

# Overactivation of S6 Kinase 1 as a Cause of Human Insulin Resistance During Increased Amino Acid Availability

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To examine the molecular mechanisms by which plasma amino acid elevation impairs insulin action, we studied seven healthy men twice in random order during infusion of an amino acid mixture or saline (total plasma amino acid ~6 vs. ~2 mmol/l). Somatostatin-insulin-glucose clamps created conditions of low peripheral hyperinsulinemia (~100 pmol/l, 0–180 min) and prandial-like peripheral hyperinsulinemia (~430 pmol/l, 180–360 min). At low peripheral hyperinsulinemia, endogenous glucose production (EGP) did not change during amino acid infusion but decreased by ~70% during saline infusion ( $EGP_{150-180 \text{ min}} 11 \pm 1$  vs.  $3 \pm 1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ,  $P = 0.001$ ). Prandial-like peripheral hyperinsulinemia completely suppressed EGP during both protocols, whereas whole-body rate of glucose disappearance ( $R_d$ ) was ~33% lower during amino acid infusion ( $R_{d \text{ 330-360 min}} 50 \pm 4$  vs.  $75 \pm 6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ,  $P = 0.002$ ) indicating insulin resistance. In skeletal muscle biopsies taken before and after prandial-like peripheral hyperinsulinemia, plasma amino acid elevation markedly increased the ability of insulin to activate S6 kinase 1 compared with saline infusion (~3.7- vs. ~1.9-fold over baseline). Furthermore, amino acid infusion increased the inhibitory insulin receptor substrate-1 phosphorylation at Ser312 and Ser636/639 and decreased insulin-induced phosphoinositide 3-kinase activity. However, plasma amino acid elevation

failed to reduce insulin-induced Akt/protein kinase B and glycogen synthase kinase 3 $\alpha$  phosphorylation. In conclusion, amino acids impair 1) insulin-mediated suppression of glucose production and 2) insulin-stimulated glucose disposal in skeletal muscle. Our results suggest that overactivation of the mammalian target of rapamycin/S6 kinase 1 pathway and inhibitory serine phosphorylation of insulin receptor substrate-1 underlie the impairment of insulin action in amino acid-infused humans. *Diabetes* 54:2674–2684, 2005

Recent data strongly support the notion that nutrient excess plays a predominant role in the development of insulin resistance and manifestation of type 2 diabetes (1–5). Meat consumption has increased by one third in industrialized countries since the year 1960 (6), resulting in excess nutrient supply of fat and proteins. The hypothesis that increased availability of amino acids might modulate glucose metabolism is supported by recent data showing that a rise in plasma amino acids directly inhibits insulin-stimulated glucose transport/phosphorylation into skeletal muscle, leading to impaired muscle glycogen synthesis and reduced insulin-stimulated whole-body glucose disposal in healthy humans (7). Furthermore, plasma amino acid elevation enhances gluconeogenesis sufficiently to induce hyperglycemia in the presence of impaired insulin secretion (8). Thus, increased availability of amino acids directly modulates hepatic and skeletal muscle glucose metabolism both in the presence of hyperinsulinemia and insulin deficiency. Of note, the effects of short-term amino acid elevation on glucose metabolism in the presence of low peripheral hyperinsulinemia have not yet been evaluated under standardized conditions.

In vitro studies have shown that amino acids generate signals transduced by the serine/threonine kinase mammalian target of rapamycin (mTOR) to regulate protein synthesis and cell proliferation as mediated by S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (9). Thus, mTOR appears to sense the availability of nutrients, most notably amino acids. Interestingly, activation of the mTOR pathway by prolonged insulin stimulation or increased amino acid availability induces insulin resistance in muscle cells and adipocytes

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Received for publication 13 December 2004 and accepted in revised form 27 May 2005.

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4E-BP1, eukaryotic initiation factor 4E-binding protein 1; EGP, endogenous glucose production; FFA, free fatty acid; GSK, glycogen synthase kinase; IRS, insulin receptor substrate; MEM, minimum essential medium; mTOR, mammalian target of rapamycin; PI, phosphoinositide; PKB, protein kinase B; S6K1, S6 kinase 1.

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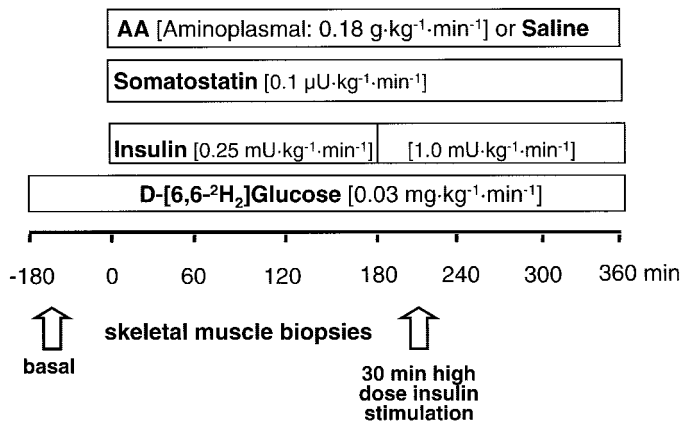


FIG. 1. Experimental protocol.

in vitro (10–12). Activation of mTOR and/or its downstream target, S6K1, leads to serine/threonine phosphorylation and consequently proteosomal degradation of insulin receptor substrate (IRS)-1 (10–12). This in turn results in inhibition of phosphoinositide (PI) 3-kinase activity (13,14), an essential step in insulin-mediated glucose metabolism. We further demonstrated that amino acid-induced mTOR/S6K1 activation speeds up insulin-dependent PI 3-kinase deactivation in cultured myocytes even before detectable IRS-1 degradation, suggesting that uncoupling of IRS-1/PI 3-kinase signaling is an early event in the interaction between amino acids and insulin action (13).

This study was therefore designed to determine the effects of short-term elevation of plasma amino acids on whole-body rate of glucose disappearance ( $R_d$ ) as well as endogenous glucose production (EGP) in the presence of low peripheral hyperinsulinemia as well as during prandial-like peripheral hyperinsulinemia. Furthermore, the insulin-dependent activation of early steps in insulin signaling was simultaneously assessed under these conditions both in biopsies of human skeletal muscle and in cultured L6 skeletal muscle cells.

## RESEARCH DESIGN AND METHODS

Seven healthy male volunteers (age  $25 \pm 0.7$  years, BMI  $23.4 \pm 1.0$  kg/m<sup>2</sup>, body weight  $74.4 \pm 3.5$  kg) without family history of diabetes or dyslipidemia were included in this study. They were neither glucose intolerant nor suffering from conditions related to insulin resistance or 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 behaviors with regard to timing of meal ingestion or dietary composition such as extremely low (vegetarians) or high protein intake were not included. 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.

Seven participants were studied twice during infusion of amino acids and control saline infusion in random order. The two study days were separated by 4–14 weeks during which time their body weight and lifestyle remained unchanged.

After a 12-h overnight fast, all studies began at 7:30 A.M. (–150 min) 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.

