Original Article

The Mammalian Target of Rapamycin Pathway Regulates Nutrient-Sensitive Glucose Uptake in Man

Michael Krebs,[1] Barbara Brunmair,[1] Attila Brehm,[1] Michaela Artwohl,[1] Julia Szendroedi,[1] Peter Nowotny,[1] Erich Roth,[2] Clemens Fürnsinn,[1] Miriam Promintzer,[1] Christian Anderwald,[1] Martin Bischof,[1] and Michael Roden[3]

The nutrient-sensitive kinase mammalian target of rapamycin (mTOR) and its downstream target S6 kinase (S6K) are involved in amino acid–induced insulin resistance. Whether the mTOR/S6K pathway directly modulates glucose metabolism in humans is unknown. We studied 11 healthy men (29 years old, BMI 23 kg/m^2) twice in random order after oral administration of 6 mg rapamycin, a specific mTOR inhibitor, or placebo. An amino acid mixture was infused to activate mTOR, and somatostatin-insulin-glucose clamps created conditions of low peripheral hyperinsulinemia (~100 pmol/l, 0–180 min) and prandial-like peripheral hyperinsulinemia (~450 pmol/l, 180–360 min). Glucose turnover was assessed using D-[6,6-$^2H_2$]glucose infusion (n = 8). Skeletal muscle biopsies were performed at baseline and during prandial-like peripheral hyperinsulinemia (n = 3). At low peripheral hyperinsulinemia, whole-body glucose uptake was not affected by rapamycin. During prandial-like peripheral hyperinsulinemia, rapamycin increased glucose uptake compared with placebo by 17% ($R_{d|300–360\ min}$, 75 ± 5 vs. 64 ± 5 μmol · kg^{-1} · min^{-1}, P = 0.0008). Rapamycin affected endogenous glucose production neither at baseline nor during low or prandial-like peripheral hyperinsulinemia. Combined hyperaminoacidemia and prandial-like hyperinsulinemia increased S6K phosphorylation and inhibitory insulin receptor substrate-1 (IRS-1) phosphorylation at Ser312 and Ser636 in the placebo group. Rapamycin partially inhibited this increase in mTOR-mediated S6K phosphorylation and IRS-1 Ser312 and Ser636 phosphorylation. In conclusion, rapamycin stimulates insulin-mediated glucose uptake in man under conditions known to activate the mTOR/S6K pathway. *Diabetes* 56:1600–1607, 2007

From the [1]Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria; the [2]Department of Surgery, Medical University of Vienna, Vienna, Austria; and the [3]Medical Department, Hanusch Hospital, Vienna, Austria.

Address correspondence and reprint requests to Michael Krebs, MD, Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. E-mail: michael.krebs@meduniwien.ac.at.

Received for publication 21 July 2006 and accepted in revised form 20 February 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 28 February 2007. DOI: 10.2337/db06-1016.

EGP, endogenous glucose production; FFA, free fatty acid; GIR, glucose infusion rate; IRS-1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; S6K, S6 kinase.

Type 2 diabetes is closely linked to obesity and insulin resistance (1–3). In addition to polygenic predisposition, environmental factors including quality and quantity of food supply, dietary behavior, and physical activity are of major importance for the development of type 2 diabetes (4,5). The availability of nutrients plays a pivotal role in the modulation of insulin action (6,7). In industrialized countries, nutrient excess comprises high intake of not only fat but also proteins (8). A chronic excess in protein intake is associated with insulin resistance, glucose intolerance, and type 2 diabetes (9–12). We have shown that a short-term rise in plasma free fatty acids (FFAs) (13–15) or amino acids (16–18) leads to decreased insulin-stimulated whole-body glucose disposal, which is preceded by an impaired rise in intramuscular glucose-6-phosphate concentrations and followed by reduction in rates of glycogen synthesis. These findings indicate that both FFAs and amino acids directly inhibit skeletal muscle glucose transport/phosphorylation (17).

The mammalian target of rapamycin (mTOR) pathway (19) could be involved in sensing of nutrient availability and modulation of insulin action in vivo. Recent reports indicate that the activity of the mTOR pathway is increased in rodent models of obesity and that mice deficient in S6 kinase (S6K), a downstream effector of mTOR, are protected against diet-induced insulin resistance (20,21). Insulin activation of S6K is potentiated by increased amino acid availability, which leads to increased inhibitory serine phosphorylation of IRS-1 and insulin resistance of glucose uptake in muscle cells in vitro (22). We have shown that combined hyperinsulinemia and hyperaminoacidemia *1*) results in overactivation of S6K, *2*) stimulates inhibitory IRS-1 phosphorylation at Ser312 and Ser636, and *3*) induces insulin resistance in humans (18). However, whether these are unrelated responses or whether mTOR/S6K activation is causally linked to skeletal muscle insulin resistance in vivo in humans is unclear.

In addition to impaired insulin-stimulated skeletal muscle glucose uptake (3,23,24), increased endogenous glucose production (EGP) (25–27) contributes to hyperglycemia in type 2 diabetic patients. Enhanced EGP by stimulation of hepatic gluconeogenesis has been observed in response to elevated plasma amino acid concentrations (28,29). Recently, it has been shown that the mTOR/S6K pathway is activated not only in skeletal muscle and adipose tissue but also in the liver of fat-fed obese rats (21). However, so far it is not known whether

the mTOR pathway plays a role in the modulation of hepatic glucose metabolism in humans.

This study was therefore designed to determine whether a causal link exists between nutrient-dependent mTOR activation and modulation of glucose metabolism in man. To this end, the effects of rapamycin, a very specific inhibitor of mTOR (30), on rates of EGP and glucose disappearance (R_d) at baseline and under conditions known to activate mTOR in humans were measured.

RESEARCH DESIGN AND METHODS

Eleven healthy male volunteers (age, 28.3 ± 1.4 years; BMI, 22.8 ± 0.7 kg/m^2; body wt, 75.3 ± 2.8 kg) without family history of diabetes or dyslipidemia were studied. They were neither suffering from conditions related to insulin resistance nor taking any medication. None of the study participants were on intensive exercise training, and they stopped regular moderate exercising at least 3 days before the experiments to exclude its acute metabolic effects. They were instructed to ingest an isocaloric diet (carbohydrate:protein:fat 55%:15%:30%) during the 2 days preceding the studies. Subjects with unusual dietary behaviors like extremely low (vegetarians) or high protein intake were not included. The 2 study days were separated by 2–6 weeks during which their body weight and lifestyle remained unchanged. The protocol was approved by the local ethical board, and informed consent was obtained from all subjects after the nature and possible consequences of the procedures had been explained to them.

