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Research Article

Post-transcriptional gene silencing of ribosomal protein S6 kinase 1 restores insulin action in leucine-treated skeletal muscle

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Abstract. Excessive nutrients, especially amino acids, impair insulin action on glucose metabolism in skeletal muscle. We tested the hypothesis that the branched-chain amino acid leucine reduces acute insulin action in primary myotubes *via* a negative feedback mechanism involving ribosomal protein S6 kinase 1 (S6K1). The effect of S6K1 on glucose metabolism was determined by applying RNA interference (siRNA). Leucine (5 mM) reduced glucose uptake and incorporation to glycogen by 13 % and 22 %, respectively, compared to the scramble siRNA-transfected control at the basal level. Leucine also reduced insulin-stimulated Akt phosphorylation, glucose uptake and glucose incorporation to glycogen (39 %, 39 % and 37 %, respectively), and this reduction was restored after S6K1 silencing. Depletion of S6K1 enhanced basal glucose utilization and protected against the development of impaired insulin action, in response to excessive leucine. In conclusion, S6K1 plays an important role in the regulation of insulin action on glucose metabolism in skeletal muscle.

Keywords. Glucose uptake, glycogen synthesis, signal transduction, branched chain amino acids, nutrient sensing.

Introduction

The branched-chain amino acid (BCAA) leucine plays a key role in signaling pathways that promote protein synthesis [1–3]. Plasma amino acid levels, in particular BCAAs, are increased in obesity and have been implicated in the development of peripheral insulin resistance [4–7]. However, leucine also has "anabolic-like effects" and suppresses atrophy and proteolysis in skeletal muscle *in vivo* and *in vitro* at physiological and super-physiological concentrations in human and rodents [1, 2].

Leucine activates translational stimulation of protein synthesis through the mammalian target of rapamycin (mTOR)-dependent signaling cascade [8]. Ribosomal protein S6 kinase 1 (S6K1) (also known as, 70-kDa S6 protein kinase) is an effector of mTOR [9, 10], and is responsive to glucose [11], insulin [4, 12], and amino acids [13]. mTOR and S6K1 play a role in the nutrient–hormonal signaling network in obesity, diabetes and cancer (for review see [14]). S6K1 activation is regulated by multiple site phosphorylation, especially the phosphorylation of Thr^{389}, which appears to be a critical and rate-limiting step in the activation.

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The main S6K1 substrate, ribosomal S6 protein (rpS6), plays an important role in glucose homeostasis, cell size and growth regulation [15] and links RNA translation and protein synthesis *via* mTOR and S6K1 (for review see [16]).

The interaction between amino acids and insulin at the level of phosphatidylinositol 3-kinase (PI3K) implicates cross-talk between nutrient sensing pathways and peripheral insulin action [17]. Supplemental amino acids induce insulin resistance due to inhibition of glucose transport and glucose-6 phosphate phosphorylation [6, 18, 19], which can occur through excessive S6K1 signaling along the mTOR pathway [20–22]. This in turn leads to excessive insulin receptor substrate-1 (IRS1) serine phosphorylation and reduced PI3K activity [17, 23, 24]. The insulin signaling impairment on PI3K and IRS-1 is restored pharmacologically by inhibiting mTOR activity with rapamycin [20, 22, 25, 26] and thereby reducing phosphorylation of IRS1 on Ser^{307} and IRS1 $Ser^{636/639}$ [21, 23, 27]. Moreover, genetic ablation of S6K1 in knockout mice protects against the development of dietary-induced insulin resistance, due to enhanced insulin-stimulated PKB/Akt phosphorylation, and reduced phosphorylation of IRS1 at Ser^{307} and IRS1 $Ser^{636/639}$ [21]. Although whole body S6K1 knockout mice display mild glucose intolerance in response to amino acid stimulation, normal fasting and fed glucose levels are maintained [28]. These findings have potential clinical relevance, since insulin-induced Akt phosphorylation is reduced [29] and IRS1 serine phosphorylation is elevated in obesity and type 2 diabetes [30].

Given the link between S6K1 and nutrient–hormonal signaling in the development of peripheral insulin resistance, we tested the hypothesis that leucine impairs insulin action on glucose uptake and metabolism due to a negative feedback regulation of S6K1 in skeletal muscle. Thus, we determined the direct role of S6K1 on glucose utilization, using RNA interference (siRNA) to knockdown the S6K1 protein in human myotubes. We provide evidence that depletion of S6K1 protects against the development of insulin resistance in response to leucine.

Materials and methods

Materials. Dulbecco's modified Eagles medium (DMEM), DMEM/F-12 medium, fetal bovine serum (FBS), penicillin/streptomycin (PeSt), and Fungizone were obtained from Gibco-BRL (Invitrogen, Stockholm, Sweden). General laboratory reagents were obtained from Sigma (St Louis, MO), and radioactive reagents were purchased from Amersham (Sweden).

Subject characteristics. Muscle biopsies were obtained with the informed consent of the donors during scheduled abdominal surgery (four male and four female, age 55±5 years, BMI 25.6±1.5 kg/m^2; results are expressed as mean ± SEM). None of the subjects had any known metabolic disease. Informed consent was received and the ethical committee at Karolinska Institutet and the regional ethical vetting (EPN) at Stockholm approved all protocols.