Pancreatic clamps were performed as described (7,8,15,16) (Fig. 1). Briefly, somatostatin (UCB Pharma, Vienna, Austria) was infused (–5 to 360 min,  $0.1$  nmol · kg<sup>-1</sup> · min<sup>-1</sup>) to suppress amino acid-induced secretion of glucoregulatory hormones. From 0 to 180 min, insulin (Actrapid; Novo Nordisk,

Bagsvaerd, Denmark) was continuously replaced at a rate of  $0.25$  mU · kg<sup>-1</sup> · min<sup>-1</sup>, and from 180 to 360 min, a primed-continuous insulin infusion ( $1$  mU · kg<sup>-1</sup> · min<sup>-1</sup>) was administered to create standardized conditions of a two-step (peripheral) hyperinsulinemic clamp (0–180 min, low peripheral hyperinsulinemia  $\sim 100$  pmol/l; 180–360 min, prandial-like peripheral hyperinsulinemia  $\sim 430$  pmol/l). Plasma glucose was maintained at  $\sim 5.5$  mmol/l using a variable D-glucose infusion (20%). Glucose turnover rates were determined using D-[6,6-<sup>2</sup>H<sub>2</sub>] glucose (99% enrichment, Cambridge Isotope Laboratories, bolus  $16.7$  μmol/kg, continuous infusion from –120 min to +360 min,  $0.17$  μmol · kg<sup>-1</sup> · min<sup>-1</sup>). To maintain stable enrichments of D-[6,6-<sup>2</sup>H<sub>2</sub>]glucose during the clamp tests, the variable glucose infusion was enriched to 2% with D-[6,6-<sup>2</sup>H<sub>2</sub>]glucose (7). The enrichment of D-[6,6-<sup>2</sup>H<sub>2</sub>]glucose in the variable glucose infusion was adjusted to prevent tracer dilution during the first step (low peripheral hyperinsulinemia) of the clamp, when EGP was expected to rise during amino acid infusion (8). This led to slightly higher enrichment of the variable glucose infusion so that tracer-to-tracee ratios similarly increased within 60 min by  $\sim 44\%$  (amino acid  $42 \pm 0.09$  vs. control  $45 \pm 0.04\%$ ) and remained stable thereafter during both protocols. On one study day, plasma amino acid concentrations were raised by infusion (0–330 min) of a mixture of amino acids ( $0.18$  g · kg<sup>-1</sup> · h<sup>-1</sup>; Aminoplasma 10% without electrolytes; Braun, Melsungen, Germany) which is commonly used for parenteral nutrition (7,8). This solution contains the following L-amino acids: isoleucine (5.1 g/l), leucine (8.9 g/l), valine (4.8 g/l), lysine (5.6 g/l), methionine (3.8 g/l), phenylalanine (5.1 g/l), tryptophan (1.8 g/l), arginine (9.2 g/l), histidine (5.2 g/l), N-acetyl-cysteine (0.7 g/l), proline (8.9 g/l), threonine (4.1 g/l), glutamate (4.6 g/l), serine (2.4 g/l), glycine (7.9 g/l), alanine (13.7 g/l), asparagine (3.3 g/l), ornithine (3.2 g/l), tyrosine (0.3 g/l), N-acetyl-tyrosine (1.3 g/l), aspartate (1.3 g/l). During control studies, normal saline was infused at identical infusion rates.

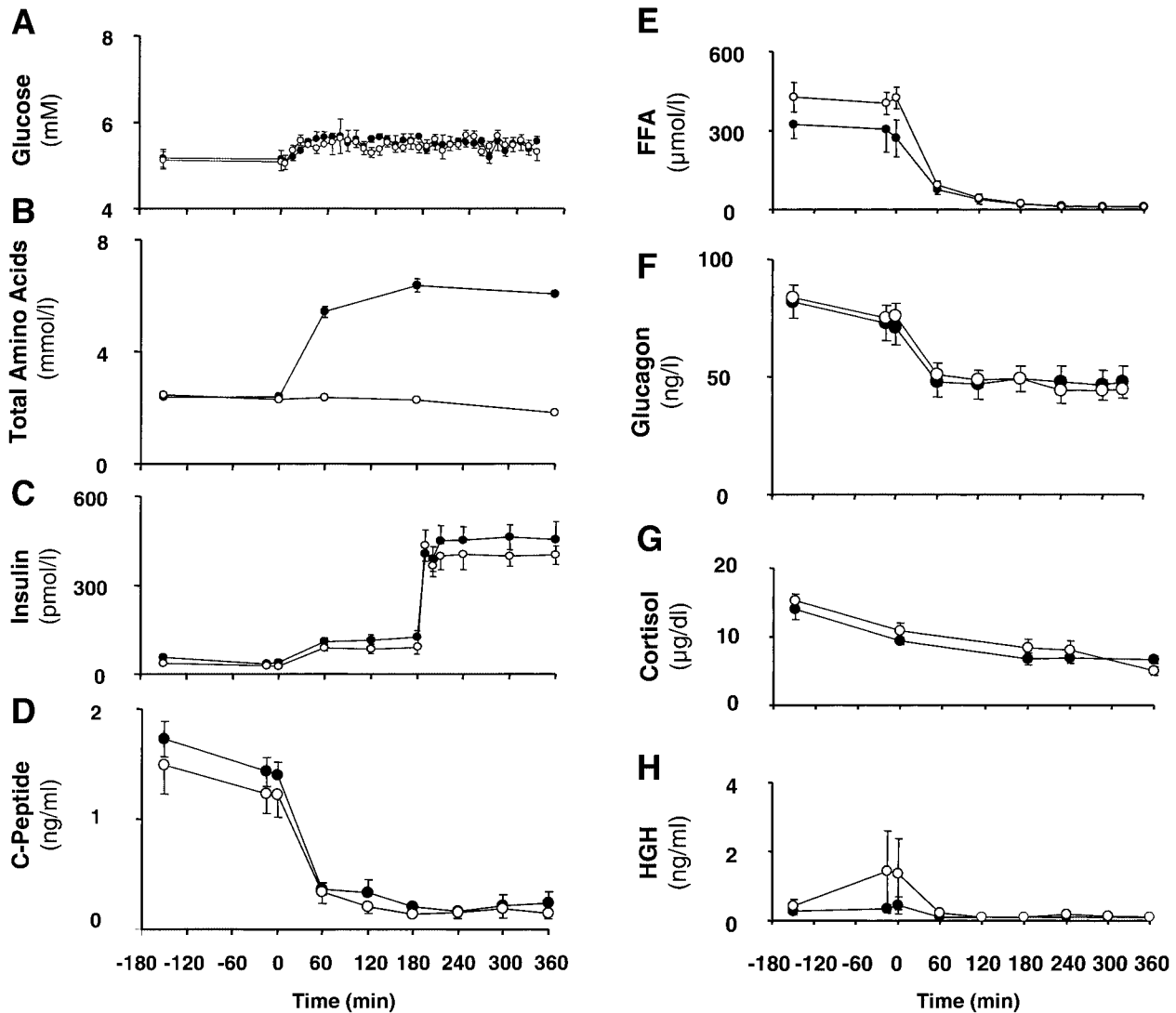
Blood samples for determination of glucoregulatory hormones, enrichments of D-[6,6-<sup>2</sup>H<sub>2</sub>] glucose, free fatty acids (FFAs), and amino acids were drawn at defined time points.

**Needle biopsies of the vastus lateralis muscle.** The subjects were supine and resting quietly for at least 60 min, and the right vastus lateralis muscle was prepared sterilely under subcutaneous lidocaine (Xylocain 2%; Astra, Linz, Austria) anesthesia (17). At baseline (–120 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 hyperinsulinemia (210 min), a repeat muscle biopsy was taken at a site  $\sim 4$  cm proximal of the first biopsy. All samples were stored in liquid nitrogen until analysis. Muscle biopsies were homogenized with a polytron in six volumes of lysis buffer containing 50 mmol/l HEPES, pH 7.5, 137 mmol/l NaCl, 1 mmol/l CaCl<sub>2</sub>, 1 mmol/l MgCl<sub>2</sub>, 10% glycerol, 2 mmol/l EDTA, 10 mmol/l NaF, 2 mmol/l Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor cocktail. Muscle homogenates were solubilized in 1% NP-40 for 1 h at 4°C and centrifuged at 14,000g for 10 min. The supernatant was used for insulin signaling studies as described below.

**Cell culture and treatment.** A line of L6 skeletal muscle cells (gift of Dr. Amira Klip, Hospital for Sick Children, Toronto, ON, Canada) clonally selected for high fusion potential was used in the present study. The L6 cell line was derived from neonatal rat thigh skeletal muscle cells and retains many morphological, biochemical, and metabolic characteristics of skeletal muscle. Cells were grown and maintained in monolayer culture in  $\alpha$ -minimum essential medium (MEM) containing 10% (vol/vol) fetal bovine serum and 1% (vol/vol) antibiotic/antimycotic solution (10,000 U/ml penicillin, 10,000 nmol/l streptomycin and 25 nmol/l amphotericin B) in an atmosphere of 5% CO<sub>2</sub> at 37°C. L6 myoblasts were allowed to differentiate into myotubes in  $\alpha$ -MEM containing 2% (vol/vol) fetal bovine serum and antibiotics for 7 days. Then, serum-deprived (4 h) cells were incubated either in an amino acid-free (Earle's balanced salt solution) or amino acid-containing medium (Earle's balanced salt solution + 2× MEM amino acid solution) as previously described (13). Vehicle (0.01% DMSO) or rapamycin (25 nmol/l) was added during the 1-h incubation and stimulated with or without insulin as indicated in the figure legends.

