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Journal of Nutritional Biochemistry 24 (2013) 32–38

Journal of Nutritional Biochemistry

Phosphorylated S6K1 (Thr389) is a molecular adipose tissue marker of altered glucose tolerance☆

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Received 16 August 2011; received in revised form 5 December 2011; accepted 3 January 2012

Abstract

Molecular tissue markers of altered glucose metabolism will be useful as potential targets for antidiabetic drugs. S6K1 is a downstream signal of insulin action. We aimed to evaluate ^{pThr389}S6K1 and total S6K1 levels in human and rat fat depots as candidate markers of altered glucose metabolism. ^{pThr389}S6K1 and total S6K1 levels were measured using enzyme linked immune sorbent assay (ELISA) in 49 adipose tissue samples from subjects with morbid obesity and in 18 peri-renal white adipose tissue samples from rats. The effects of high glucose and rosiglitazone have been explored in human preadipocytes. ^{pThr389}S6K1/$_{total}$S6K1 in subcutaneous adipose tissue was significantly increased subjects with Type 2 diabetes (0.78±0.26 *vs.* 0.55±0.14, *P*=.02) and associated with fasting glucose (r=0.46, *P*=.04) and glycated hemoglobin (r=0.63, *P*=.02) in SAT. Similar associations with fasting glucose (r=0.43, *P*=.03) and *IRS1* (r=-0.41, *P*=.04) gene expression were found in visceral adipose tissue. In addition, rat experiments confirmed the higher ^{pThr389}S6K1/totalS6K1 levels in adipose tissue in association with obesity-associated metabolic disturbances. ^{pThr389}S6K1/totalS6K1 was validated using western blot in rat adipose tissue. Both ELISA and western blot data significantly correlated (*r*=0.85, *P*=.005). In human preadipocytes, high glucose medium led to increased ^{pThr389}S6K1/total S6K1 levels in comparison with normal glucose medium, which was significantly decreased under rosiglitazone administration. In conclusion, in human and rat adipose tissue, phosphorylated S6K1 is a marker for increased glucose levels.

Keywords: S6K1; Adipose tissue; Type 2 diabetes

1. Introduction

Adipose tissue plays a central role in the management of systemic energy stores as well as in many other processes. The loss of insulin sensitivity in adipose tissue impaired its capacity for lipid storage, which contributed in obesity-associated metabolic disturbances [1,2].

Recently, some evidences have demonstrated that ribosomal protein S6 kinase 1 (S6K1), a component of the insulin signal transduction pathway, is a key molecular element in driving insulin resistance under conditions of nutrient overload [3-5]. Such a system has been speculated to have evolved to prevent cells from actively taking up nutrients in response to insulin under conditions of nutrient overload, such as in the obese setting. The incessant supply of nutrients associated with the obese state would lead to constitutive activation of mTOR/S6K1 and desensitization of insulin signaling. S6K1 contributes to insulin resistance by phosphorylation of IRS-1 at several serine residues. The S6K-IRS1 interaction represents a molecular mechanism by insulin, other hormones, and amino acids induce insulin resistance [6,7]. Global inactivation of S6K1 in knock-out mice protects mice from diet-induced insulin resistance [8]. In 3T3-L1 cells, inhibition of the S6K1 activity by rapamycin leads to protection from insulin resistance [7]. Phosphorylation of Thr389 in the linker domain of S6K is critical for kinase function [9,10].

To our knowledge, no studies of S6K1 activity in human adipose tissue are available. In the search of molecular adipose tissue markers of altered glucose metabolism, we aimed to investigate ^{pThr389}S6K1 and total S6K1 in human subcutaneous and visceral fat depots in association with metabolic parameters by enzyme linked immune sorbent assay (ELISA) that has been validated by western blot. Finally, to study the effects of hyperglycemia on S6K1

☆ This work was partially supported by research grants from the Ministerio de Educación y Ciencia (SAF2008-02073). CIBEROBN Fisiopatología de la Obesidad y Nutrición is an initiative from the Instituto de Salud Carlos III from Spain.