Myotube cultures. Satellite cells were isolated and cultured from muscle biopsies (rectus abdominus) as described [31]. Myoblasts were grown in growth medium (DMEM/F12 supplemented with 20% FBS, 1% PeSt and 1% Fungizone) until 70% confluent, cells were trypsinized to be frozen or grown to higher passages. Myoblasts were seeded in 6-well plates and were grown to confluence (70%) and switched to differentiation medium (DMEM plus 2% FBS, 1% PeSt and 1% Fungizone). Myotubes were incubated with serum-free DMEM plus 1% PeSt and 1% Fungizone overnight before the day of experiment. Myotubes were incubated in serum-free DMEM under the basal condition, or in the presence of 0.05, 0.5 or 5 mM leucine, and 5 mM glucose in serum-free DMEM under leucine-stimulated conditions for 20 min or 2 h. Osmotic differences were adjusted by the addition of mannitol.

A leucine concentration of 5 mM was chosen to study glucose metabolism in skeletal muscle cell culture. Due to the low expression of several proteins involved in glucose metabolism (such as GLUT4, insulin receptor and IRS1) in cultured myotube compared to adult skeletal muscle, higher insulin doses are generally used to elicit maximal effects on glucose uptake [31–33]. Our previous studies provide evidence that a maximal effect of insulin on glucose transport is observed in the presence of 60 nM insulin [32]. Insulin was added (for the insulin-stimulated condition) during the last 20 min for the protein phosphorylation analysis, the last 60 min for studies of the glucose uptake analysis or at the last 90 min for the glycogen synthesis analysis.

S6K1 siRNA transfection. siRNA transfection of myotubes has been described previously [34]. siRNA against S6K1 or a scramble sequence (Dharmacon, USA) (1 μg/ml) was mixed in serum/antibiotic-free DMEM (final volume 50 μl/ml) for 5 min, and 1 μl of the transfection agent, Lipofectamine 2000 (Invitrogen, Sweden) was mixed and incubated with 49 μl DMEM in a separate tube for 5 min. The two mixtures were combined and mixed gently with agitation at room temperature for 30 min. Myocytes were freshly seeded 4–5 days before differentiation

initiation. Differentiated myotubes (2 days) were washed with sterile PBS twice and thereafter 1 ml serum/antibiotic-free DMEM was added to each well and samples were incubated at 37°C. siRNA transfection complexes (100 µl) were added to each well and incubated for >16 h. Myotubes were washed with sterile PBS and 2 ml/well 2% FBS supplemented DMEM was added. Gene expression profiles and metabolic responses are similar between non-transfected and scramble siRNA-transfected myotubes (scramble siRNA control). Myotubes were used 4 days after transfection. Less than 5% cell death was observed in cultures exposed to siRNA/Lipofectamine 2000 as determined by the cell death ratio using reversed light microscopy. All data are presented from scramble and S6K1-transfected myotubes.

RNA purification and quantitative real-time RT-PCR. Myoblasts were cultured in 100-mm dishes, and the differentiation was initiated at >80% confluence. At 5 days after differentiation, myotubes were FBS-starved for >16 h and then incubated with leucine 5 mM for 2 h. At the end of the incubation, cells were washed three times with RNase-free phosphate-buffered saline and then harvested directly for RNA extraction (RNAeasy minikit, Qiagen, Crawley, UK). All RNA was DNase-treated before reverse transcription (RQ1 RNase-free DNase, Promega, Southampton, UK). Total RNA concentration was measured, and cDNA was generated using a reverse-transcription reaction with random hexamer primers (Invitrogen, Sweden). Real-time PCR (ABI-PRISMA 7000 Sequence Detector, Perkin-Elmer Life Sciences) was performed for quantification of specific mRNA content, and data were collected and analyzed by ABI Prism 7000 SDS software version 1.1. mRNA content was normalized for β-actin or 18S (unchanged in both genes) mRNA. All TaqMan primer/probes were purchased from Applied Biosystems (Sweden).

Western blot analysis. Non-transfected myotubes were treated for 20 min or 2 h with 0.05, 0.5 or 5 mM leucine for dose-response studies. For the siRNA experiments, myotubes were treated for 2 h with 5 mM leucine. Myotubes were harvested into ice-cold homogenizing buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, 30 mM $Na_4P_2O_7$, 10% (v/v) glycerol, 1 mM benzamidine, 1 mM dithiothreitol, 10 µg/mL leupeptin, 200 mM phenylmethanesulfonyl fluoride and 1 µM microcystin). Homogenates were rotated for 60 min at 4°C and subjected to centrifugation (20 000 *g* for 15 min at 4°C). Cell lysate were adjusted to equal protein concentration, and boiled in Laemmli-buffer and loaded on 10% or 6–12% gradient gels and transferred to a polyvinylidene difluoride membrane (Immobilon Transfer Membrane, MilliporeA/S). Membranes were blocked in TBST buffer (10 mM Tris-base, 150 mM NaCl, 0.25% Tween 20) containing 5% low fat milk protein for 2 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C, washed with TBST buffer followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Richmond, CA) for 1 h at room temperature. To determine the efficiency of the siRNA transfection, S6K1 protein was detected by Western blot analysis using a rabbit polyclonal antibody against S6K1 (Cell Signaling, Boston, MA). Phosphorylation of various proteins were determined by using following antibodies against phospho IRS1 Ser^{1101}, phospho Akt Ser^{473}, phospho S6K Thr^{389}, phospho rpS6 $Ser^{240/244}$, phospho rpS6 $Ser^{235/236}$ (Cell Signaling Technology), phospho IRS1 Ser^{307}, phospho IRS1 Ser^{318}, and phospho IRS1 $Ser^{636/639}$ (Upstate Biotechnology). A GAPDH (Cell Signaling Technology) antibody was used to confirm equal loading. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL or ECL plus; Amersham, Arlington Heights, IL) and quantified by densitometry using Molecular Analyst Software (Bio-Rad). Results are normalized to total protein and expressed as relative units compared with basal samples loaded on the same gel.