**Western blotting.** Muscle homogenates were subjected to SDS-PAGE (18). Antibodies were obtained from Cell Signaling (phospho-IRS-1 [Ser312 and Ser636/639], phospho-GSK-3 $\alpha/\beta$  [Ser21/9] and phospho-S6K1 [Thr421/Ser424]), Santa Cruz (IRS-1 and S6K1), and Upstate Biotechnology (IRS-1 and p85 subunit of PI 3-kinase).

**S6K1 activity.** A total of 500 μg muscle homogenates were immunoprecipitated with 2 μg anti-S6K1 coupled to protein A-Sepharose for 2 h at 4°C. Immune complexes were washed three times in 50 mmol/l Tris-acetate, pH 8.0, 5 mmol/l  $\beta$ -glycerophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.1%  $\beta$ -mercaptoethanol, 2 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/l NaF, and twice in kinase buffer (50 mmol/l MOPS, pH 7.5, 25 mmol/l  $\beta$ -glycerophosphate, 5 mmol/l EGTA, 2 mmol/l EDTA, 20 mmol/l MgCl<sub>2</sub>, 1 mmol/l DTT, 2 mmol/l Na<sub>3</sub>VO<sub>4</sub>, and 10



**FIG. 2. Metabolites and hormones.** Plasma concentrations of glucose (A), total amino acids (B), insulin (C), C-peptide (D), FFAs (E), glucagon (F), cortisol (G), and growth hormone (H) during saline (low amino acids, control, ○) and amino acid (high amino acids, amino acid, ●) infusion. Data are given as means  $\pm$  SE of seven healthy subjects (errors bars smaller than the symbol size are not visible).

mmol/l NaF). The reaction was started by adding 50  $\mu$ l kinase buffer (containing 100  $\mu$ mol/l ATP, 2  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP, and 100  $\mu$ mol/l S6K1 substrate [Santa Cruz]) for 10 min at 30°C. Reaction product was spotted on p81 filter paper (Whatman) and washed 3  $\times$  15 min with 1% phosphoric acid. Incorporated radioactivity was determined by liquid scintillation counting.

**IRS-1-associated PI 3-kinase activity.** PI 3-kinase activity was measured in IRS-1 immunoprecipitates based on the technique used for rat muscle (13). In brief, muscle homogenates (1 mg) were incubated with protein A-Sepharose beads (Amersham Bioscience, Uppsala, Sweden) precoupled to IRS-1 antibody (H-165; Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were washed, and beads were resuspended in 70  $\mu$ l kinase buffer (8 mmol/l Tris, pH 7.5, 80 mmol/l NaCl, 0.8 mmol/l EDTA, 15 mmol/l MgCl<sub>2</sub>, 180  $\mu$ mol/l ATP, and 5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP) and 10  $\mu$ l sonicated PI mixture (20  $\mu$ g L- $\alpha$ -PI, 10 mmol/l Tris, pH 7.5, and 1 mmol/l EGTA) for 15 min at 30°C. Reaction was stopped by the addition of 20  $\mu$ l of 8 mol/l HCl, mixed with 160  $\mu$ l CHCl<sub>3</sub>:CH<sub>3</sub>OH (1:1) and centrifuged. Lower organic phases were spotted on oxalate-treated silica gel TLC plate and developed in CHCl<sub>3</sub>:CH<sub>3</sub>OH:H<sub>2</sub>O:NH<sub>4</sub>OH (60:47:11.6:2). The plate was dried and visualized by autoradiography with intensifying screen at -80°C.

**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 acids were measured by high-performance liquid chromatography (19). Plasma FFA concentrations were determined with a microfluorimetric method (Wako, Richmond, VA). Plasma immunoreactive insulin, C-peptide, glucagon, and growth hormone were measured by commercially available radioimmunoas-

says (7,8,15,16,20) (insulin, Pharmacia, Uppsala, Sweden; C-peptide, Cis, Gif-Sur-Yvette, France; glucagon, Linco, St. Charles, MO; growth hormone, Sorin Biomedica, Saluggia, Italy). Plasma cortisol was determined following extraction and charcoal-dextran separation by radioimmunoassay (21).

Gas chromatography-mass spectrometry for the determination of tracer-to-tracee ratios of  $^2$ H in glucose was performed as described (7,8). The glucose-pentaacetate was analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with a CP-Sil5 25 m  $\times$  0.25 mm  $\times$  0.12- $\mu$ m capillary column (Chrompack, Middelburg, Netherlands) 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- $^2$ H<sub>2</sub> (M+2) enrichments in glucose were assessed using fragments of C3-C6 with their masses of 187 and 189, respectively.

**Calculations.** Rates of glucose appearance ( $R_a$ ) and disappearance ( $R_d$ ) were calculated using Steele's non-steady-state equations modified for the use of stable isotopes (7,8) with EGP given as the difference between  $R_a$  and glucose infusion rates.

**Data analysis and statistics.** Autoradiographs were analyzed by laser scanning densitometry using a tabletop Agfa scanner (Arcus II; Agfa-Gavaert, Morstel, Belgium) and quantified with the National Institutes of Health Image program (available from <http://rsb.info.nih.gov/nih-image/>). Immunoblots and kinase activity determinations were expressed relative to those obtained from a single muscle biopsy to allow comparisons between samples processed during different experiments.

Hormone profiles and glucose turnover data from both protocols (i.e.,



amino acid and control) were compared by a two-way ANOVA with two within repeated-measures factors for the basal period, the low- and the high-insulin period of the clamp tests. The first within-subject repeated-measures factor with two levels accounted for the paired crossover study design and the second for repetitive measurements during each period of the clamp test. Differences between protocols during each period were assessed from the main effect of the first repeated-measures factor, and differences between amino acid and controls at singular time points were calculated with post hoc Tukey honest significant difference correction for multiple comparisons. Changes of sequential (time-dependent) data within protocols were analyzed by ANOVA for repeated measurements and Dunnett's post hoc testing. Muscle insulin signaling data were analyzed by ANOVA followed by Bonferroni/Dunn's post hoc testing. Differences were considered significant at  $P < 0.05$ .

## RESULTS

**Metabolites and hormones.** Fasting ( $-150$  min) plasma glucose (amino acid  $5.17 \pm 0.21$ , control  $5.12 \pm 0.19$  mmol/l), total amino acid (amino acid  $2.38 \pm 0.06$ , control  $2.45 \pm 0.06$  mmol/l), insulin (amino acid  $57 \pm 9$ , control  $37 \pm 5$  pmol/l), C-peptide (amino acid  $1.73 \pm 0.16$ , control  $1.49 \pm 0.27$  ng/ml), FFAs (amino acid  $324 \pm 56$ , control  $426 \pm 55$   $\mu$ mol/l), glucagon (amino acid  $82 \pm 7$ , control  $83 \pm 6$  ng/l), cortisol (amino acid  $387 \pm 42$ , control  $422 \pm 28$  nmol/l), and growth hormone (amino acid  $0.26 \pm 0.09$ , control  $0.42 \pm 0.20$   $\mu$ g/l) were not different between amino acid infusion and control studies (Fig. 2).

During the clamp tests, plasma glucose remained at baseline and was not different between the studies (Fig. 2A). Fasting plasma amino acid concentrations were comparable in both studies (Fig. 2B, Table 1). During amino acid infusion, total plasma amino acid concentration increased by  $\sim 2.5$ -fold, whereas it decreased by  $\sim 20\%$  during control studies (Fig. 2B). Plasma concentrations of individual amino acids and branched-chain amino acids (valine, leucine, isoleucine) at baseline and during steady-state conditions are presented in Table 1. Except for glutamate and tyrosine, all amino acids contributed to the rise in plasma amino acid concentrations observed during amino acid infusion. Whereas most amino acids decreased during control studies, alanine, glutamine, glycine, and taurine remained at their basal fasting levels. Plasma insulin concentrations rose similarly to  $\sim 100$  pmol/l during low-dose insulin replacement and thereafter quickly increased further to  $\sim 430$  pmol/l during high-dose insulin infusion (Fig. 2C), whereas plasma C-peptide was equally suppressed in both studies (Fig. 2D). Plasma FFA and glucagon similarly decreased during the pancreatic clamp studies (Fig. 2E and F). Plasma cortisol and growth hormone concentrations were similar between the two protocols (Fig. 2G and H).