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http://dx.doi.org/10.1016/j.jnutbio.2012.01.005

phosphorylation status, we explored in human preadipocytes the effects of high glucose levels and rosiglitazone (a PPARg agonist, that improve adipogenesis and insulin action, with glucose reducing effects) on ^{pThr389}S6K1/S6K1 levels.

2. Methods and materials

2.1. Participants' recruitment

Twenty-two subcutaneous [14 (5 men and 9 women) with normal glucose tolerance and 8 (1 man and 7 women) with Type 2 diabetes] and 27 visceral [17 (5 men and 12 women) with normal glucose tolerance and 10 (2 men and 8 women) with Type 2 diabetes] adipose tissue samples were obtained from fat depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia and gastric by-pass surgery), washed, fragmented and immediately flash-frozen in liquid nitrogen before be stored at −80°C. All patients underwent a clinical assessment including medical history, physical examination, comorbidity evaluation as well as nutritional interviews performed by a multidisciplinary consultation team. All subjects were of Caucasian origin, being Spanish from the same area (Girona). Exclusion criteria for those patients included the following: (1) clinically significant hepatic, neurological, or other major systemic disease, including malignancy; (2) history or current clinical evidence of hemochromatosis; (3) history of drug or alcohol abuse, defined as >80 g/day, or serum transaminase activity more than twice the upper limit of normal; (4) an elevated serum creatinine concentration; (5) acute major cardiovascular event in the previous 6 months; (6) acute illnesses and current evidence of high grade chronic inflammatory or infective diseases; and (7) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of the study. All subjects gave written informed consent after the purpose of the study was explained to them. The hospital ethics committee (Clinical Investigation Ethic Committee, CIEC) approved the protocol.

2.2. Anthropometric measurements and analytical determinations

Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used and the first and fifth phases were recorded. Values used in the analysis are the average of three readings taken at 5-min intervals. Patients were requested to withhold alcohol and caffeine during at least 12 h prior to the different tests.

The serum glucose levels were measured in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Beckman Co., Brea, CA). The coefficient of variation was 1.9%. HbA1c was measured by the high-performance liquid chromatography method (Bio-Rad, Muenchen, Germany, and autoanalyser Jokoh HS-10, respectively). Intraassay and interassay coefficients of variation were less than 4% for all these tests. Serum insulin was measured in duplicate by monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The intra-assay coefficient of variation was 5.2% at a concentration of 10 mU/l and 3.4% at 130 mU/l. The interassay coefficients of variation were 6.9 and 4.5% at 14 and 89 mU/l, respectively. Total serum cholesterol was measured through the reaction of cholesterol esterase/oxidase/peroxidase, using a BM/Hitachi 747. high-density lipoprotein (HDL) cholesterol was quantified after precipitation with polyethylene glycol at room temperature. Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase.

2.3. Animals and experimental design

Wistar female rats bred in the vivarium of the University of Córdoba were used. The animals were maintained under constant conditions of light (14 h of light, from 07.00 h) and temperature (22°C) and, after weaning, were housed in groups of four rats per cage, with free access to pelleted food and tap water. The experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation, and were conducted in accordance with the European Union normative for care and use of experimental animals.

Blood and adipose tissue sampling for different analyses was carried out in adult (approx. 120 days old) female rats. Animals were fasted overnight (<12 h) before sampling. In order to induce different degrees of overweight and metabolic perturbations, three experimental groups were generated by sequential combination of various metabolic insults. Thus, Group 1 consisted of control rats and raised in litters of 12 pups per lactating dam (normal litters, NL) as a means to allow normal feeding during lactation, which was followed by ad libitum feeding with standard low fat diet (LF <10% fat content; D12450B; Research Diets, New Brunswick, NJ, USA) during puberty and adulthood. Group 2 consisted of moderately overweighed rats, as caused by postnatal overnutrition due to breeding in litters of 4 pups per lactating dam (small litters, SL), which was followed by feeding on a high fat diet (HF >45% fat content; D12451, Research Diets) from weaning onwards. Finally, Group 3 consisted of overtly overweighed rats, in which the metabolic insults of Group 2 were preceded by neonatal injection of a pharmacological dose of testosterone propionate (TP): one bolus of 1.25 mg/rat on day 1 postpartum. Protocols of perinatal androgenization analogous to this reported here have been previously shown to induce an obesogenic and diabetogenic state in rodents [11], associated to perturbed ovarian cyclicity and anovulation.