Glucose incorporation to glycogen (glycogen synthesis). Glycogen synthesis was assessed by incorporation of ^{14}C-labeled glucose into glycogen as previously described [31]. Overnight serum-starved myotubes, in 6-well dishes, were treated for 2 h with 0.5 or 5 mM leucine in the absence or presence of 60 nM insulin for 2 h. During the last 90 min, cells were incubated with 5 mM glucose-DMEM, supplemented with D-[U-^{14}C]glucose (final specific activity, 1 µCi/ml). Each experiment was performed on duplicate wells.

Glucose uptake. Glucose uptake was determined as previously described [33]. Overnight serum-starved myotubes were stimulated with or without 5 mM leucine for 2 h in 5 mM glucose-DMEM and insulin (60 nM) was added during the last 60 min. Cells were washed with room temperate PBS and incubated with 10 µM 2-deoxy glucose and [^3H]2-deoxy glucose (2 µCi/ml) for 15 min. Each experiment was performed on duplicate wells.

Statistics. Data are presented as mean ± SEM. Statistical differences for metabolic responses, protein phosphorylation, and content and mRNA expression

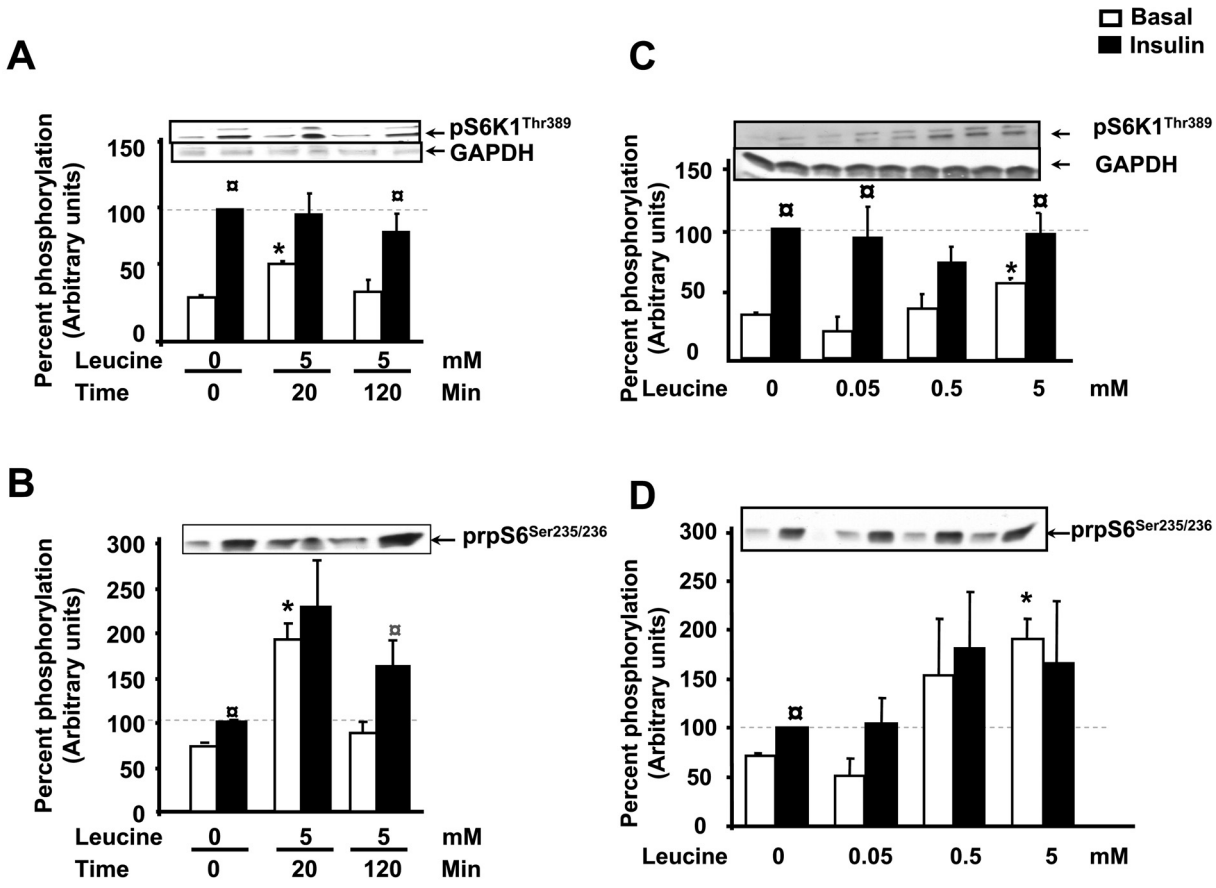

Figure 1. Time-course (*A, B*) and dose-response (*C–E*) effects of leucine in human skeletal muscle myotubes. Effect of 20-min and 2-h treatment with 5 mM leucine on (*A*) S6 kinase 1 (S6K1) phosphorylation or (*B*) S6K1 substrate ribosomal S6 protein (rpS6) phosphorylation. Effect of 20-min treatment with 0.05, 0.5 and 5 mM leucine on (*C*) phosphorylation of S6K1 or (*D*) phosphorylation of rpS6 and (*E*) glucose incorporation to glycogen (2 h with 0.5 and 5 mM leucine). Human skeletal myotubes were incubated with 0, 0.05, 0.5 and 5 mM leucine for 20 min and 2 h, cells were harvested for Western blot analysis (see Materials and methods) for time-course and dose-response studies. Data are mean ± SEM for four to six subjects. * $p<0.05$ vs basal, and ¤ $p<0.05$ insulin-stimulated vs basal.

were determined using student's *t*-test or ANOVA as appropriate. Significant differences were accepted at $p<0.05$

Results

Dose response and time course. Differentiated myotubes were incubated for 20 min or 2 h with 0.05, 0.5 or 5 mM leucine alone, or in combination with 60 nM insulin. Incubation of myotubes for 20 min with 5 mM leucine was associated with increased S6K1 phosphorylation (1.8-fold vs basal, $p<0.05$) but no additive effect on insulin-stimulated S6K1 phosphorylation was seen (Fig. 1A). Moreover, leucine exposure for 20 min also increased rpS6 protein phosphorylation (2.7-fold vs basal, $p<0.05$) (Fig. 1B). S6K1 and rpS6 phosphorylation was vaguely observable after 2-h incubation with 5 mM leucine (Fig. 1A, B). Lower concentrations of leucine (0.05 and 0.5 mM) showed no effect on the phosphorylation of S6K1 and rpS6 protein (Fig. 1C, D).