**Glucose turnover.** Glucose infusion rates required to maintain euglycemia were  $\sim 71\%$  (150–180 min, amino acid  $7.6 \pm 3.7$ , control  $26.2 \pm 6.5$   $\mu$ mol  $\cdot$  kg $^{-1}$   $\cdot$  min $^{-1}$ ,  $P < 0.01$ ) and  $\sim 34\%$  (330–360 min, amino acid  $50.8 \pm 5.6$ , control,  $76.7 \pm 6.1$   $\mu$ mol  $\cdot$  kg $^{-1}$   $\cdot$  min $^{-1}$ ,  $P < 0.01$ ) lower during amino acid infusion compared with control studies in the presence of low peripheral hyperinsulinemia and prandial-like peripheral hyperinsulinemia, respectively (data not shown). During amino acid infusion, rates of glucose disappearance ( $R_d$ ) were lower compared with control studies at the end of the low peripheral hyperinsulinemic period and in the presence of prandial-like peripheral hyperinsulinemia (33%) (Fig. 3A).

EGP was comparable between both studies at baseline (amino acid  $10.0 \pm 0.7$ , control  $9.6 \pm 0.6$   $\mu$ mol  $\cdot$  kg $^{-1}$   $\cdot$  min $^{-1}$  (Fig. 3B).

TABLE 1

Plasma concentrations of individual amino acids at baseline and at the end of amino acid (high amino acids) and saline (low amino acids, control) infusion

Amino acids ( $\mu$ mol/l)	Time (min)	High amino acids (fold change vs. 0 min)	Low amino acids, control (fold change vs. 0 min)
Alanine	0	330 $\pm$ 37	299 $\pm$ 44
	360	1,231 $\pm$ 50* (3.7)	349 $\pm$ 18† (1.2)
Arginine	0	99 $\pm$ 7	92 $\pm$ 7
	360	430 $\pm$ 27* (4.3)	60 $\pm$ 5†‡ (0.6)
Asparagine	0	47 $\pm$ 3	48 $\pm$ 3
	360	159 $\pm$ 7* (3.4)	33 $\pm$ 2*† (0.7)
Citrulline	0	35 $\pm$ 2	32 $\pm$ 2
	360	65 $\pm$ 4* (1.9)	17 $\pm$ 1*† (0.5)
Glutamine	0	654 $\pm$ 25	623 $\pm$ 27
	360	905 $\pm$ 20* (1.4)	549 $\pm$ 27† (0.9)
Glutamate	0	51 $\pm$ 10	39 $\pm$ 4
	360	74 $\pm$ 9 (1.5)	23 $\pm$ 3*† (0.6)
Glycine	0	229 $\pm$ 20	218 $\pm$ 18
	360	860 $\pm$ 58* (3.8)	201 $\pm$ 15† (0.9)
Histidine	0	75 $\pm$ 1	80 $\pm$ 4
	360	248 $\pm$ 9* (3.3)	67 $\pm$ 2†¶ (0.8)
Isoleucine	0	63 $\pm$ 3	65 $\pm$ 5
	360	161 $\pm$ 12* (2.5)	14 $\pm$ 1*† (0.2)
Leucine	0	115 $\pm$ 5	123 $\pm$ 6
	360	333 $\pm$ 22* (2.9)	51 $\pm$ 2*† (0.4)
Methionine	0	34 $\pm$ 2	34 $\pm$ 2
	360	229 $\pm$ 10* (6.7)	17 $\pm$ 1*† (0.5)
Phenylalanine	0	54 $\pm$ 2	55 $\pm$ 3
	360	182 $\pm$ 5* (3.4)	33 $\pm$ 2*† (0.6)
Serine	0	122 $\pm$ 9	118 $\pm$ 6
	360	253 $\pm$ 12* (2.1)	76 $\pm$ 5*† (0.6)
Taurine	0	38 $\pm$ 2	39 $\pm$ 3
	360	57 $\pm$ 2* (1.5)	36 $\pm$ 3† (0.9)
Threonine	0	122 $\pm$ 7	123 $\pm$ 10
	360	368 $\pm$ 17* (3.0)	86 $\pm$ 8†¶ (0.7)
Tryptophan	0	44 $\pm$ 2	46 $\pm$ 3
	360	95 $\pm$ 6* (2.2)	28 $\pm$ 1*† (0.6)
Tyrosine	0	60 $\pm$ 3	57 $\pm$ 5
	360	59 $\pm$ 3 (1.0)	33 $\pm$ 2*† (0.6)
Valine	0	213 $\pm$ 9	219 $\pm$ 12
	360	396 $\pm$ 23* (1.9)	131 $\pm$ 7*† (0.6)
Branched-chain amino acid	0	392 $\pm$ 6	408 $\pm$ 8
	360	891 $\pm$ 19* (2.3)	196 $\pm$ 3*† (0.5)

Data are given as means  $\pm$  SE. Seven healthy subjects underwent somatostatin-insulin clamp tests. \* $P < 0.001$  vs. corresponding baseline value; † $P < 0.001$  vs. amino acid; ‡ $P < 0.01$ ; ¶ $P < 0.05$ .

During control studies, EGP decreased by 70% compared with baseline percentage (150–180 min,  $P < 0.01$ ) in the presence of low peripheral hyperinsulinemia (Fig. 3B). However, during amino acid infusion, the decrease in EGP was completely blunted under these conditions. In the presence of prandial-like peripheral hyperinsulinemia (180–360 min), EGP declined close to zero in both protocols (Fig. 3B).

**Insulin signaling.** To assess the molecular mechanisms underlying amino acid–induced insulin resistance in human skeletal muscle, activation status of mTOR/S6K1 and that of the IRS-1/PI 3-kinase/Akt pathways was measured. Before insulin infusion (basal), S6K1 activity was similar between saline and amino acid–infused subjects (Fig. 4). However, although raising insulinemia to prandial-like

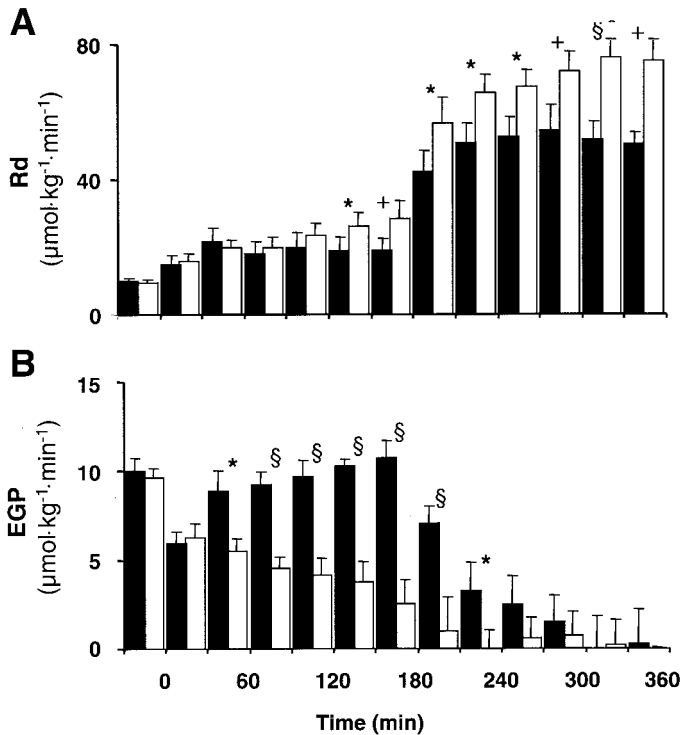


FIG. 3. Amino acids infusion inhibits insulin-stimulated glucose uptake and suppression of EGP.  $R_d$  (A) and EGP (B) during infusion of saline (low amino acids, control,  $\square$ ) and amino acids (high amino acids,  $\blacksquare$ ) infusion. Data are given as means  $\pm$  SE of seven healthy subjects (error bars smaller than the symbol size are not visible). \* $P < 0.05$ , + $P < 0.01$ , § $P < 0.001$  vs. amino acid.