To determine the effects of mTOR inhibition, eight participants (age, 29 ± 1.2 years; BMI, 22.8 ± 1.8 kg/m^2) were studied twice in random order. On 1 day, rapamycin (Rapamune; Wyeth, Berkshire, United Kingdom; 6 mg p.o.) was administered, and on another day, placebo was ingested at −120 min. All studies began at 7:30 A.M. (−150 min) after a 12-h overnight fast, with the insertion of catheters (Vasofix; Braun, Melsungen, Germany) into one antecubital vein of the left and one of the right arm for blood sampling and infusions, respectively. On both days, plasma amino acid concentrations were raised by infusion (0–360 min) of a mixture of amino acids (0.18 g · kg^{-1} · h^{-1}) (Aminoplasmal 10% without electrolytes; Braun) that is commonly used for parenteral nutrition (16,18,28). Pancreatic clamps were performed as described previously (13,16,18,28,31). Somatostatin (UCB Pharma, Vienna, Austria) was infused (−5 to 360 min; 0.1 μg · kg^{-1} · min^{-1}) to suppress amino acid–induced endogenous secretion of glucoregulatory hormones. From 0 min to 180 min, insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was continuously replaced at a rate of 0.2 mU · kg^{-1} · min^{-1}, and from 180 to 360 min, a primed-continuous insulin infusion (1 mU · kg^{-1} · min^{-1}) was administered to create standardized conditions of a two-step (peripheral) hyperinsulinemic clamp (0–180 min: low peripheral hyperinsulinemia, ~100 pmol/l; 180–360 min: prandial-like peripheral hyperinsulinemia, ~450 pmol/l). Plasma glucose was maintained at ~5.5 mmol/l using a variable D-glucose infusion (20%). Glucose turnover rates were determined using D-[6,6-$^2H_2$]glucose (99% enrichment; Cambridge Isotope Laboratories; bolus, 16.7 μmol/kg; continuous infusion from −150 to 360 min, 0.17 μmol · kg^{-1} · min^{-1}). To maintain stable enrichments of D-[6,6-$^2H_2$]glucose during the clamp tests, the variable glucose infusion was enriched to 2% with D-[6,6-$^2H_2$]glucose (16,18). Blood samples for determination of glucoregulatory hormones, enrichments in plasma glucose, FFAs, and amino acids were drawn at defined time points.

Three participants (age, 26.0 ± 0.6 years; BMI, 22.8 ± 1.5 kg/m^2) were studied twice in random order after administration of rapamycin or placebo identically as described above, except that no D-[6,6-$^2H_2$]glucose was administered. Instead of measuring glucose turnover, needle biopsies of the vastus lateralis muscle were performed as described previously (18). Briefly, the subjects were resting supine, and the vastus lateralis muscle was prepared sterilely under subcutaneous lidocaine anesthesia (Xylocain 2%; Astra, Linz, Austria) (32). At baseline (−140 min), a muscle sample was obtained using a modified Bergström biopsy needle with suction; blotted free of blood, fat, and connective tissue; and snap frozen within 30 s in liquid nitrogen. After 30 min of prandial-like hyperinsulinemia (210 min), a repeat muscle biopsy was taken at a site ~4 cm proximal of the first biopsy. All samples were stored in liquid nitrogen until analysis.

Isolated rat muscle strips. Male Sprague-Dawley rats were purchased from the breeding facilities of the Medical University of Vienna (Himberg, Austria). They were kept at an artificial 12-h-light/12-h-dark cycle at constant room temperature and provided with conventional laboratory diet and tap water ad libitum. All experiments were performed according to local law and to the principles of good laboratory animal care. Food, but not water, had been withdrawn 3–4 h before 8- to 10-week-old rats were killed by cervical dislocation between 10:00 and 11:00 A.M. Immediately after killing, two longitudinal strips of extensor digitorum longus muscle per leg were prepared, weighed (~25 mg/strip), and tied under tension on stainless steel clips. Four muscle strips were thus available per rat allowing the paired examination of three concentrations of rapamycin (FRAP/mTOR Inhibitor; Cell Signaling, Beverly, MA) along with an intraindividual control.

According to procedures used earlier (33), muscles were immediately put into coated Erlenmeyer flasks and provided with Cell Culture Medium 199 (pH 7.35, 5.5 mmol/l glucose; Sigma, St. Louis, MO). The medium was supplemented with 0.3% (wt/vol) fatty acid–free BSA, 5 mmol/l HEPES, 300 μmol/l palmitate (dissolved in ethanol; final concentration 0.25% [vol/vol]), 25,000 units/l penicillin G, 25 mg/l streptomycin, and 0.2 mg/l ciprofloxacine. Rapamycin was dissolved in methanol and added to achieve the indicated concentrations of 10, 20, and 40 nmol/l as indicated. The final concentration of methanol was always the same as in the respective control experiment (0.1% [vol/vol]). The flasks were placed into a shaking water bath (37°C; 130 cycles/min) for a pretreatment with rapamycin for 0.5 h (one strip in 3 ml medium per flask). Immediately after pretreatment, muscles were transferred into the identical medium additionally supplemented with a maximally effective concentration of human insulin (100 nmol/l Actrapid; Novo Nordisk). Insulin stimulation lasted 1 h, after which muscles were quickly removed from the flasks, blotted, and frozen in liquid nitrogen. Throughout the whole experiment, an atmosphere of 95% O_2/5% CO_2 was provided within the flasks.