Basal glucose incorporation to glycogen was reduced 65 % after 2-h stimulation with 5 mM leucine ($P < 0.05$), whereas 0.5 mM leucine was without effect on basal and insulin-stimulated glycogen synthesis (Fig. 1E). These data provide evidence that high concentrations of leucine play a role in glucose metabolism in cultured skeletal muscle; therefore, 5 mM leucine was selected for further experiments, as this gave the strongest indication of impaired glucose metabolism.

S6K1 siRNA transfection. We determined the efficiency of S6K1 siRNA transfection in myotubes and its involvement in regulating mRNA expression of other genes important for glucose metabolism. After 2 days of differentiation, myotubes were transfected with siRNA against S6K1 or a scramble sequence (scramble siRNA control), as described in materials

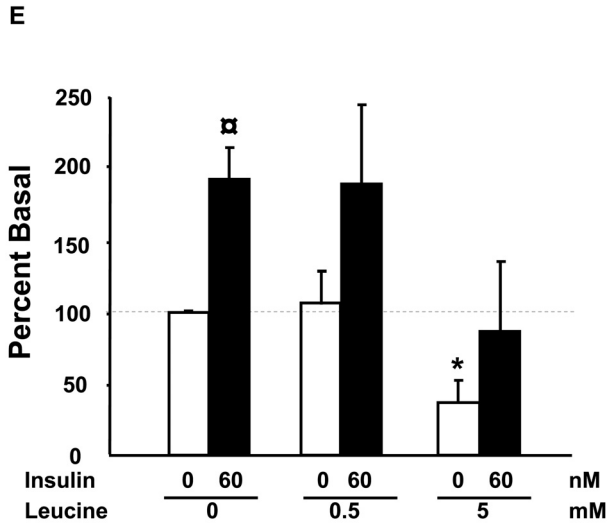

Figure 1. *(continued)*

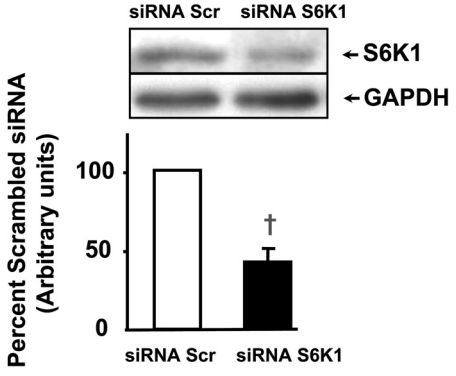

Figure 2. Expression of S6K1 protein after S6K1 RNA interference (siRNA) transfection. Cells were transfected with siRNA against S6K1 or a scrambled sequence. Top panel shows a representative immunoblot. S6K1 protein levels were determined as arbitrary units. Results for protein determination are expressed as percentage arbitrary units for S6K1 protein content in myotubes transfected with scramble siRNA. GAPDH, has been used to confirm loading. Data are presented as mean ± SEM for five to six subjects. † $p<0.05$ vs myotubes transfected with S6K1 siRNA against a scrambled (Scr) sequence.

and methods. Transfection of S6K1 siRNA did not affect myotube morphology, differentiation, or the rate of myotube formation during the 4 days following transfection, as assessed by microscopic examination when compared to either non-transfected or scramble siRNA control myotubes. The efficiency of the S6K1 transfection was determined by Western blot analysis (Fig. 2) and real-time PCR (Table 1) at day 4 after transfection. The siRNA against S6K1 reduced protein and mRNA expression in myotubes (67 % and 78 %, respectively, $p<0.05$), as compared to scramble siRNA control myotubes (Fig. 2 and Table 1).

