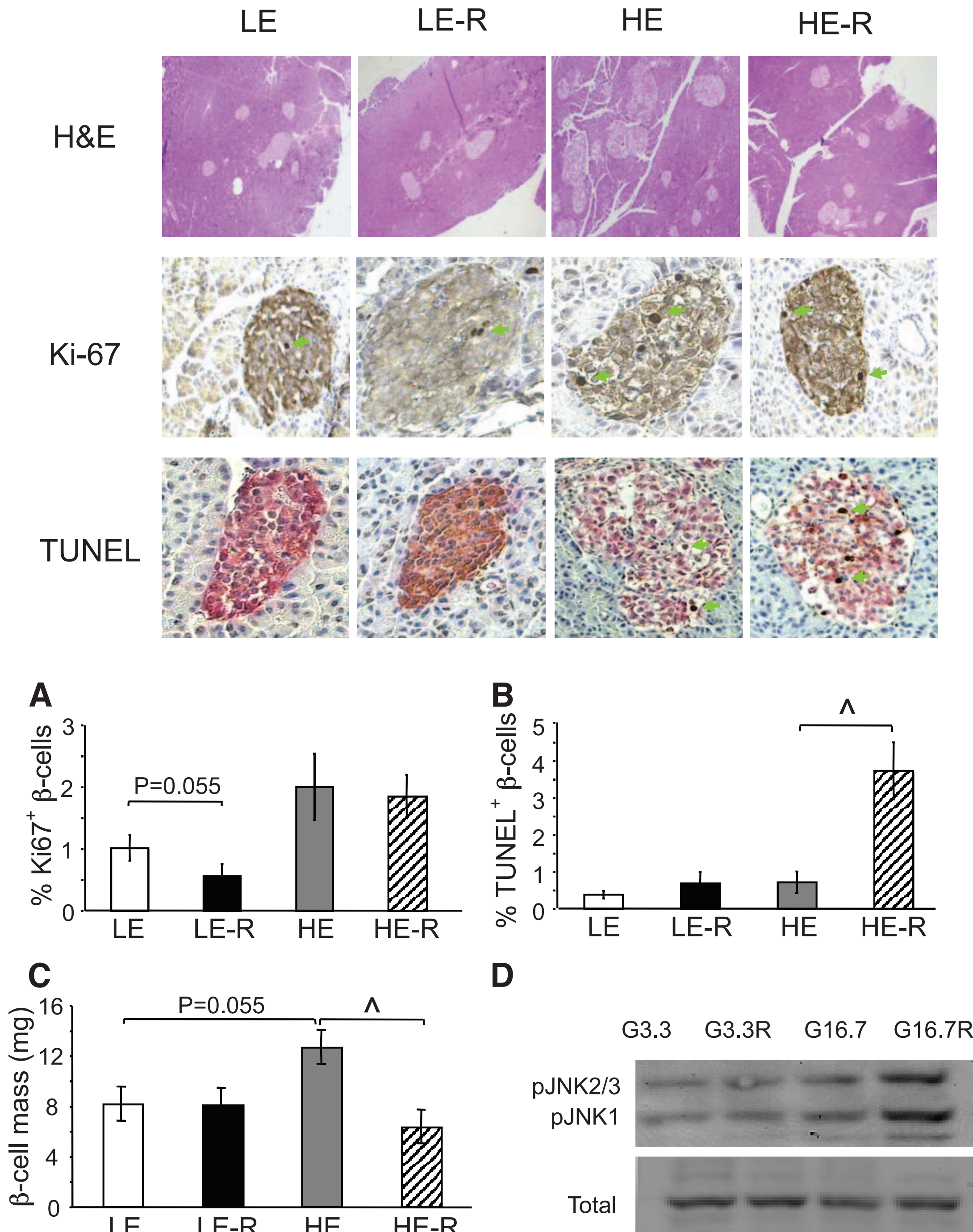


**FIG. 8. Immunostaining and morphometric analysis of pancreatic sections.** ***Top panel*****: Photomicrographs of pancreatic sections of normoglycemic (low-energy diet [LE]) and diabetic (high-energy diet [HE])** ***P. obesus*** **treated for 14–16 days with vehicle or rapamycin (R). Hematoxylin and eosin (H&E)-stained pancreatic sections are shown in the** ***top panel*****. Pancreatic sections double immunostained for insulin (brown) and Ki-67 antigen (dark brown/black;** ***middle panel*****) or with insulin (red/pink) and DNA fragmentation (TUNEL) (dark brown;** ***lower panel*****). Arrows denote Ki-67 – and TUNEL-positive β-cells. Original magnification of pictures in the** ***top panel*** **was ×100 and in the** ***middle*** **and** ***lower panels*** **×200. Quantification of β-cell proliferation (*A*) and apoptosis (*B*) expressed as the percentage of Ki-67 – and TUNEL-positive β-cells and β-cell mass in the different treatment groups (*C*). Results are expressed as means ± SE of 5–10 animals in each group. ^$P < 0.001$ for the differences between the indicated groups.** ***D*****: Rapamycin effect on JNK phosphorylation in islets in vitro. Islets were isolated from normoglycemic** ***P. obesus*** **and incubated at 3.3 and 16.7 mmol/l glucose (denoted as G3.3 and G16.7, respectively) with and without 50 nmol/l rapamycin (R) for 48 h. JNK phosphorylation was analyzed by Western blot. A representative gel of five separate experiments is shown.**

pathway by insulin. Other hormones, growth factors, and nutrients that are increased in type 2 diabetes may also be involved in the steady-state activation of this pathway.

Elevated mTOR/S6K activity induces insulin resistance (3,4); therefore, its inhibition was expected to alleviate the insulin resistance of obesity and type 2 diabetes. Surprisingly, treatment with rapamycin, which efficiently inhibited S6K1 phosphorylation, increased rather than decreased the insulin resistance of *P. obesus*. This contrasts with the observation that S6K1 knockout mice are protected from diet-induced insulin resistance (16). The apparent discrepancy between the effects of prolonged