Western blotting. Frozen muscle specimens were crushed into pieces and homogenized with a polytron homogenizer in Weinberg lysis buffer containing 50 mmol/l HEPES, pH 7.0; 0.5% NP-40; 250 mmol/l NaCl; 5 mmol/l EDTA, 5 mmol/l NaF, and protease inhibitors (1 mmol/l sodium orthovanadate, 50 mmol/l β-glycerophosphate, 10 mg/l aprotinine, 2 mmol/l benzamidine, 10 mg/l leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride). Homogenates were centrifuged (3,000*g* for 15 min), and protein concentration of the supernatants was measured by BCA-protein assay (Pierce, Rockford, IL) using a BSA standard curve. Thirty micrograms protein was diluted with SDS-sample buffer and electrophoresed on an 8% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Kassel, Germany). After transfer, nonspecific binding sites were blocked using a blocking reagent from Roche (Mannheim, Germany) followed by exposure to primary antibodies (rabbit anti-S6K IgG [p70 S6K (C-18): sc-230; Santa Cruz Biotechnology]; rabbit phospho-IRS-1 [Ser636/639] IgG [Cell Signaling, Danvers, MA]; rabbit phospho-IRS-1 [Ser312, equivalent to Ser307 and in mice] or rabbit Anti-IRS-1 IgG [Upstate, Lake Placid, NY] overnight under gentle agitation) and secondary antibodies (peroxidase-labeled anti-rabbit IgG [Amersham, Buckinghamshire, United Kingdom]; 90 min, room temperature). Thereafter, membranes were washed and developed using Super Signal System (Pierce). Blots were exposed to Kodak XAR-Omat films, and semiquantitative analysis of visualized bands was performed by densitometry (Gel Documentation System; MWG Biotech, Ebersberg, Germany) using Gene Profiler 3.56 for Windows.

To quantify the phosphorylation status of S6K, the different electrophoretic mobilities of the hyperphosphorylated and hypophosphorylated forms of S6K were exploited as described previously (34–37). The ratio of the more heavily phosphorylated and thus more slowly migrating forms to the total immune reactivity found in the sample reflects the activity of S6K. The amount of serine-phosphorylated IRS-1 was related to total IRS-1 protein by homologous immunoblotting to minimize effects of variation in loading of the SDS gels.

Plasma metabolites and hormones. Plasma glucose concentrations were measured by the glucose oxidase method (Glucose analyzer II; Beckman Instruments, Fullerton, CA). Concentrations of individual plasma amino acid were measured by high-performance liquid chromatography (38). Plasma FFA concentrations were determined with a microfluorimetric method (Wako Chem USA, Richmond, VA). Plasma immunoreactive insulin (Pharmacia, Uppsala, Sweden), C-peptide (Cis, Gif-Sur-Yvette, France), glucagons (Linco, St. Charles, MO), and growth hormone (Sorin Biomedica, Saluggia, Italy) were measured by commercially available radioimmunoassays (13,16,28,31,39). Plasma cortisol was determined by RIA (40).

Gas chromatography–mass spectrometry for the determination of tracer-to-tracee ratios of ^2H in glucose was performed as described previously (16,28). The glucose-pentaacetate was analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with a CP-Sil5 25-m × 0.25-mm × 0.12-μm capillary column (Chrompack, Middelburg, NL) interfaced to a Hewlett-Packard 5971A Mass Selective Detector operating in the electron impact ionization mode. Selective ion monitoring was used to determine tracer enrichments in various molecular mass ion fragments of glucose. 6,6-$^2H_2$ (M+2) enrichments in glucose were assessed using fragments of C3-C6 with their masses of 187 and 189, respectively.

Calculations. Rate of glucose appearance (R_a) and R_d were calculated using Steele's non–steady-state equations modified for the use of stable isotopes (16,28) with EGP given as the difference between R_a and glucose infusion rates (GIRs).