peripheral hyperinsulinemia only led to a modest increase in S6K1 activity in saline-infused subjects ( $\sim 1.9$ -fold above basal), combined hyperinsulinemia and hyperaminoacidemia resulted in a highly significant 3.7-fold increase in the enzymatic activity of S6K1. Given the prominent role of S6K1 in mediating serine phosphorylation of IRS-1 (22), we assessed whether phosphorylation of IRS-1 using phospho-specific antibodies against Ser312 and Ser636/639 (equivalent to Ser307 and Ser632/635 in mice, respectively) was increased in skeletal muscle of amino acid-infused subjects. Whereas immunoreactivity to both antibodies was barely detectable in skeletal muscle isolated from saline-infused subjects, amino acid infusion increased the phosphorylation state of IRS-1 on Ser312 (Fig. 5A) and Ser636/639 (Fig. 5B) in the insulin-stimulated state. Quantification of immunoblots from several muscle biopsies revealed that insulin increased IRS-1 Ser636/639 phosphorylation up to sixfold in amino acid-infused subjects. Furthermore, we found that the heavily serine phosphorylated form of IRS-1 detected in biopsies from amino acid-infused subjects was not prone to a greater rate of degradation because its expression level in muscle homogenates was similar to that of saline-infused humans (Fig. 5C).

To evaluate the functional consequences of increased phosphorylation of IRS-1 on serine residues, PI 3-kinase activity was determined in human muscle biopsies. When plasma insulin was raised to prandial-like peripheral hyperinsulinemia, IRS-1-associated PI 3-kinase activity in saline-infused subjects increased by  $\sim$ twofold above basal, whereas under similar conditions, infusion of amino

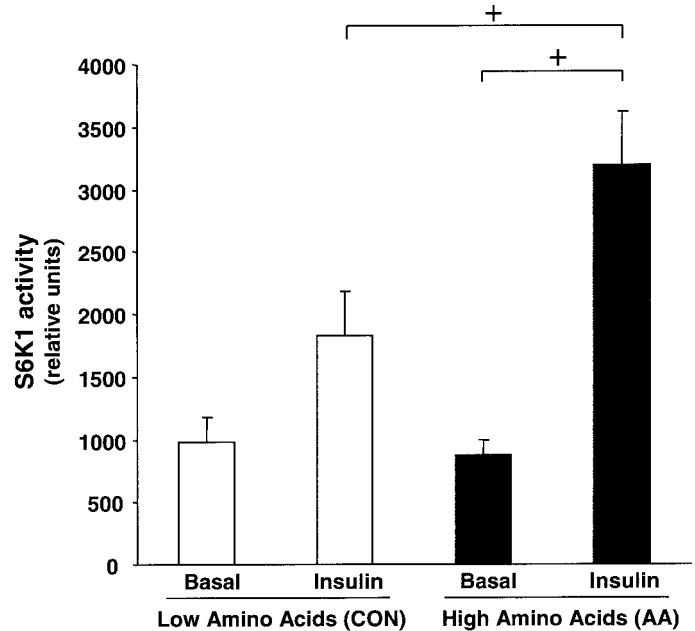
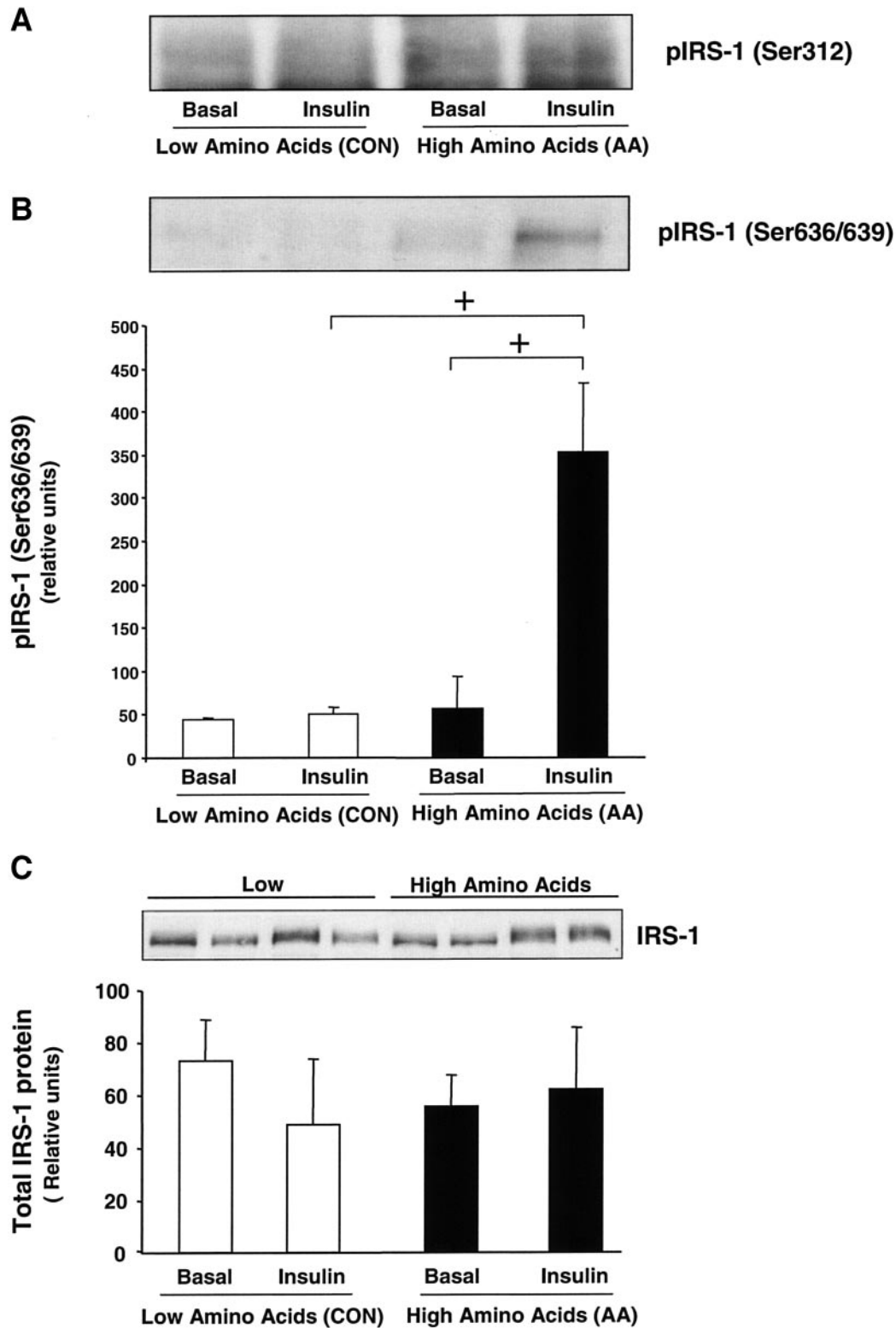


FIG. 4. Amino acids infusion increases activation of S6K1 by insulin in human skeletal muscle. Human skeletal muscle biopsies were obtained during saline (low amino acids,  $\square$ ) and amino acids (high amino acids,  $\blacksquare$ ) infusion. Biopsies were sampled 120 min before insulin infusion (basal) and following 30 min of hyperinsulinemia (insulin). S6K1 kinase activity was determined in S6K1 immunoprecipitates as described in RESEARCH DESIGN AND METHODS. Data are given as means  $\pm$  SE of seven healthy subjects. + $P < 0.01$ .

acid completely blunted the activation of the enzyme ( $P < 0.05$ ; Fig. 6A). Importantly, changes in PI 3-kinase activity between saline and amino acid-infused subjects could not be attributed to altered expression of the p85 subunit of PI 3-kinase since it was found to be similar among all experimental groups (Fig. 6B).