Upon becoming adults, experimental animals were daily monitored for vaginal cytology as to assess the stage of the ovarian cycle, except for rats in Group 3 because of the anovulatory features described above for perinatal TP administration. To control for the potential bias of dynamic changes in endogenous sex steroid milieu linked to the different phases of the ovarian cycle, all the cyclic animals (Groups 1 and 2) were sampled in the morning (10:00–12:00) of the stage of diestrus-1. Animals from Group 3 were sampled in parallel. Of note, perinatal androgenization is known to prevent the preovulatory surges of gonadotropins and sex steroids so that reproductive hormone variability within this group is very low.

Animals from the three experimental groups were sampled upon decapitation: trunk blood was taken for glucose, leptin and insulin determinations, while a block of peri-renal white fat tissue was excised free of blood upon necropsy from each animal, snap-frozen in liquid N2 and stored at −80 C until used for ^{pThr389}S6K1and total S6K1 determinations. TP was purchased from Sigma Chemical (St Louis, MO, USA) and dissolved in olive oil for androgenization experiments; non-androgenized groups (1 and 2) received an equivalent injection of vehicle (oil, 100 μL/rat).

Serum glucose concentrations were determined using an automatic glucose analyzer (Accu-Chek; Roche Diagnostics, Barcelona, Spain). Serum leptin and insulin levels were assayed using commercial radioimmunoassay kits from LINCO Research (St. Charles, MO, USA), following the instructions of the manufacturer. The sensitivities of the assays were 0.05 and 0.01 ng per tube for leptin and insulin, respectively; the intra-assay coefficients of variation were <5%.

Oral glucose tolerance tests (OGTT) were electively conducted in a subset of adult animals from the three experimental groups (n=8/group). After overnight fasting, the animals were submitted to a bolus of a glucose solution (2 g/kg) via oral gavage, preceded by basal glucose determinations. Thereafter, blood samples were drawn for glucose measurements at 20-, 60-, 120- and 180-min after glucose administration. Insulin concentrations were also determined at basal conditions, and at 20- and 60-min after the oral glucose bolus. In order to ease comparison of results, integral glycemic responses, defined as the net increase of serum glucose concentrations over the 180-min period following the oral glucose bolus, were calculated as area under the curve (AUC) using the trapezoidal rule. Similarly, integral insulin levels during the first 60 min after the oral glucose challenge were estimated using a similar AUC approach.

2.4. Adipose tissue protein preparation

Proteins were extracted from subcutaneous and visceral adipose tissue (200 mg) by using a Polytron PT-1200C homogenizer (Kinematica, Lucerne, Switzerland) directly in radioimmuno precipitation assay (RIPA) buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150mM NaCl, and 50 mM Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 g/ml aprotinin and 2 g/ml leupeptin). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 13 000 rpm for 60 min at 4°C, recovering the soluble fraction below the fat supernatant and avoiding the non-homogenized material at the bottom of the centrifuge tube. Protein concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The same amount of protein (20 μg) was carried in ELISA.

2.5. Experiments in human preadipocytes

Isolated preadipocytes (Zen-Bio, Research Triangle Park, NC, USA) were plated on T-75 cell culture flasks and cultured at 37°C and 5% CO2 in DMEM/Nutrient Mix F-12 medium (1:1, v/v) supplemented with 10 U/ml P/S, fetal bovine serum (FBS) 10%, HEPES 1% and glutamine 1% (all from GIBCO, Invitrogen, Barcelona, Spain). One week later, the isolated and expanded human subcutaneous pre-adipocytes were cultured (~40.000 cells/cm^2) in 12-well plates with pre-adipocyte medium (Zen-Bio, Research Triangle Park, NC, USA) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, penicillin and streptomycin in a humidified 37°C incubator with 5% CO2. Twenty-four hours after plating, cells were checked for complete confluence. When complete confluence was achieved, we performed the following experiments: NG, normal glucose (3.15 g/L); HG, high glucose (18 g/L); and HG + rosiglitazone (1 μM). All these treatments were performed in triplicate during 48 h.