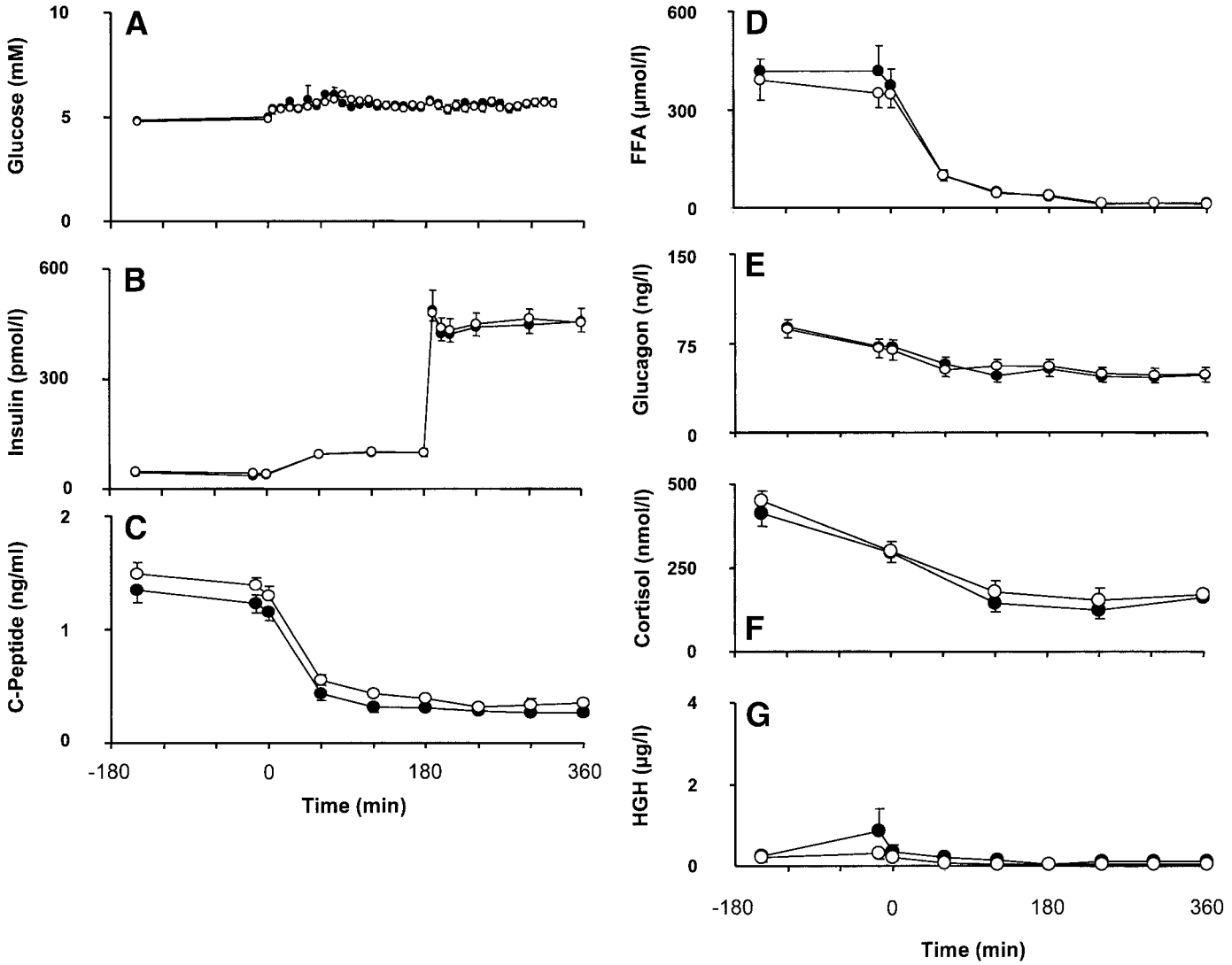

FIG. 1. Metabolites and hormones. Plasma concentrations of glucose (*A*), insulin (*B*), C-peptide (*C*), FFAs (*D*), glucagon (*E*), cortisol (*F*), and growth hormone (HGH) (*G*) after administration of placebo (○) and rapamycin (●). Data are given as means ± SE of eight healthy subjects (error bars smaller than the symbol size are not visible).

Data analysis and statistics. Changes of sequential (time-dependent) data within protocols were analyzed by ANOVA for repeated measurements and Dunnetts' post hoc test, and differences between the paired groups were analyzed with the paired Student's *t* test. Differences were considered significant at P values <0.05.

RESULTS

Effects of rapamycin on glucose metabolism

Metabolites and hormones. Fasting (−150 min) plasma glucose (rapamycin, 4.84 ± 0.11 mmol/l; placebo, 4.76 ± 0.10 mmol/l), insulin (rapamycin, 46 ± 6 pmol/l; placebo, 47 ± 5 pmol/l), C-peptide (rapamycin, 1.35 ± 0.11 ng/ml; placebo, 1.49 ± 0.10 ng/ml), FFAs (rapamycin, 417 ± 36 µmol/l; placebo, 391 ± 64 µmol/l), glucagon (rapamycin, 89 ± 7 ng/l; placebo, 87 ± 7 ng/l), cortisol (rapamycin, 413 ± 17 nmol/l; placebo, 449 ± 32 nmol/l), and growth hormone (rapamycin, 0.23 ± 0.12 µg/l; placebo, 0.22 ± 0.11 µg/l) were not different between amino acid infusion and control studies (Fig. 1).

During the clamp tests, plasma glucose remained at baseline and was not different between the studies (Fig. 1*A*). Plasma insulin concentrations rose similarly to ~100 pmol/l during low-dose insulin replacement and thereafter quickly increased further to ~450 pmol/l during high-dose insulin infusion (Fig. 1*B*), whereas plasma C-peptide was equally suppressed in both studies (Fig. 1*C*). Plasma FFAs and glucagon similarly decreased during the pancreatic clamps without any difference between the two protocols (Fig. 1*D* and *E*). Plasma cortisol and growth hormone concentrations were also similar between the two protocols (Fig. 1*F* and *G*).

Fasting plasma amino acid concentrations were comparable in both studies (rapamycin, 2.69 ± 0.06 mmol/l; placebo, 2.53 ± 0.06 mmol/l; Fig. 2; Table 1). Total plasma amino acid concentrations comparably increased by ~2.5-fold during both studies (Fig. 2) with all individual amino acids, except for tyrosine, contributing to this rise in total plasma amino acid (Table 1).

Rapamycin was detectable only after oral administration of the drug (0 min: rapamycin, 10.8 ± 1.3 nmol/l, placebo, <2 nmol/l; 360 min: rapamycin, 8.0 ± 1.0 nmol/l, placebo, <2 nmol/l).

Glucose turnover. GIRs required to maintain euglycemia were comparable in the presence of low peripheral hyperinsulinemia (120–180 min: rapamycin, 14.0 ± 1.8 µmol · kg^{-1} · min^{-1}; placebo, 10.9 ± 3.5 µmol · kg^{-1} · min^{-1}; NS). However, in the presence of prandial-like peripheral hy-

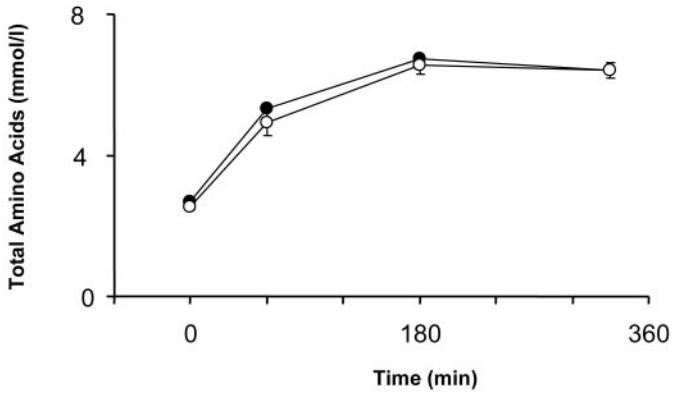

FIG. 2. Total amino acid concentrations. Plasma concentrations of total amino acids after administration of placebo (○) and rapamycin (●). Data are given as means ± SE of eight healthy subjects (error bars smaller than the symbol size are not visible).

perinsulinemia, GIRs were ~15% higher after rapamycin administration compared with placebo (300–360 min: rapamycin, 69.1 ± 4.3 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; placebo, 60.1 ± 4.9 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; $P < 0.01$). Tracer-determined rates of R_d were similar between both studies at baseline and in the presence of low peripheral hyperinsulinemia, but in the presence of prandial-like peripheral hyperinsulinemia, R_d was ~17% higher ($P < 0.001$) after ingestion of rapamycin compared with control studies (Fig. 3*A*).

EGP was comparable between both studies at baseline (rapamycin, 10.1 ± 0.4 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; placebo, 11.0 ± 0.6 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) as well as in the presence of low peripheral hyperinsulinemia and prandial-like peripheral hyperinsulinemia (Fig. 3*B*).