To determine the effect of insulin and/or amino acid infusion on the activation status of the PI 3-kinase downstream effector Akt in skeletal muscle biopsies, we first examined the phosphorylation status of Akt on both regulatory sites, Thr308 and Ser473. We showed that infusion of insulin during both the saline and amino acid clamps led to a similar increase in the phosphorylation of Akt on Thr308 (about twofold above basal; Fig. 7A) and Ser473 (about five- to sixfold above basal; Fig. 7B). Similarly, when GSK-3 $\alpha$  and  $\beta$  phosphorylation were determined as an index of in vivo Akt activation, amino acid infusion was found to neither affect basal nor insulin-stimulated GSK-3 $\alpha$  phosphorylation on Ser21 when compared with saline-infused subjects (about twofold above basal for both saline and amino acid clamp; Fig. 7C). Consistent with a previous human study (23), physiological hyperinsulinemia failed to increase the phosphorylation of GSK-3 $\beta$  on Ser9 in both saline and amino acid-infused subjects (data not shown).

The involvement of mTOR/S6K1 in mediating inhibitory phosphorylation of IRS-1 was further examined in L6 myocytes since this in vitro model allows to test the involvement of the mTOR pathway using rapamycin, a very specific inhibitor of this pathway (24). We first observed that amino acid potentiates insulin-induced activation of S6K1 as revealed by its phosphorylation on Thr421/Ser424 and in vitro kinase activity (data not

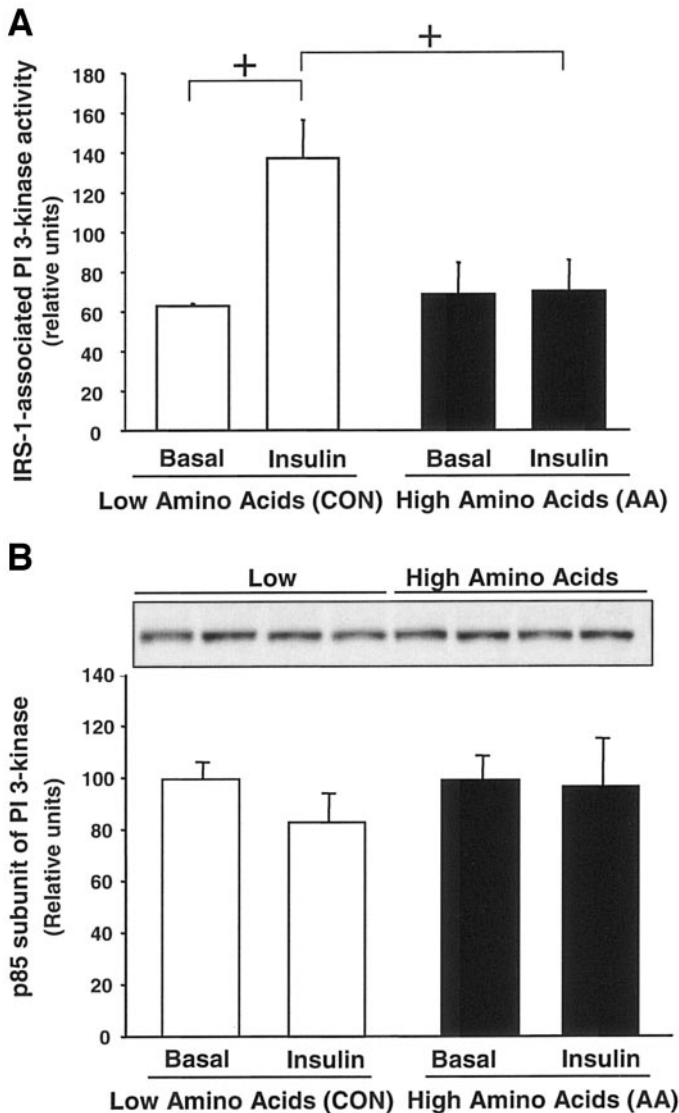


**FIG. 5.** Amino acids infusion increases insulin-induced serine phosphorylation of IRS-1 in human skeletal muscle. Phosphorylation status of IRS-1 was determined in human skeletal muscle biopsies obtained during saline (low amino acids, □) and amino acids (high amino acids, ■) infusion. Biopsies were sampled 120 min before insulin infusion (basal) and following 30 min of hyperinsulinemia (insulin). Phosphorylation status of IRS-1 on Ser312 (**A**) and IRS-1 on Ser636/639 (**B**) were determined using specific antibodies as described in RESEARCH DESIGN AND METHODS. Total IRS-1 expression (**C**) was measured in whole-tissue extracts as described in RESEARCH DESIGN AND METHODS. Data are given as means  $\pm$  SE of four and seven healthy subjects (for **A** and for **B** and **C**, respectively).  $+P < 0.01$ .

shown). Further amino acid infusion concomitantly increased phosphorylation of IRS-1 on Ser636/639 in L6 myocytes (Fig. 8A). Of note, inhibition of the mTOR/S6K1

pathway by rapamycin completely blocked phosphorylation of these sites following amino acid and insulin infusion (Fig. 8A). Amino acid exposure also severely reduced





**FIG. 6.** Amino acids infusion impairs insulin-stimulated IRS-1-associated PI 3-kinase activity in human skeletal muscle. PI 3-kinase activity (**A**) and p85 regulatory subunit expression (**B**) were determined in human skeletal muscle biopsies obtained during saline (low amino acids, □) and amino acids (high amino acids, ■) infusion. Biopsies were sampled 120 min before insulin infusion (basal) and following 30 min of hyperinsulinemia (insulin). PI 3-kinase activity was determined in IRS-1 immunoprecipitates as described in RESEARCH DESIGN AND METHODS. Data are given as means  $\pm$  SE of four and seven healthy subjects (for **A** and **B**, respectively). \* $P < 0.05$ , + $P < 0.01$ .

the ability of insulin to increase PI 3-kinase activity measured in IRS-1 immunoprecipitates (Fig. 8B) and glucose transport (Fig. 8C), which again was fully prevented by rapamycin treatment (Fig. 8B and C). Insulin-stimulated Akt phosphorylation and kinase activity were not affected by amino acid or rapamycin treatment (data not shown).

## DISCUSSION

In cross-sectional studies, high protein intake is associated with insulin resistance and glucose intolerance in humans (25,26), protein ingestion increases plasma glucose concentrations as well as insulin requirements in patients with insulin-dependent diabetes (27,28), and a diet high in processed meats increases the risk for developing type 2 diabetes (29). We have shown that elevation of plasma

amino acids impair glucose metabolism in vivo by directly inhibiting insulin-stimulated glucose transport/phosphorylation in skeletal muscle (7). Furthermore, plasma amino acid elevation directly increases gluconeogenesis and glucose production in healthy humans but does not affect glycemia because of simultaneous amino acid-induced stimulation of insulin secretion (30), which counteracts the direct effects of amino acid on glucose production (8). Thus, the mechanisms by which amino acids modulate glucose metabolism in liver and skeletal muscle were examined during somatostatin administration to inhibit the endogenous secretion of the major glucoregulatory hormones (1,7,8). Short-term studies showed that a protein-enriched diet does not impair or may even improve glucose metabolism in type 2 diabetes (31). These observations are not necessarily in contrast to the results of the present study. Protein ingestion will give rise to plasma amino acid concentrations, which will stimulate insulin secretion and consequently decrease postprandial glycemia. Thus, it is possible that amino acid-induced stimulation of endogenous insulin release obscures the direct effects of amino acid on glucose metabolism (8).

In the present study, the effects of plasma amino acid elevation on endogenous glucose production and whole-body  $R_d$  were evaluated in the presence of low (0–180 min) and high (180–360 min) peripheral hyperinsulinemia corresponding to fasting portal vein insulin concentrations and prandial peripheral hyperinsulinemia, respectively. This study demonstrates that low peripheral hyperinsulinemia suppressed endogenous glucose production by  $\sim 70\%$  during control studies. However, this insulin-mediated decline in glucose production was completely blunted during amino acid infusion. This can likely be attributed to direct stimulation of gluconeogenesis by amino acid (8,32) since amino acid-induced endogenous release of glucoregulatory hormones, including glucagon, was inhibited by continuous somatostatin infusion. Impaired postprandial suppression of endogenous glucose production contributes to hyperglycemia in patients with type 2 diabetes (33). Increased amino acid availability could, therefore, play a role in the impairment of insulin-mediated suppression of endogenous glucose production observed in obese patients with type 2 diabetes (34).