To determine the direct role of S6K1 on target genes and the role of acute leucine stimulation on gene expressions in skeletal muscle, mRNA expression of S6K1, GLUT1, GLUT4 and IRS1 was determined after 2-h stimulation with 5 mM leucine in scramble siRNA control and S6K1 siRNA-transfected myotubes. Under these conditions, mRNA levels of GLUT1, GLUT4 and IRS1 were unaltered in either leucine-treated myotubes or siRNA S6K1-transfected myotubes compared to scramble siRNA control myotubes (Table 1).

Effect of S6K1 on upstream and downstream signaling. To determine the direct role of S6K1 on downstream kinase phosphorylation, differentiated myotubes were incubated for 20 min or 2 h with 5 mM leucine alone, or in combination with 60 nM insulin for the last 20 min. Myotubes were transfected 4 days before S6K1 or scramble siRNA transfection as described in materials and methods. In myotubes, where S6K1 was silenced, S6K1 Thr^{389} phosphorylation was decreased under basal (80 %, $p<0.05$) and insulin (42 %, $p<0.05$) stimulated conditions vs scramble siRNA control myotubes (Fig. 3A). The $rpS6Ser^{235/236}$ basal phosphorylation was reduced in S6K1 siRNA-transfected myotubes (50 %, $p<0.05$, vs scramble siRNA control myotubes) with no alteration in insulin-induced $rpS6Ser^{235/236}$ phosphorylation compared to the insulin-stimulated scramble siRNA control myotubes (Fig. 3B). Phosphorylation of $rpS6Ser^{240/244}$ showed similar results as the $pS6Ser^{235/236}$ in myotubes (data not shown). Leucine (5 mM) stimulation for 20 min showed a tendency to decrease (40 %, $p<0.13$) insulin-stimulated Akt phosphoryla-

Table 1. mRNA expression profile in primary human skeletal muscle myotubes transfected with scramble (Scr) or S6K1 siRNA (S6K1) after 2-h exposure to leucine. The RNA expression data were determined as percentage quantity over the house keeping gene β-actin mRNA. Data are presented as mean ± SEM for five subjects. * $p<0.05$ vs S6K1 siRNA-transfected myotubes against a scrambled sequence.

Condition	S6K1	GLUT1	GLUT4	IRS1
Scr-basal	100	100	100	100
Scr-leucine	88±12	71±12	110±10	145±32
S6K1-basal	22±9*	89±24	76±16	156±34
S6K1-leucine	12±3*	75±26	132±23	85±35