Effects of rapamycin on S6K phosphorylation and inhibitory serine phosphorylation of IRS-1

To confirm that rapamycin inhibits stimulated mTOR activity, the phosphorylation status of S6K, a downstream target of mTOR, was measured in skeletal muscle. Incubation of rat skeletal muscle with insulin potently in-

TABLE 1
Plasma concentrations of individual amino acids at baseline (0 min) and during amino acid infusion after administration of rapamycin or placebo (360 min)

Amino acids	Time (min)	Rapamycin		Placebo	
		μmol/l	Fold change vs. 0 min	μmol/l	Fold change vs. 0 min
Alanine	0	362 ± 37		372 ± 38	
	360	1,414 ± 53*	3.9	1,291 ± 55*	3.5
Arginine	0	113 ± 7		108 ± 7	
	360	423 ± 13*	3.7	431 ± 13*	4.0
Asparagine	0	57 ± 2		51 ± 1†	
	360	171 ± 7*	3.0	165 ± 7*	3.2
Citrulline	0	41 ± 3		42 ± 3	
	360	78 ± 4*	1.9	79 ± 5*	1.9
Glutamine	0	652 ± 21		638 ± 22	
	360	883 ± 91‡	1.4	940 ± 20*	1.5
Glutamate	0	27 ± 3		28 ± 4	
	360	57 ± 8§	2.1	64 ± 8§	2.3
Glycine	0	252 ± 15		259 ± 24	
	360	928 ± 36*	3.7	922 ± 43*	3.6
Histidine	0	90 ± 3		89 ± 3	
	360	262 ± 12*	2.9	267 ± 11*	3.0
Isoleucine	0	80 ± 3		70 ± 2†	
	360	169 ± 8*	2.1	168 ± 12*	2.4
Leucine	0	154 ± 6		139 ± 5	
	360	357 ± 16*	2.3	354 ± 20*	2.6
Methionine	0	40 ± 2		35 ± 2	
	360	205 ± 8*	5.1	202 ± 9*	5.8
Phenylalanine	0	58 ± 2		53 ± 3	
	360	181 ± 6*	3.1	180 ± 8*	3.4
Serine	0	129 ± 8		117 ± 5	
	360	264 ± 19*	2.0	253 ± 14*	2.2
Taurine	0	35 ± 1		38 ± 2	
	360	46 ± 1*	1.3	50 ± 3*	1.3
Threonine	0	156 ± 11		135 ± 8	
	360	416 ± 17*	2.7	380 ± 7*	2.8
Tryptophan	0	51 ± 2		49 ± 2	
	360	93 ± 5*	1.8	91 ± 4*	1.9
Tyrosine	0	67 ± 2		62 ± 3	
	360	66 ± 5	1.0	53 ± 4	0.9
Valine	0	273 ± 19		247 ± 10	
	360	444 ± 17*	1.6	431 ± 21*	1.7

Data are means ± SE of eight healthy subjects who underwent somatostatin-insulin clamp tests. *$P < 0.001$ vs. corresponding baseline value. †$P < 0.05$ vs. RAPA. ‡$P < 0.05$ vs. corresponding baseline value. §$P < 0.01$ vs. corresponding baseline value.

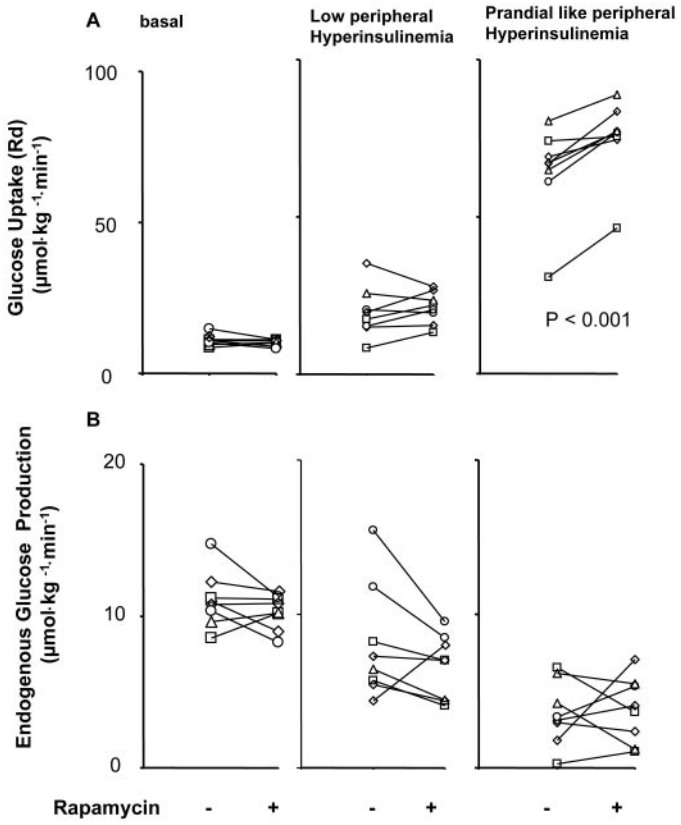

FIG. 3. Effects of rapamycin on glucose turnover. Individual rates of glucose uptake (R_d) (*A*) and EGP (*B*) at baseline (−15 to 0 min) and in the presence of low (120–180 min) and prandial-like (300–360 min) peripheral hyperinsulinemia after administration of placebo or rapamycin. Individual data of eight healthy subjects are given.

creased S6K phosphorylation in vitro (~2.4-fold increase over baseline, $P < 0.001$). As expected, rapamycin dose dependently inhibited insulin-stimulated S6K phosphorylation. Preincubation of skeletal muscle strips with 10 nmol/l rapamycin decreased insulin-stimulated S6K phosphorylation by ~50% (NS), whereas an increase in rapamycin concentration to 40 nmol/l inhibited the effect of insulin on S6K phosphorylation by ~70% ($P < 0.01$). In the absence of insulin, rapamycin did not affect S6K phosphorylation in explanted rat muscle (data not shown).

An approximate fourfold increase in S6K phosphorylation over baseline ($P < 0.01$; Fig. 4*A*) was observed in biopsies of skeletal muscle of placebo-treated humans obtained during combined prandial-like peripheral hyperinsulinemia and hyperaminoacidemia in placebo studies in vivo. Rapamycin did not affect basal S6K phosphorylation. However, stimulated S6K phosphorylation tended to be lower ($P = 0.06$) after rapamycin administration (Fig. 4*A*).