In the presence of prandial-like peripheral hyperinsulinemia (180–360 min), whole-body  $R_d$  was  $\sim 33\%$  lower during amino acid infusion compared with control studies. Since under such insulin-stimulated conditions the majority of glucose is taken up by skeletal muscle (35), this can be attributed to amino acid-induced skeletal muscle insulin resistance. We have previously shown that amino acids directly inhibit both insulin-stimulated glucose transport/phosphorylation into skeletal muscle and glycogen synthesis reflecting reduced insulin-stimulated whole-body  $R_d$  in healthy humans (7). However, the molecular mechanisms involved are still unclear in vivo.

Our first effort to identify the molecular events linked to skeletal muscle insulin resistance under physiological hyperaminoacidemia pointed on the mTOR nutrient-sensing pathway (13). Insulin activation of S6K1, a downstream effector of mTOR, is potentiated by increased amino acid availability, leading to increased inhibitory serine/threonine phosphorylation of IRS-1 and rapid time-dependent

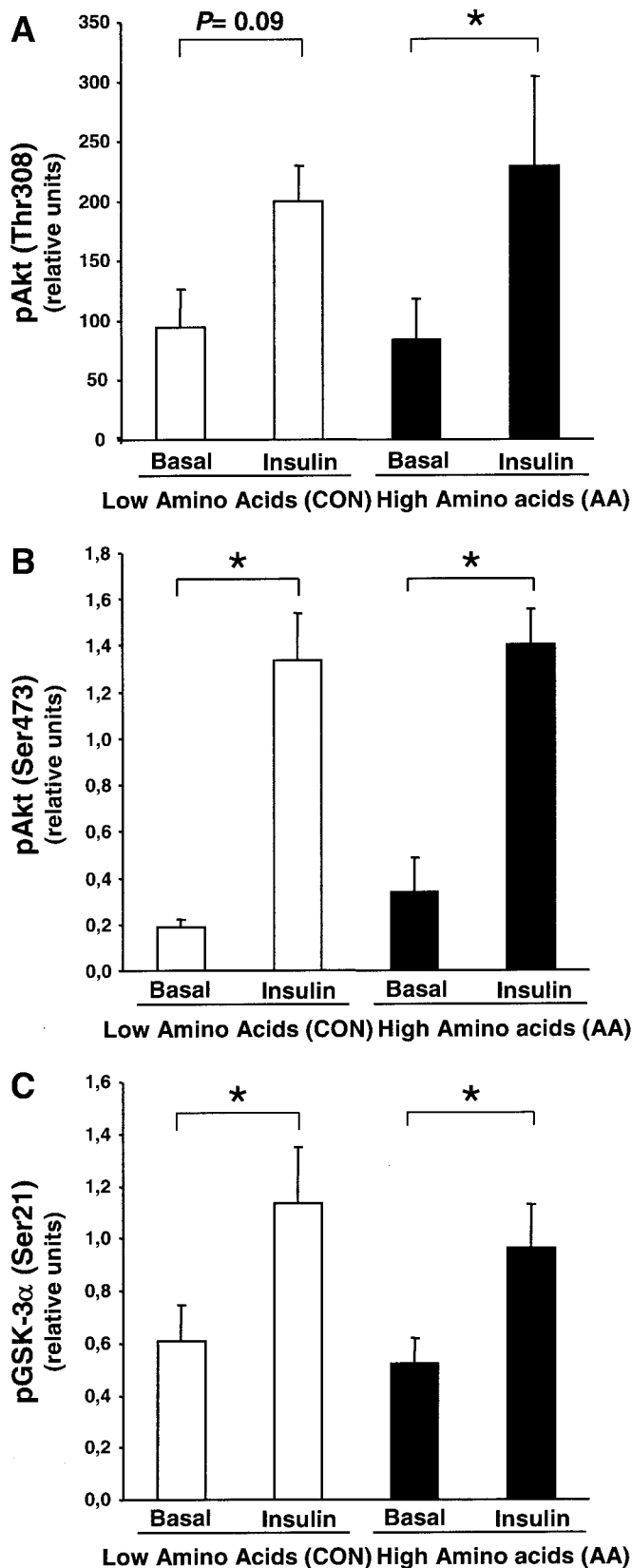


FIG. 7. Amino acids infusion fails to modulate insulin-induced phosphorylation of Akt/GSK-3 in human skeletal muscle. Phosphorylation status of Akt and GSK-3 $\alpha$  was determined in human skeletal muscle biopsies obtained during saline (low amino acids,  $\square$ ) and amino acids (high amino acids,  $\blacksquare$ ) infusion. Biopsies were sampled 120 min before insulin infusion (basal) and following 30 min of hyperinsulinemia (insulin). Phosphorylation status of Akt on Thr308 (A), Akt on Ser473

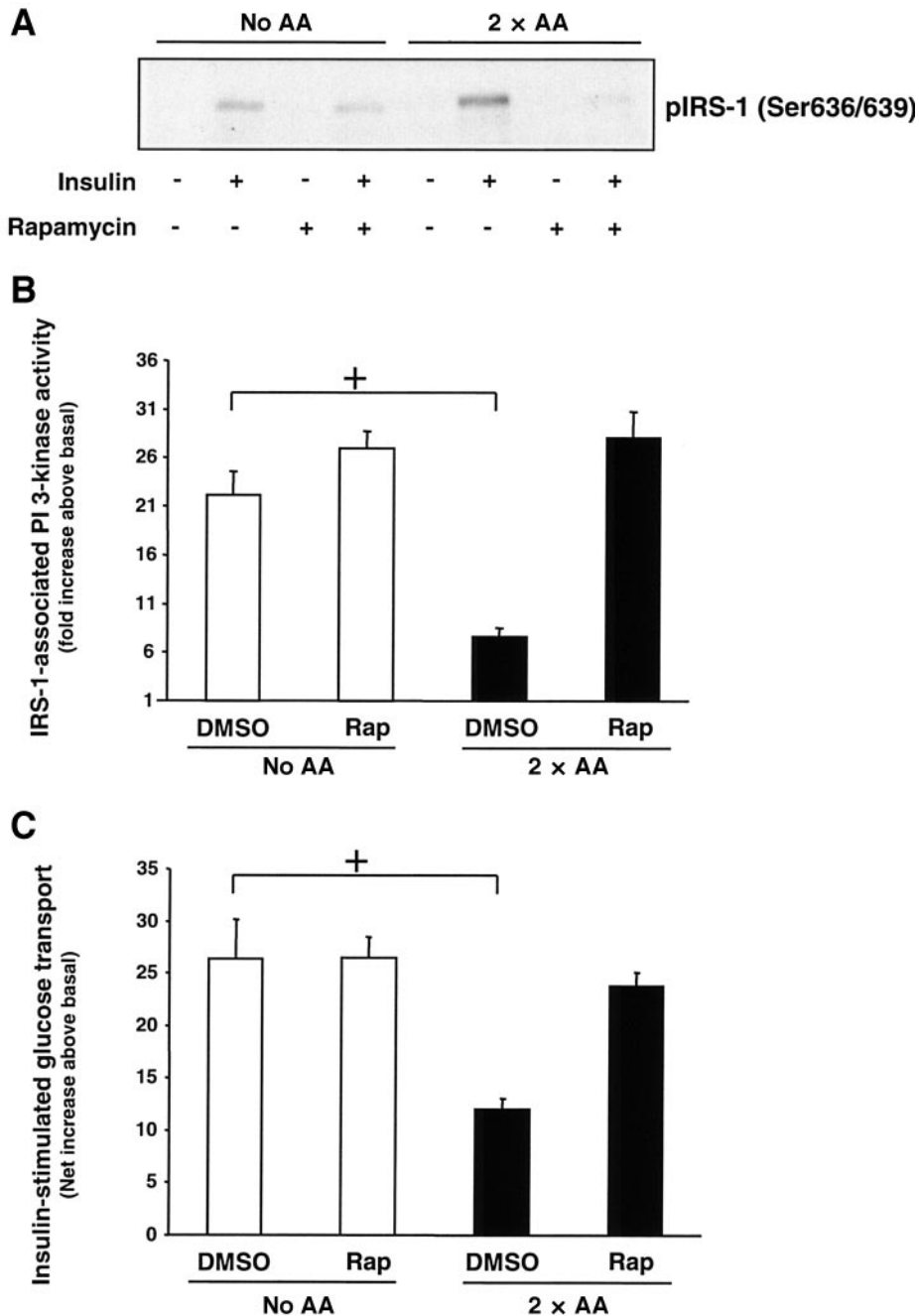
deactivation of PI 3-kinase activity (13). In this study, we present evidence that this negative feedback loop is also operative in human skeletal muscle under parenteral nutrient satiation in vivo. Indeed, our data show that amino acid infusion increases activation of S6K1 and phosphorylation of IRS-1 on multiple serine residues, leading to impaired stimulation of PI 3-kinase by insulin in human muscle. Further studies in L6 myocytes confirmed that inhibition of mTOR/S6K1 by rapamycin prevents amino acid-induced hyperphosphorylation of IRS-1 on Ser636/639 and restored insulin activation of PI 3-kinase associated with IRS-1 and glucose transport. In support of these findings is the recent observation that S6K1-deficient mice are protected against diet-induced insulin resistance by a mechanism that involves, at least in part, reduced phosphorylation of IRS-1 on Ser307 and Ser636/639 (22), thereby placing S6K1 as a major player involved in the development of insulin resistance under nutrient abundance in both animals and humans.