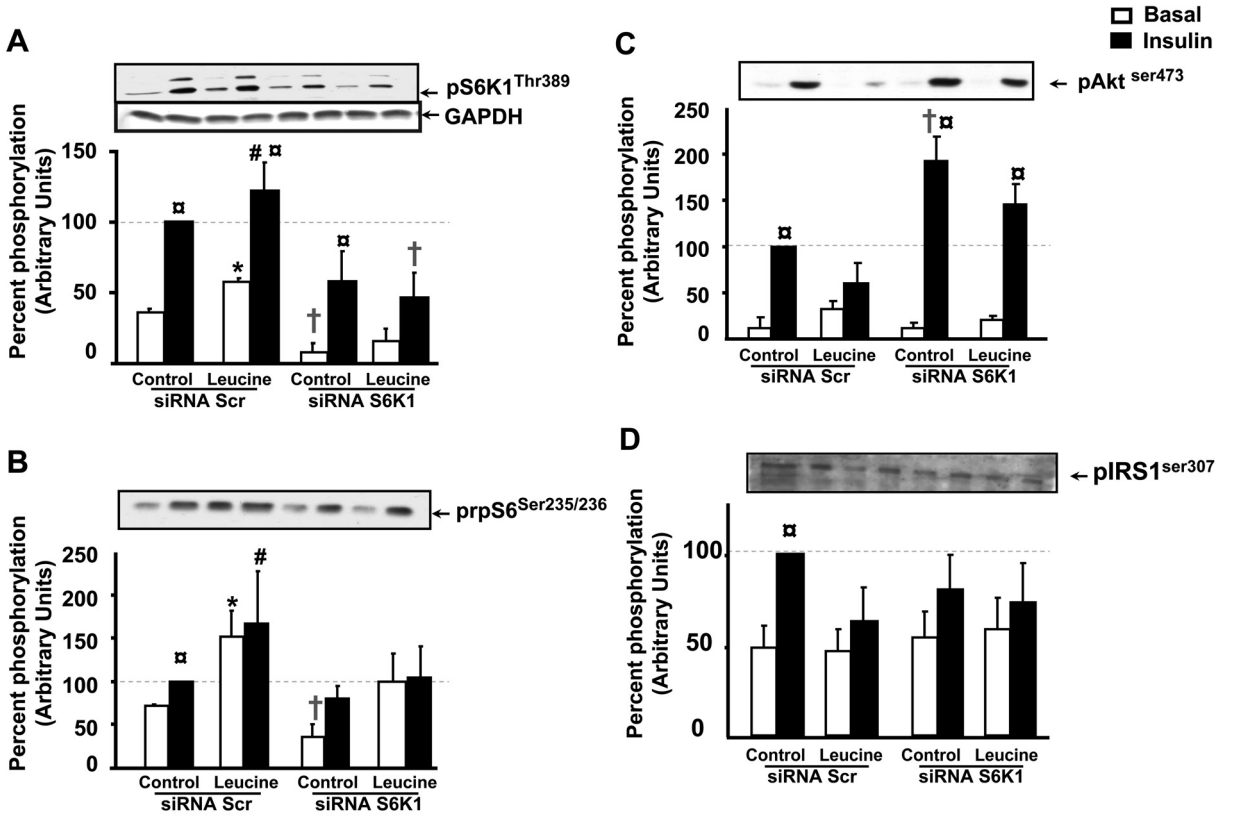

Figure 3. Effect of S6K1 silencing on (*A*) phosphorylation of S6K1 Thr^{389} (*B*) phosphorylation of rpS6 Ser$^{235/236}$, (*C*) phosphorylation of Akt Ser^{473}, (*D*) phosphorylation of IRS1Ser ^{307} and (*E*) phosphorylation of IRS1Ser $^{636/639}$ after leucine stimulation. Human skeletal muscle myotubes were transfected with siRNA against S6K1 or a scrambled sequence. Cells were incubated for 20 min with 5 mM leucine, in the absence or presence of 60 nM insulin during the last 20 min. Results are expressed as percentage arbitrary unit over basal condition for myotubes transfected with a scrambled sequence. Results are presented as mean ± SEM for five to eight subjects. * $p<0.05$ vs scramble siRNA control, ¤ $p<0.05$ control/leucine vs own insulin-stimulated, # $p<0.05$ leucine-insulin vs control-insulin cells and † $p<0.05$ S6K1 siRNA vs scramble siRNA control myotubes.

tion (Fig. 3C), and this effect was more clear after 2-h exposure to leucine (data not shown). Insulin-induced Akt phosphorylation was increased in S6K1 siRNA-transfected myotubes (80%, $p<0.05$ vs scramble siRNA insulin-stimulated myotubes). S6K1 silencing prevented the leucine-induced impairment on insulin action at the level of Akt phosphorylation (Fig. 3C). Phosphorylation of IRS1Ser^{1101} was not detected in human skeletal muscle cell culture. Phosphorylation of IRS1Ser^{318} and IRS1Ser^{302} was unaltered in myotubes treated with leucine for 20 min or 2 h in S6K1 siRNA-transfected myotubes compared to scramble siRNA control myotubes (data not shown). Insulin enhanced IRS1Ser^{307} phosphorylation and this effect tended to be reduced after leucine stimulation for 20 min (42%, $p=0.07$ vs insulin-treated scramble siRNA myotubes; Fig. 3D). This reduction in IRS1Ser^{307} phosphorylation was not found after 2-h leucine treatment (data not shown). Another IRS1 phosphorylation site Ser$^{636/639}$ was also measured. Phosphorylation of IRS1Ser$^{636/639}$ was significantly decreased after S6K1 silencing but not after leucine treatment in either scramble or S6K1-transfected myotubes (Fig. 3E). These data suggest that the IRS1 serine phosphorylation sites tested in this study are not directly involved in the negative regulation of leucine on glucose metabolism in cultured human myotubes.

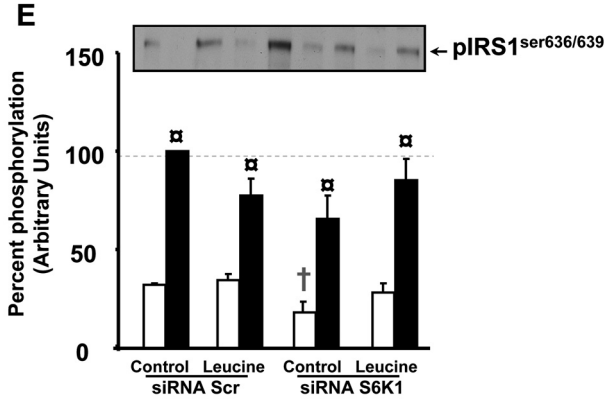

Figure 3. (*continued*)

Glucose incorporation to glycogen (glycogen synthesis). To determine the effect of an acute exposure to leucine on glucose metabolism, glucose incorporation to glycogen was determined (Fig. 4A). Differentiated myotubes were incubated for 2 h with 5 mM leucine alone, or in combination with 60 nM insulin for the final 90 min. Myotubes were transfected 2 days after differentiation with siRNA against S6K1 or a scramble siRNA control as described in materials and methods. In scramble siRNA control myotubes, insulin increased glycogen synthesis 2.1-fold ($p<0.05$), compared to the basal state. This effect of insulin was reduced (38% reduction vs. insulin-stimulated scramble siRNA myotubes, $p<0.05$) after 2-h incubation with 5 mM leucine. Leucine treatment also decreased basal glycogen synthesis 22% (vs scramble siRNA under basal conditions, $p<0.05$). Silencing S6K1 increased glycogen synthesis in basal and leucine-treated myotubes, 40% and 39%, respectively ($p<0.05$, compared to control and leucine-treated myotubes transfected with scramble siRNA; Fig. 4A). The leucine-induced impairment on insulin action (37% reduction vs insulin-stimulated scramble siRNA myotubes, $p<0.05$), was prevented by depletion of S6K1 protein (increased 74% $p<0.05$, compared to leucine-insulin-stimulated myotubes transfected with scramble siRNA).