To evaluate the effects of high glucose and rosiglitazone in insulin action, we measured insulin-induced ^{pSer473}AKT and total AKT by PathScan Phospho-Akt1 (Ser473) Sandwich ELISA kit and PathScan Total Akt1 Sandwich ELISA kit (Cell Signaling Technology, Izasa, Barcelona, Spain). The analysis was performed following manufacturer's instructions. Insulin (100 nM) administration was performed during 10 min at the end of the experiment (after 48 h).

2.6. ^{pThr389}S6K1, total S6K1, ^{pSer2448}mTOR and total mTOR determination

Human and rat ^{pThr389}S6K1 and total S6K1 were measured by ELISA (KHO0581, Invitrogen, Barcelona, Spain; and DYC8962-2, R & D, Vitro, Madrid, Spain) according to the manufacturer instructions. ^{pSer2448}mTOR and total mTOR by PathScan Phospho-mTOR (Ser2448) Sandwich ELISA kit and PathScan Total mTOR Sandwich ELISA kit (Cell Signaling Technology, Izasa SA, Barcelona, Spain). The analysis was performed following manufacturer's instructions. The average recovery was 90%.The specificity

of these assays was confirmed by peptide competition and Western blot analysis. Intra- and interassay coefficients of variation for all these determinations were between 5–10%.

The same amount of protein (20 μg) was carried in ELISA.

2.7. Western blot analysis

Nine RIPA protein extracts (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes by conventional procedures. Membranes were immunoblotted with ^{pThr389}S6K1 (Invitrogen, Barcelona, Spain) S6K1 and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-rabbit IgG and anti-mouse IgG coupled to horseradish peroxidase (HRP) was used as secondary antibody. HRP activity was detected by chemiluminescence and quantification of protein expression was performed using scion image software.

2.8. Gene expression analyses

RNA was prepared from adipose tissue fragments using RNeasy Lipid Tissue Mini Kit (QIAgen, Valencia, CA, USA). The integrity of each RNA sample was checked by either agarose gel electrophoresis or with an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA was quantified by means of spectrophotometer (GeneQuant, GE Health Care, Piscataway NJ, USA) or with the bioanalyzer. 3 μg of RNA from each fat sample were then reverse-transcribed to cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol.

Gene expression was assessed by real time PCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) and TaqMan technology suitable for relative gene expression quantification. The reaction was performed following manufacturers' protocol in a final volume of 25 μl. The cycle program consisted of an initial denaturing of 10 min at 95°C then 40 cycles of 15-s denaturizing phase at 92°C and a 1-min annealing and extension phase at 60°C. Positive and negative controls were included in all the reactions.

The commercially available and pre-validated TaqMan primer/probe sets used were as follows: cyclophilin A (*PPIA; Hs99999904_m1*) was used such as endogenous control for all target genes in each reaction and fatty acid synthase (*FASN; Hs00188012_m1*) and Insulin Receptor Substrate-1 (*IRS1;* Hs00178563_m1) were the target genes.

A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human Cyclophilin A (PPIA) cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta Ct}$, so gene expression results are expressed in all cases as expression ratio relative to PPIA gene expression according to manufacturers' instructions.

2.9. Statistical analysis

Unpaired and paired *t* tests and Pearson's correlation were used to evaluate the differences or associations between continuous variables. Non-parametric Mann-Whitney test and Spearman's correlation were used because some parameters did not display a normal distribution. One-way analysis of variance were used to performed statistical comparisons in rat experiments. Multiple regression models were used to assess the influence of selected variables on S6K1 activity. Levels of statistical significance were set at $P<.05$.

3. Results

3.1. Findings in humans

Anthropometrical and biochemical parameters from the studied subjects are shown on Table 1. ^{pThr389}S6K1 and ^{pThr389}S6K1/total S6K1 in subcutaneous adipose tissue significantly increased in patients with Type 2 diabetes (Table 1).