Because S6K plays a prominent role in mediation of inhibitory serine phosphorylation of IRS-1 (18,20), we assessed whether phosphorylation of IRS-1 was affected by treatment with rapamycin using phospho-specific antibodies against Ser312 and Ser636 (equivalent to Ser307 and Ser632 in mice, respectively). In placebo studies, combined prandial-like peripheral hyperinsulinemia and hyperaminoacidemia was associated with a significant increase in the phosphorylation state of IRS-1 on Ser312 (Fig. 4*B*) and Ser636 (Fig. 4*C*). Rapamycin administration did not affect phosphorylation of these sites at baseline. However, partial inhibition of stimulated mTOR/S6K activity by rapamycin was sufficient to block the increase in inhibitory phosphorylation of IRS-1 in response to combined prandial-like peripheral hyperinsulinemia and hyperaminoacidemia (Fig. 4*C* and *D*).

DISCUSSION

We have recently shown that increased amino acid availability results in overactivation of S6K and inhibitory serine-phosphorylation of IRS-1 as well as reduction of insulin-stimulated glucose metabolism in human skeletal muscle (18). The present study was performed to distinguish whether these responses are independent consequences of increased amino acid availability or are in causal relationship. The latter has been suggested by in vitro studies, which indicated that the mTOR/S6K pathway could operate a feedback loop toward IRS-1, causing inhibition of phosphatidylinositol 3-kinase and, consequently, muscle glucose uptake (22,41).

The mTOR inhibitor rapamycin was the ideal tool to answer the posed question because it is one of the most potent and specific kinase inhibitors known (30) and absorbed rapidly with peak plasma concentrations reached after ~60 min (42).

This study shows that mTOR regulates skeletal muscle glucose uptake in vivo in humans. Rapamycin administration augments insulin-stimulated glucose uptake in the presence of combined hyperaminoacidemia and prandial-like peripheral hyperinsulinemia. In the presence of hyperinsulinemia, the majority of glucose is taken up by skeletal muscle (3). Therefore, the observed increase in glucose disposal is likely to relate at least in part to a rapamycin-induced increase in skeletal muscle glucose uptake. The determination of S6K phosphorylation in biopsies of human skeletal muscle confirmed a marked stimulation of the mTOR/S6K pathway under these conditions (18). However, at baseline and during combined hyperaminoacidemia and low peripheral hyperinsulinemia, no effect of rapamycin on glucose uptake was observed. This likely indicates that rapamycin affects glucose metabolism only in the presence of pronounced mTOR/S6K activation. This is in line with the observation that rapamycin did not affect S6K phosphorylation and inhibitory IRS-1 phosphorylation under basal (unstimulated) conditions.

It has been suggested that nutrient-dependent stimulation of the mTOR/S6K pathway could induce insulin resistance not only in skeletal muscle (18) but also in the liver (21). However, rapamycin affected EGP in healthy humans neither at baseline nor in the presence of combined hyperaminoacidemia and hyperinsulinemia, indicating no effect on hepatic glucose metabolism.

Some limitations of our study must be considered. First, because of the long half-life of the drug and potential adverse effects, the dose of rapamycin administered to healthy volunteers was not higher than generally recommended for initiation of immunosuppressive therapy after renal transplantation (43). It turned out that this single oral dose leads to circulating concentrations of rapamycin (~9 nmol/l) that were very likely not sufficient to completely inhibit the mTOR/S6K pathway. Our experiments on explanted rat skeletal muscle suggested that approximate fourfold higher doses of rapamycin are necessary for ~70% inhibition of stimulated S6K phosphorylation. Therefore, it appears likely that our results underestimate the contribution of mTOR to the modulation of insulin-stimulated glucose uptake.