Glucose uptake. To determine the effect of acute exposure to leucine on glucose transport, human skeletal myotubes were transfected with either siRNA against a scramble sequence or S6K1, and, 4 days after transfection, myotubes were exposed to 5 mM leucine for 2 h with or without 60 nM insulin (for the final 60 min) and glucose uptake was determined. Similar to the data on glycogen synthesis, leucine decreased basal glucose uptake by 22% ($p<0.05$ vs scramble siRNA control myotubes) and prevented insulin-stimulated glucose uptake (reduced 39%, $p<0.05$ vs scramble siRNA myotubes; Fig. 4B). In myotubes transfected with siRNA against S6K1, basal glucose uptake was increased 42% compared to scramble siRNA control myotubes ($p<0.05$). S6K1 silencing increased insulin-stimulated glucose uptake 52% ($p<0.05$) compared with insulin-stimulated scramble siRNA myotubes. In leucine-treated cells, the siRNA-mediated reduction of S6K1 protein restored basal and insulin-stimulated glucose uptake to the level of basal and insulin-stimulated scramble siRNA myotubes (Fig. 4B).

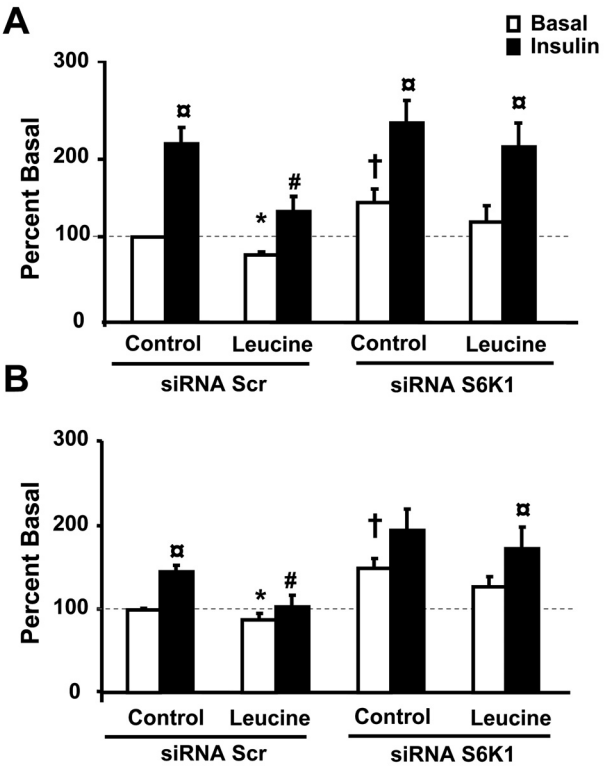

Figure 4. Effect of S6K1 silencing on (*A*) glucose incorporation into glycogen (*B*) glucose transport. Human skeletal muscle myotubes were transfected with siRNA against S6K1 or a scrambled sequence. Cells were incubated for 2 h with 5 mM leucine as indicated, with or without the addition of 60 nM insulin at the last 90 min. The rate of D-[U-^{14}C]glucose incorporation to glycogen was determined (pmol glucose/mg protein/h). For glucose uptake, cells were incubated for 2-h treatment with 5 mM leucine as indicated, with or without the addition of 60 nM insulin at the last 60 min. The rate of [^{3}H]2-deoxyglucose uptake was determined (pmol glucose/mg protein/h). Results are expressed as percentage basal effect in myotubes transfected with a scrambled sequence and presented as mean ± SE for seven to eight subjects. * $p<0.05$ vs scramble siRNA-transfected control, ¤ $p<0.05$ control/leucine vs own insulin-stimulated, # $p<0.05$ leucine-insulin vs control-insulin cells and † $p<0.05$ S6K1 siRNA vs scramble siRNA control myotubes.

Discussion

Excess nutrients from fat-rich diets [35–38] and high-protein diets [20, 39, 40] induce peripheral insulin resistance. Amino acids and BCAA overload, in particular leucine, impairs forearm and whole body insulin-stimulated glucose uptake and hepatic insulin sensitivity in healthy human subjects [7, 19, 41]. Here we tested the hypothesis that acute treatment of skeletal muscle cells with a super-physiological leucine concentration impairs insulin action on glucose uptake and metabolism due to a negative feedback regulation of S6K1. Gene silencing of S6K1 enhanced basal glucose utilization and protected against the development of an acute impairment in insulin action

in response to leucine. Our findings provide evidence for a direct and essential role of S6K1 in the regulation of skeletal muscle glucose metabolism.