In subcutaneous adipose tissue (SAT), the ratio ^{pThr389}S6K1/total S6K1 was associated with fasting glucose ($r=0.46$, $P=.04$), and HbA1c ($r=0.63$, $P=.02$) (Fig. 1), and tended to be associated with HDL-cholesterol ($r=-0.42$, $P=.05$). In visceral adipose tissue (VAT), the ratio ^{pThr389}S6K1/total S6K1 was associated with fasting glucose ($r=0.43$, $P=.03$) (Fig. 1), *FASN* (r=−0.43, $P=.03$) and *IRS1* ($r=-0.41$, $P=.04$) gene expression (Table 2).

In multiple regression analysis, fasting glucose ($P=.04$) contributed independently to ^{pThr389}S6K1/total S6K1 variance in SAT after controlling for the effects of age, sex and BMI in SAT. In the same model, only fasting glucose ($P=.045$) contributed independently to ^{pThr389}S6K1/total S6K1 variance in VAT.

Table 1
Anthropometrical and biochemical variables of studied subjects

	NGT	Type 2 diabetes	*P*
Number of participants	31	18	-
Age (years)	43±5.9	44.7±2.9	.45
BMI (kg/m^2)	39.7±10.3	43.2±4.4	.38
Fasting glucose (mg/dl)	82.6±7.8	162.4±31.6	**<.001**
HbA1c (%)	4.8±0.37	6.3±1.5	**.01**
Fasting triglycerides (mg/dl)	96.3±37.2	124.2±45.6	**.01**
HDL-cholesterol (mg/dl)	51.6±8.5	54.1±9	.6
Findings in SAT	*N*=14	*n*=8	
^{pThr389}S6K1 (EU/ml)	19.9±7.6	29.3±9.2	**.004**
^{pThr389}S6K1/Total S6K1 (EU/ng)	0.55±0.14	0.78±0.26	**.02**
Total S6K1 (ng/ml)	36.1±18.5	37.2±9.4	.8
IRS1 gene expression (R.U.)	0.016±0.011	0.009±0.002	.1
FASN gene expression (R.U.)	0.11±0.06	0.082±0.08	.4
Findings in VAT	*N*=17	*n*=10	
^{pThr389}S6K1 (EU/ml)	18.9±7.8	22.3±4.35	.15
^{pThr389}S6K1/ Total S6K1 (EU/ng)	0.63±0.26	0.75±0.4	.3
Total S6K1 (ng/ml)	29.6±12.4	29.9±9.9	.9
IRS1 gene expression (R.U.)	0.012±0.005	0.007±0.002	**.01**
FASN gene expression (R.U.)	0.116±0.1	0.039±0.02	**.02**

Values in bold indicate statistical significance ($P<.05$). Data are presented as means±S.D.
NGT, normal glucose tolerance; R.U., relative units; VAT, visceral adipose tissue.