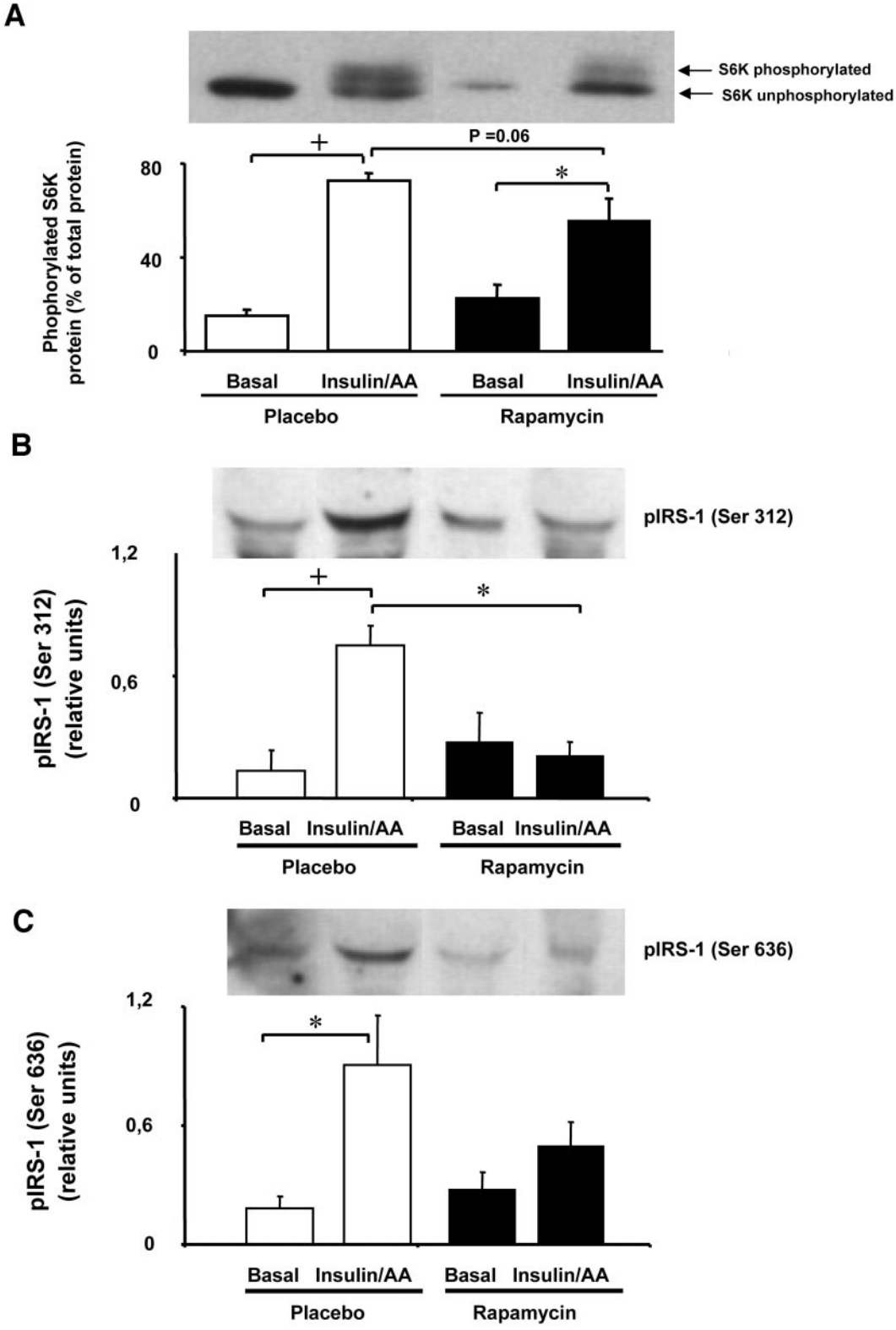

FIG. 4. Effects of rapamycin on S6K phosphorylation and on serine phosphorylation of IRS-1. *A*: S6K phosphorylation in human skeletal muscle in vivo under basal conditions versus combined hyperaminoacidemia and hyperinsulinemia in the absence and presence of rapamycin. Phosphorylation status of IRS-1 on Ser312 (*B*) and Ser636 (*C*) as determined using specific antibodies. Data are given as means ± SE of three healthy subjects studied twice after administration of rapamycin or placebo. Representative autoradiograms (*A–C*) were obtained from muscle biopsies from a single individual. *$P < 0.05$, +$P < 0.01$.

Second, we have studied short-term effects of rapamycin on glucose metabolism. Hence, the results are difficult to extrapolate to a clinical setting of chronic immunosuppressive therapy. Rapamycin (also known as Rapamune or sirolimus) is widely used to prevent graft rejection in transplant recipients (44). Because rapamycin counteracts the proposed inhibitory effect of mTOR/S6K activation on IRS-1 signaling, it has been suggested that this drug might

be effective for the treatment of metabolic disorders including insulin resistance (41). However, a major side effect of rapamycin is hyperlipidemia that could be secondary to inhibition of adipocyte differentiation and inhibition of peroxisome proliferator–activated receptor-γ activity (45). Furthermore, rapamycin has deleterious effects on pancreatic islets in vitro (46). Although both mechanisms could facilitate the development of impaired glucose tolerance and diabetes in humans (14,47), loss of mTOR/S6K activity correlates with decreased fat accumulation and protects against diet-induced obesity and insulin resistance in mice (20). In light of such versatile findings, further studies are needed to clearly delineate chronic effects of rapamycin on glucose metabolism in vivo.

Third, in the present study, total plasma amino acid concentrations observed during amino acid infusion are ~40% higher than those seen after ingestion of a large-size (50 g) protein meal (48). Furthermore, plasma amino acid concentrations were maintained at this elevated level throughout the infusion. Thus, it cannot be ruled out that stimulation of the mTOR/S6K pathway might have been attenuated when a shorter and somewhat lower increase in plasma amino acid concentrations would have been present.

Fourth, epidemiological studies showing that a chronic excess in protein intake is associated with insulin resistance, glucose intolerance, and type 2 diabetes (9–12) suggest that various states of insulin resistance might be associated with a chronic activation of the mTOR/S6K pathway. However, in the present study, this pathway was acutely activated by short-term elevation of plasma insulin and amino acid concentrations in healthy insulin-sensitive subjects. Therefore, it cannot be excluded that insulin resistance associated with a more chronic activation of the mTOR/S6K pathway does not quickly respond to mTOR inhibition.

In summary, we show that the activity state of the mTOR pathway can modulate insulin sensitivity in humans. Our results suggest that the mTOR/S6K pathway could have an important role in insulin desensitization under nutrient abundance and, therefore, could be a target in the pharmacological prevention and treatment of nutrient-induced insulin resistance.

ACKNOWLEDGMENTS

This work was in part supported by an unrestricted research grant from Novo Nordisk (Vienna). M.K. has received grants from the Austrian Diabetes Association and the Austrian Association of Internal Medicine (Josef Skoda Award).

We gratefully acknowledge the excellent cooperation with F. Garo, A. Hofer, and the Endocrine Research Laboratory.