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rapamycin treatment and of genetic deletion of S6K1 on insulin resistance could result from the inhibition by rapamycin of TORC1 targets other than S6K1, such as 4EBP1, which does not occur in S6K1 knockout mice. In the short-term in vivo study (20) showing that rapamycin alleviated insulin resistance in humans, insulin resistance was induced by infusion of a high concentration of amino acids, which markedly augment mTOR activity. This metabolic setup is probably very different from that induced by the chronic ingestion of high-energy diets in common models of obesity and insulin resistance. Moreover, the long-term metabolic effects of rapamycin treatment could not be reported in this study. It is noteworthy that the only report on long-term effects of rapamycin in humans showed that insulin resistance increased and basal and insulin-stimulated Akt phosphorylation decreased (39), in accordance with our data.

The augmentation of insulin resistance by rapamycin was more pronounced in diabetic animals. This is based on the observation that rapamycin reduced Akt phosphorylation by 50% in diabetic animals versus 28% in normoglycemic *P. obesus* (Fig. 5). Moreover, in the fed state, rapamycin markedly decreased steady-state Akt phosphorylation in muscle of diabetic, but not normoglycemic, animals (online appendix Figure S2). Thus, the rapamycin effects on insulin sensitivity and its metabolic implications depend on the metabolic context in which they are studied.

The mechanisms underlying the inhibition of Akt phosphorylation by rapamycin are not clear. Intriguingly, IRS1 tyrosine phosphorylation was not reduced by rapamycin. We cannot exclude that small changes in tyrosine phosphorylation were overlooked due to variability among animals. However, Akt phosphorylation was consistently decreased in the very same animals, suggesting that factors other than IRS1 impair insulin signaling in response to rapamycin. It is noteworthy that the mTOR-rictor complex (i.e., TORC2) functions as a phosphorylating kinase of Akt on serine 473 (40). Prolonged rapamycin treatment was reported to reduce Akt phosphorylation (41). It is possible that rapamycin inhibition of TORC2 activity contributes to the observed decrease of Akt phosphorylation in peripheral tissues of *P. obesus*.