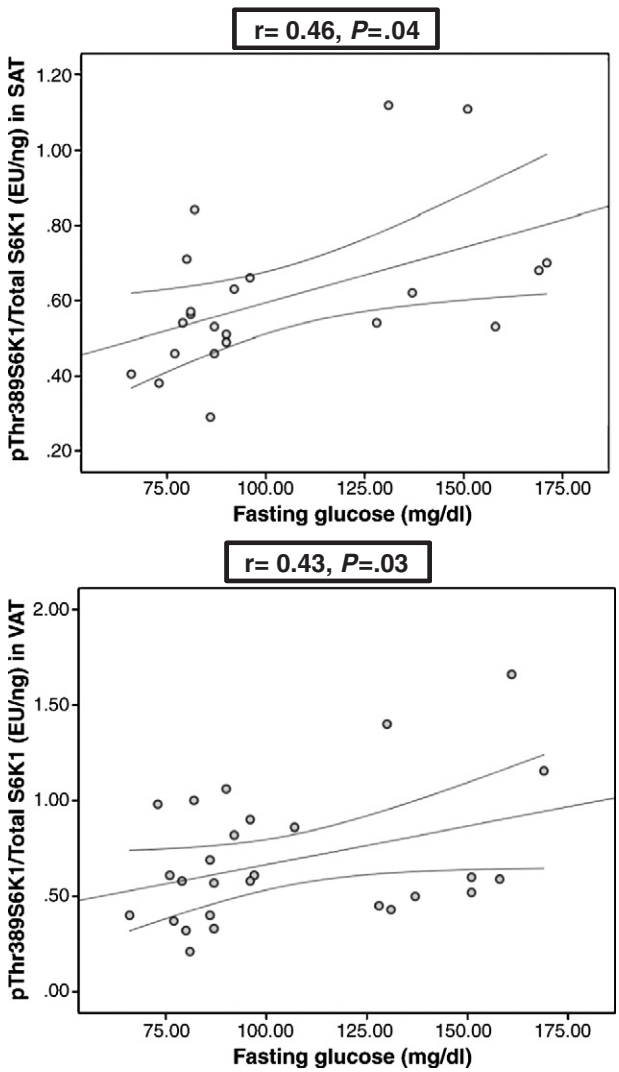

Fig. 1. Correlation between ^{pThr389}S6K1/ Total S6K1 and fasting glucose in SAT and in VAT in human cross-sectional study.

Table 2
Associations between ^{pThr389}S6K1/Total S6K1 and metabolic variables

	SAT ^{pThr389}S6K1/ Total S6K1		VAT ^{pThr389}S6K1/ Total S6K1	
	R	*P*	r	*P*
Age (years)	0.13	.5	-0.13	.4
BMI (Kg/m^2)	0.1	.6	0.29	.1
Fasting glucose (mg/dl)	0.46	**.04**	0.43	**.03**
HbA1c (%)	0.63	**.02**	0.38	.07
Fasting triglycerides (mg/dl)	0.36	.1	0.25	.2
HDL-cholesterol (mg/dl)	-0.42	.05	-0.19	.4
SAT *IRS1* gene expression (R.U.)	-0.32	.2	-	-
SAT *FASN* gene expression (R.U.)	0.3	.2	-	-
VAT *IRS1* gene expression (R.U.)	-	-	-0.41	**.04**
VAT *FASN* gene expression (R.U.)	-	-	-0.43	**.03**

Values in bold indicate statistical significance (*P* value <.05).

Finally, in a subsample of 25 visceral and 13 subcutaneous fat depots, mTOR phosphorylation (activation) status was studied. ^{pSer2448}mTOR/$_{total}$mTOR correlated significantly with ^{pThr389}S6K1/ ^{total}S6K1 in both visceral and subcutaneous adipose tissues (Fig. 2).

3.2. Findings in rats

Female rats submitted to early overnutrition followed by high-fat diet (SL/HF) became modestly overweighed at adulthood (<10% increased BW); yet, diet-induced adiposity was evidenced by a substantial rise in the circulating levels of leptin (nearly 2-fold increase over controls) (Fig. 3A and B). In addition, a nonsignificant increase in serum basal glucose levels was detected in this group, which nonetheless failed to display an overt rise of basal insulin concentrations (Fig. 3C and D). In contrast, neonatally androgenized female rats, submitted to postnatal overfeeding followed high-fat diet (A/SL/HF), showed a marked increase in final BW (approx. 40% rise) and serum leptin levels (>2.5-fold elevation over controls) in adulthood (Fig. 3A and B). In addition, basal glucose levels rose by 30% and basal insulin concentrations were doubled in this extreme group (Fig. 3C and D). As further index of the metabolic compromise of these animals, the glycemic mass estimated as AUC during the first 180-min following an oral overload of glucose was approximately four times higher in overtly overweight (A/SL/HF) animals, as compared to controls. A similar fourfold increase in integral (AUC) insulin levels after the oral glucose bolus was detected in this group (Fig. 3E and F). Likewise, moderately overweighed (SL/HF) females displayed a perturbed glucose tolerance, as evidenced by integral glycemic and insulin responses, which were >2.75 times and 60% higher than in controls, respectively (Fig. 3E and F).

In parallel to these metabolic phenotypes, the ratio ^{pThr389}S6K1/ S6K1 and ^{pSer2448}mTOR/$_{total}$mTOR significantly increased in subcutaneous adipose tissue from A/SL/HF rat compared with SL/HF rats. In both A/SL/HF and SL/HF groups, the ratio ^{pThr389}S6K1/S6K1 and ^{pSer2448}mTOR/$_{total}$mTOR also were consistently increased in comparison with control rats (Fig. 4A).