REFERENCES

1. Reaven GM: Role of insulin resistance in human disease. *Diabetes* 37:1595–1607, 1988
2. DeFronzo R: Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. *Diabetes Rev* 5:177–269, 1997
3. Shulman G, Rothman D, Jue T, Stein P, DeFronzo R, Shulman R: Quantification of muscle glycogen synthesis in normal subjects and subjects with non-insulin dependent diabetes by C-13 nuclear magnetic resonance spectroscopy. *N Engl J Med* 322:223–228, 1990
4. Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Laasko M, Louheranta A, Rastas M, Salminen V, Uusitupa M: Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 344:1343–1350, 2001
5. Group DPPR: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346:393–403, 2002
6. Patti M: Nutrient modulation of insulin action. *Ann NY Acad Sci* 892:187–203, 1999
7. Krebs M, Roden M: Nutrient-induced insulin resistance in human skeletal muscle. *Curr Med Chem* 11:1241–1253, 2004
8. Karg G: Nutrition in Germany. In *German Nutrition Report*. Society GN, Ed. Frankfurt, Henrich, 2000, p. 17–77
9. Linn T, Geyer R, Prassek S, Laube H: Effect of dietary protein intake on insulin secretion and glucose metabolism in insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 81:3938–3943, 1996
10. Linn T, Grönemeyer D, Aygen S, Scholz N, Busch M, Bretzel R: Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia* 43:1257–1265, 2000
11. Schulze MB, Manson JE, Willett WC, Hu FB: Processed meat intake and incidence of type 2 diabetes in younger and middle-aged women. *Diabetologia* 46:1465–1473, 2003
12. Promintzer M, Krebs M: Effects of dietary protein on glucose homeostasis. *Curr Opin Clin Nutr Metab Care* 9:463–468, 2006
13. Krebs M, Krssak M, Nowotny P, Weghuber D, Gruber S, Mlynarik V, Bischof M, Stingl H, Furnsinn C, Waldhausl W, Roden M: Free fatty acids inhibit the glucose-stimulated increase of intramuscular glucose-6-phosphate concentration in humans. *J Clin Endocrinol Metab* 86:2153–2160, 2001
14. Krebs M, Roden M: Molecular mechanisms of lipid-induced insulin resistance in muscle, liver and vasculature. *Diabetes Obes Metab* 7:621–632, 2005
15. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI: Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97:2859–2865, 1996
16. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, Nowotny P, Roth E, Waldhausl W, Roden M: Mechanism of amino acid–induced skeletal muscle insulin resistance in humans. *Diabetes* 51:599–605, 2002
17. Krebs M, Roden M: Nutrient-induced insulin resistance in human skeletal muscle. *Curr Med Chem* 11:901–908, 2004
18. Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, Nowotny P, Waldhausl W, Marette A, Roden M: Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* 54:2674–2684, 2005
19. Chen J, Fang Y: A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochem Pharmacol* 64:1071–1077, 2002
20. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G: Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431:200–205, 2004
21. Khamzina L, Veilleux A, Bergeron S, Marette A: Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology* 146:1473–1481, 2005
22. Tremblay F, Marette A: Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. *J Biol Chem* 276:38052–38060, 2001
23. Petersen KF, Hendler R, Price T, Perseghin G, Rothman DL, Held N, Amatruda JM, Shulman GI: $^{13}C/^{31}P$ NMR studies on the mechanism of insulin resistance in obesity. *Diabetes* 47:381–386, 1998
24. Cline GW, Petersen KF, Krssak M, Shen J, Hundal RS, Trajanosky Z, Inzucchi S, Dresner A, Rothman DL, Shulman GI: Impaired glucose transport as a cause of decreased insulin- stimulated muscle glycogen synthesis in type 2 diabetes. *N Engl J Med* 341:240–246, 1999
25. DeFronzo RA: The triumvirate: β-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 37:667–687, 1988
26. Anderwald C, Bernroider E, Krssak M, Stingl H, Brehm A, Bischof MG, Nowotny P, Roden M, Waldhäusl W: Effects of insulin treatment in type 2 diabetic patients on intracellular lipid content in liver and skeletal muscle. *Diabetes* 51:3025–3032, 2002
27. Magnusson I, Rothman D, Katz L, Shulman R, Shulman G: Increased rate of gluconeogenesis in type II diabetes mellitus: C-13 nuclear magnetic resonance study. *J Clin Invest* 90:1323–1327, 1992
28. Krebs M, Brehm A, Krssak M, Anderwald C, Bernroider E, Nowotny P, Roth E, Chandramouli V, Landau BR, Waldhausl W, Roden M: Direct and indirect effects of amino acids on hepatic glucose metabolism in humans. *Diabetologia* 46:917–925, 2003
29. Exton JH, Mallette LE, Jefferson LS, Wong EHA, Friedmann N, Miller TB, Park CR: The hormonal control of gluconeogenesis. *Recent Prog Horm Res* 26:411–461, 1970

30. Davies SP, Reddy H, Caivano M, Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105, 2000
31. Krebs M, Geiger M, Polak K, Vales A, Schmetterer L, Wagner OF, Waldhausl W, Binder BR, Roden M: Increased plasma levels of plasminogen activator inhibitor-1 and soluble vascular cell adhesion molecule after triacylglycerol infusion in man. *Thromb Haemost* 90:422–428, 2003
32. Hojlund K, Staer P, Hansen BF, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski FP: Increased phosphorylation of skeletal muscle glycogen synthase at NH_2-terminal sites during physiological hyperinsulinemia in type 2 diabetes. *Diabetes* 52:1393–1402, 2003
33. Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhausl W, Furnsinn C: Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes* 53:1052–1059, 2004
34. Greiwe JS, Kwon G, McDaniel ML, Semenkovich CF: Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. *Am J Physiol Endocrinol Metab* 281:E466–E471, 2001
35. Hillier T, Long W, Jahn L, Wei L, Barrett EJ: Physiological hyperinsulinemia stimulates p70(S6k) phosphorylation in human skeletal muscle. *J Clin Endocrinol Metab* 85:4900–4904, 2000
36. Liu Z, Wu Y, Nicklas EW, Jahn LA, Price WJ, Barrett EJ: Unlike insulin, amino acids stimulate p70S6K but not GSK-3 or glycogen synthase in human skeletal muscle. *Am J Physiol Endocrinol Metab* 286:E523–E528, 2004
37. Karlsson HK, Nilsson PA, Nilsson J, Chibalin AV, Zierath JR, Blomstrand E: Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. *Am J Physiol Endocrinol Metab* 287:E1–E7, 2004
38. Spittler A, Sautner T, Gornikiewicz A, Manhart N, Oehler R, Bergmann M, Függer R, Roth E: Postoperative glycyl-glutamine infusion reduces immunosuppression: partial prevention of the surgery induced decrease in HLA-DR expression on monocytes. *Clin Nutr* 20:37–42, 2001
39. Stingl H, Krssak M, Krebs M, Bischof MG, Nowotny P, Furnsinn C, Shulman GI, Waldhausl W, Roden M: Lipid-dependent control of hepatic glycogen stores in healthy humans. *Diabetologia* 44:48–54, 2001
40. Waldhäusl W, Herkner K, Nowotny P, Bratusch-Marrain P: Combined 17- and 18-hydroxylase deficiency associated with complete male pseudohermaphroditism and hypoaldosteronism. *J Clin Endocrinol Metab* 46: 236, 1978
41. Wullschleger S, Loewith R, Hall MN: TOR signaling in growth and metabolism. *Cell* 124:471–484, 2006
42. Brattstrom C, Sawe J, Jansson B, Lonnebo A, Nordin J, Zimmerman JJ, Burke JT, Groth CG: Pharmacokinetics and safety of single oral doses of sirolimus (rapamycin) in healthy male volunteers. *Ther Drug Monit* 22:537–544, 2000
43. Yakupoglu YK, Kahan BD: Sirolimus: a current perspective. *Exp Clin Transplant* 1:8–18, 2003
44. Kahan BD, The Rapamune US Study Group: Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomised multicentre study. *Lancet* 356:194–202, 2000
45. Kim JE, Chen J: Regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis. *Diabetes* 53:2748–2756, 2004
46. Bell E, Cao X, Moibi JA, Greene SR, Young R, Trucco M, Gao Z, Matschinsky FM, Deng S, Markman JF, Naji A, Wolf BA: Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets. *Diabetes* 52:2731–2739, 2003
47. Teutonico A, Schena PF, Di Paolo S: Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus. *J Am Soc Nephrol* 16:3128–3135, 2005
48. Adibi SA, Mercer DW: Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentration after meals. *J Clin Invest* 52:1586–1594, 1973