Leucine supplements have been shown to increase energy expenditure and to improve insulin sensitivity [42, 43], but effects may be dose dependent. For example, exposure of C_2C_{12} myotubes to 1 mM leucine was without effect on glucose utilization, whereas exposure to 5–10 mM leucine increased 2-deoxyglucose uptake and intracellular glycogen content [44]. Moreover, in human skeletal myotubes, addition of a balanced mixture of amino acids, including leucine in the range of 400–800 μM, promotes glycogen synthesis and S6K1 phosphorylation, which was inhibited by blocking mTOR with rapamycin [45]. Here we report that leucine increased phosphorylation of rpS6 and S6K1 in a dose-dependent manner, with the greatest effect observed at the 5 mM concentration. We also provide evidence that an acute stimulation of primary human myotubes with 0.5 mM leucine was without effect on basal or insulin-stimulated glucose uptake and glycogen synthesis. However, in the presence of 5 mM leucine, basal and insulin-stimulated responses were impaired. The differences in the effects of leucine on glucose metabolism between the present and earlier studies [44, 45] are unclear, but might be due to experimental differences in the cell systems employed or leucine concentration studied. Our results are compatible with early *in vivo* studies using the euglycemic clamp technique showing leucine infusion impairs insulin-mediated whole body glucose uptake [7, 19, 41]. Changes in gene expression or IRS1 and GLUT4 are unlikely to account for the reductions in basal and insulin-stimulated glucose metabolism observed in the present study since the leucine exposure was limited to 2 h. Rather, the transient increase S6K1 phosphorylation in response to leucine may be involved the impairment in glucose uptake and metabolism.

To directly test the hypothesis that leucine-impaired insulin action on glucose uptake and metabolism is due to a negative feedback regulation of S6K1, we applied siRNA. We studied S6K1 since its activity is elevated in several animal models of diabetes and obesity, including genetic models such as ob/ob and K/K A(y) mice or dietary-induced insulin-resistant mice [21]. Importantly, whole body S6K1 knockout mice are protected against dietary-induced obesity due to an enhancement in beta-oxidation [21], where S6K1 depletion blunts Ser^{307} and $Ser^{636/S639}$ phosphorylation [21] and improves glucose homeostasis. In response to amino acid stimulation, S6K1 knockout mice display mild glucose intolerance, with normal fasting and fed glucose levels [28]. In addition to these improvements in glucose homeostasis, S6K1 knockout mice are protected against dietary-induced insulin resistance [21]. In L6 muscle cells, S6K1 has been shown to directly phosphorylate IRS1 on Ser^{1101}, and mutations of this site blocks the inhibitory effect of amino acids on IRS-1 tyrosine and Akt phosphorylation [46]. Here we show that depletion of S6K1 primary human muscle cells increases basal glucose uptake and prevents the leucine-induced impairments in insulin action on glucose uptake and incorporation into glycogen. We also provide evidence that siRNA against S6K1 enhances insulin-stimulated Akt phosphorylation and prevents the leucine-induced impairment of insulin action. Akt plays an essential role in cellular signaling and the control of cellular metabolism, growth, proliferation and apoptosis [47, 48]. Here we provide direct evidence that S6K1 participates in the negative regulation of insulin action on Akt phosphorylation and glucose metabolism.

Since S6K1 plays a role as a critical signaling component that controls insulin sensitivity through a negative feedback loop involving IRS1 [21, 46], we assessed serine phosphorylation of IRS1. Leucine *per se* was without effect on basal or insulin-induced IRS1 Ser^{307} and IRS1 $Ser^{636/639}$ phosphorylation. Basal IRS1 Ser^{307} phosphorylation was unaltered after S6K1 silencing. We have also assessed serine phosphorylation of IRS1 on Ser^{1101}, Ser^{318}, and Ser^{302} (data not shown), but our experiments provide no conclusive evidence to implicate a role for these sites on leucine action, possibly because we were studying the endogenous protein, rather than in cells, whereby IRS1 is overexpressed. We estimated the phosphorylation of IRS1 Ser^{1101} because several lines of evidence suggest that IRS1 Ser^{1101} phosphorylation is involved in insulin resistance in mice and humans [46, 49]. Moreover, in healthy humans, amino acid infusion is associated with increased skeletal muscle S6K1 activity and IRS1 Ser^{1101} phosphorylation, concomitant with reduced IRS1 function and insulin resistance [46].

Exposure of L6 muscle cultured cells to high concentrations of amino acids impairs IRS1-associated PI3K activity, suggesting that excessive exposure to amino acids may contribute to insulin resistance [24, 25]. However in the present study, leucine, rather than a mixture of amino acids was studied, and we cannot exclude the possibility that the combination of essential amino acids and BCAAs may have a more potent effect on IRS1 serine phosphorylation. Based on our observations, we propose that the leucine-induced inhibition on insulin-stimulated PI3K activity is rapidly and temporally related to the activation of S6K1 and IRS1 serine phosphorylation. Even though we were unable to show an effect on IRS1 serine phosphorylation, our results from glucose uptake, glycogen synthesis and Akt ^{Ser473} phosphorylation

indicate that S6K1 plays a role in the negative feedback mechanism on insulin signaling and glucose metabolism.

While our data may provide insight into the role of S6K1, the usage of this *in vitro* system may not totally mimic the *in vivo* system, thus the physiological relevance of these findings need to be tempered.

In conclusion, treatment of human skeletal muscle myotubes with 5 mM leucine impairs basal and insulin-stimulated glucose utilization. Importantly, siRNA-based silencing of S6K1 restores basal glucose uptake and protects against the development of impaired insulin action induced by leucine overload. Our results indicate S6K1 plays a direct role in insulin action and the regulation of glucose metabolism in human skeletal muscle.

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