An important aspect of the present study is the observation that amino acid-induced desensitization of insulin action in vivo occurred without any detectable degradation of IRS-1 despite its elevated content in phosphoserine residues. This result was somewhat unexpected because prior in vitro work suggested that ser/thr phosphorylation of IRS-1 is a prerequisite for triggering its degradation (36), notably via the proteasomes (11). Our results suggest therefore that degradation of IRS-1 is not essential for the occurrence of muscle insulin resistance in amino acid-infused human subjects. Moreover, the observation that insulin resistance induced by chronic high-fat feeding (4 months) does not alter IRS-1 protein levels despite its elevated phosphoserine content (22) further suggests that IRS-1 ser/thr phosphorylation, but not its degradation, is the principal mechanism leading to nutrient-induced insulin resistance under both short- and long-term settings in vivo.

Identification of the molecular locus of impaired insulin signaling during the development of insulin resistance is a long-lasting quest. Although it has become obvious that impaired activation of PI 3-kinase is a hallmark of insulin resistance in both animal models and humans, the potential role of Akt in insulin resistance is far from being conclusive. In the present study, we found that Akt was normally activated, despite marked impairment in PI 3-kinase activity. These results are reminiscent of the findings of Kim et al. (37), who found Akt to be normally stimulated by insulin despite reduced PI 3-kinase activity in skeletal muscle of type 2 diabetic subjects. This may be explained by the fact that Akt requires only partial activation of PI 3-kinase to get fully activated. On the other hand, we previously observed that overactivation of the mTOR pathway during prolonged hyperinsulinemia (4 h) reduces Akt phosphorylation concomitantly with degradation of IRS-1 (13). It is therefore possible that a defect in insulin-induced Akt activation occurs only after chronic stimulation of the mTOR/S6K1 pathway by amino acid and marked reduction in IRS-1 content and associated PI 3-kinase activity.

(B), and GSK-3 $\alpha$  on Ser21(C) were determined using phospho-specific antibodies as described in RESEARCH DESIGN AND METHODS. Data are given as means  $\pm$  SE of seven healthy subjects. \* $P$  < 0.05.





**FIG. 8.** Rapamycin prevents amino acid–dependent increases in serine phosphorylation of IRS-1 and inhibition of PI 3-kinase activity in cultured muscle cells. Fully differentiated L6 skeletal muscle cells were incubated either in amino acid–free medium (saline) or in medium containing two times the amino acids found in MEM (amino acids) for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nmol/l) was added during the 1-h incubation. Cells were then stimulated with insulin (100 nmol/l) for the last 30 min of incubation, rinsed twice in ice-cold PBS, and lysed. Phosphorylation status of IRS-1 on Ser636/639 (**A**) was determined using a phospho-specific antibody as described in RESEARCH DESIGN AND METHODS. **B:** PI 3-kinase activity was determined in IRS-1 immunoprecipitates as described in RESEARCH DESIGN AND METHODS. Data are given as means  $\pm$  SE obtained from four independent experiments.  $+P < 0.01$ . Rap, rapamycin.

Some limitations of our study must be considered. First, the present study compared hyperaminoacidemic with moderately hypoaminoacidemic conditions. In the present study, total plasma amino acid concentrations observed during amino acid infusion are  $\sim 40\%$  higher than those seen after ingestion of a large size (50 g) protein meal (38). Furthermore, plasma amino acids were maintained at these elevated concentrations throughout the infusion. The moderate insulin-induced decrease in plasma amino acids during the control study is similar to what has been

observed after a carbohydrate-rich meal (39) and to control experiments of other studies (40). Thus, it cannot be ruled out that the differences observed between hyperaminoacidemic and hypoaminoacidemic conditions might be attenuated when fasting plasma amino acid concentrations would be present during the control study. In the present study, an amino acid mixture was infused so that one cannot discriminate between the impact of individual amino acids or certain combinations of amino acids on the obtained results. Furthermore, it cannot be excluded that

products of amino acid metabolism contribute to the observed effects. Because it is not feasible to completely control the diet of the participants who are not in-patients for several weeks, differences in dietary habits might have affected the results. We aimed to minimize this possible influence by including volunteers with constant and weight-maintaining dietary habits and by studying them twice during infusion of amino acids and control saline infusion in random order, which allowed intraindividual comparisons.

Second, this study was performed in the absence of endogenous secretion of glucoregulatory hormones. An amino acid-induced rise in plasma glucagon was prevented to exclude its stimulatory effects on EGP, which would likely have induced hyperglycemia in the presence of low peripheral hyperinsulinemia and thereby would have obscured direct amino acid action on hepatic glucose metabolism (8). It is of note that administration of protein or amino acid gives rise to glucagon secretion (8), and hyperglucagonemia is frequently present in type 2 diabetic patients (41), which is different from the hormonal environment created in the present study. Nevertheless, effects of glucagon on skeletal muscle insulin signaling are unlikely because glucagon receptors could not be demonstrated in skeletal muscle (42), and glucagon has no effect on metabolism of forearm tissues in humans (43). However, indirect effects of glucagon on muscle, e.g., by modulation of hepatic glucose metabolism cannot be completely excluded. Of note, the experimental design employed in the present study does not mimic the time course of plasma amino acid and glucagon concentrations in response to ingestion of a protein meal; conclusions on the effects of oral protein intake on skeletal muscle glucose metabolism and insulin signaling have to be drawn with caution.

In summary, we show that increased amino acid availability resulting in overactivation of S6K1 is tightly linked to reduction of insulin-stimulated glucose metabolism in human skeletal muscle. S6K1 seems to operate a feedback loop toward IRS-1 by targeting at least two sets of phosphorylation sites (Ser312 and Ser636/639), causing inhibition of PI 3-kinase and, consequently, muscle glucose uptake. Alternatively, the reported mechanisms could also represent a physiological response to increased amino acid availability. Nevertheless, S6K1 could be an attractive target in the prevention and treatment of nutrient-induced insulin resistance.

#### ACKNOWLEDGMENTS

This study was supported by grants from the Austrian Science Foundation (FWF, P15656), the European Foundation for the Study of Diabetes (EFSD, Novo Nordisk type 2 diabetes grant), the Herzfelder Family Trust to M.R., the Canadian Institutes for Health Research to A.M., an institutional grant from Novo Nordisk to W.W., and by grants from the Austrian Diabetes Association and the Theodor-Körner-Fund to M.K. F.T. was supported by a doctoral research award from the Canadian Institutes for Health Research.

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

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