To further characterize the functional consequences of reduced Akt activity in response to rapamycin, we studied the phosphorylation of GSK3β, a bona fide Akt target (35). We found that rapamycin decreased GSK3β phosphorylation, implying a higher GSK3β activity in muscle of rapamycin-treated animals. GSK3 is a negative regulator of insulin signaling in muscle and liver (36). This results from inhibition of its namesake substrate glycogen synthase with decreased insulin-stimulated glycogen synthesis as a consequence. In addition, GSK3β can attenuate the insulin-stimulated phosphorylation of tyrosine residues in IRS1 by directly phosphorylating serine residues (42). However, this is unlikely to play an important role in *P. obesus*, as rapamycin did not affect IRS1 tyrosine phosphorylation in this model. In addition, GSK3 regulates insulin resistance via activation of the nuclear factor κB and JNK pathways involved in inflammation-induced insulin resistance (36). We found that rapamycin increased JNK (Fig. 5) but not inhibition of nuclear factor-κβ kinase phosphorylation (not shown), suggesting that the JNK pathway might be involved in rapamycin-induced insulin resistance.

The balance between insulin resistance and β-cell function and mass determines the capacity to adequately regulate blood glucose. Increased insulin resistance turns on compensatory mechanisms to increase β-cell performance. Indeed, hyperglycemia in diabetic *P. obesus* and rapamycin treatment of normoglycemic *P. obesus* increased IRS2 expression in vivo and in vitro. The expression level of IRS2 is determined by the balance between synthesis, mostly transcriptionally regulated (43,44), and degradation, mainly dependent on serine/threonine phosphorylation (24). Glucose has dual effects on IRS2 expression; while it increases IRS2 transcription (43), it also accelerates its degradation via activation of TORC1/S6K1 signaling (24). It is likely that in *P. obesus* islets, high glucose levels increased IRS2 expression by preferentially augmenting its synthesis. As expected, inhibition of TORC1/S6K1 by rapamycin increased IRS2 expression, most probably by inhibiting its degradation. However, treatment of diabetic *P. obesus* with rapamycin had no further effect on islet IRS2, suggesting that the glucose effects on IRS2 transcription dominate over the effect of rapamycin in vivo.

Increased IRS2 expression in response to glucose and to rapamycin was associated with increased Akt phosphorylation. Akt promotes β-cell proliferation, survival, and differentiation via mTOR-dependent and independent pathways (45). In addition to activation of mTOR, Akt inhibits GSK3, Foxo1, and proapoptotic genes, such as BAD and procaspase 9, and activates cell cycle regulators, such as cyclin D1 (45). The fact that rapamycin activated Akt in normoglycemic *P. obesus* without affecting β-cell proliferation, apoptosis, and mass suggests that TORC1/S6K1 is a critical mediator of Akt effects on these parameters. This is in line with the observation that β-cell size is reduced in S6K1 knockout mice and in knockin mice resistant to S6 phosphorylation, resulting in hypoinsulinemia and glucose intolerance despite increased insulin sensitivity (17,46).

Increased IRS2 expression in islets of diabetic animals was associated with a 50% compensatory increase of β-cell mass. Rapamycin markedly increased β-cell apoptosis and reduced β-cell mass in diabetic *P. obesus*. In vitro experiments showed that exposure of *P. obesus* islets to high glucose and rapamycin impaired glucose-stimulated insulin secretion and proinsulin biosynthesis. Thus, mTOR/S6K1 has a prime role in β-cell adaptation to hyperglycemia. Rapamycin activated JNK mainly in islets exposed to high glucose, implying that mTOR-JNK signaling could be involved in the β-cell apoptosis induced by rapamycin in the diabetic milieu. Thus, increased JNK activity may account for the deleterious effects of rapamycin on both muscle insulin resistance and β-cell survival. In contrast to muscle, rapamycin did not affect GSK3β phosphorylation in islets (not shown), suggesting that in islets JNK activation by rapamycin is not mediated via GSK3β.

Our studies have important clinical implications, since rapamycin (sirolimus) is commonly used in solid-organ transplantation including islets (47) and in the treatment of cancer. Posttransplantation diabetes is very common (48); it is conceivable that in this clinical setting, rapamycin adversely affects a preexisting metabolic syndrome and is toxic to endogenous or transplanted pancreatic islets. Indeed, recent studies (49,50) showed that rapamycin impaired islet engraftment and β-cell function following islet transplantation.

In summary, the mTOR/S6K1 pathway is critical for β-cell adaptation to hyperglycemia. Chronic inhibition of

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mTOR with rapamycin augmented insulin resistance, β-cell dysfunction, and death, and it is therefore expected to exacerbate the metabolic syndrome in patients treated with rapamycin-like agents (51).

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