Finally, to validate the ELISA used, Western blot assay of nine representative samples (three from control, three from SL/HF and three from A/SL/HF rats) was performed (Fig. 4B). These data significantly correlated with the ELISA values for the same samples (r=0.85, P=.005) (Fig. 4C).

3.3. Findings in human preadipocytes

Finally, the effects of hyperglycemia and antidiabetic drugs (rosiglitazone) were evaluated in human preadipocytes. In high glucose conditions, ^{pThr389}S6K1/S6K1 levels increased in comparison with normal glucose (P=.01), and this increase was blunted under rosiglitazone administration (P=.02) (Fig. 5). In a same manner, rosiglitazone administration enhanced significantly insulin signaling (increasing insulin-induced $^{Ser\ 473}$Akt phosphorylation) in high glucose conditions (P=.03) (Fig. 5).

4. Discussion

It is well known that increased ^{pThr389}S6K1 levels are associated with insulin resistance, decreasing insulin-stimulated tyrosine phosphorylation of IRS-1 and blunting insulin signaling [6,12]. In this study, we found that ^{pThr389}S6K1 levels significantly increased in subcutaneous adipose tissue from morbidly obese patients with Type 2 diabetes. In addition, rat and in vitro experiments confirmed the higher ^{pThr389}S6K1 levels in subcutaneous adipose tissue and adipocytes in association with hyperglycemia and insulin resistance.

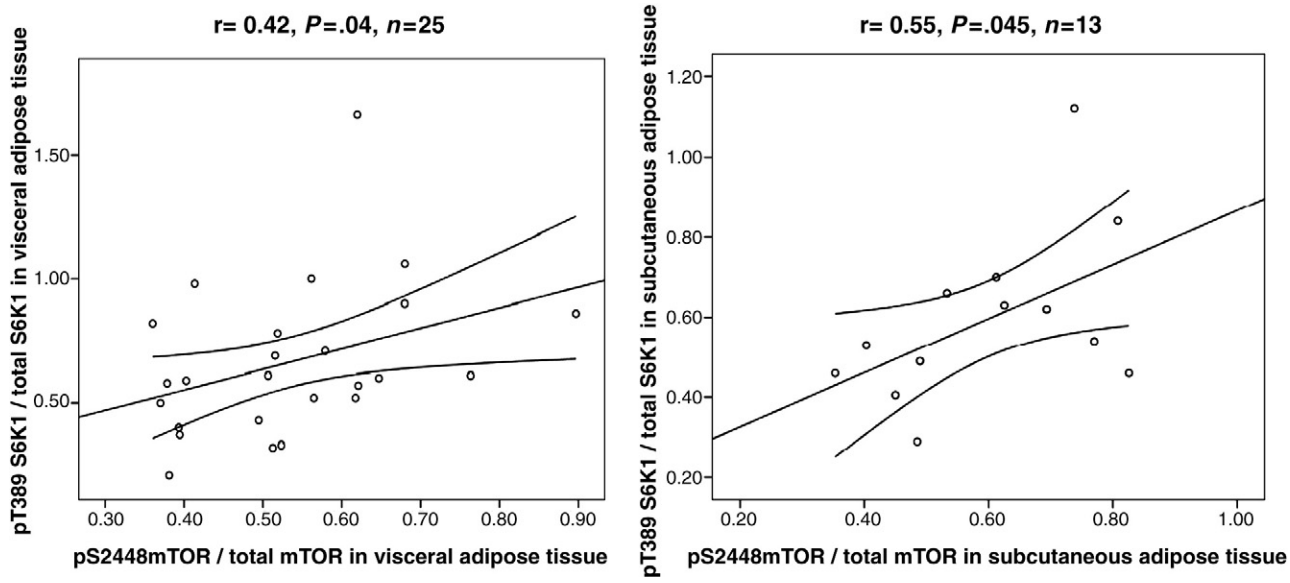

Fig. 2. Correlation between ^{pThr389}S6K1/ Total S6K1 and ^{pSer2448}mTOR/ Total mTOR in 25 visceral and 13 subcutaneous adipose tissues in human cross-sectional study.

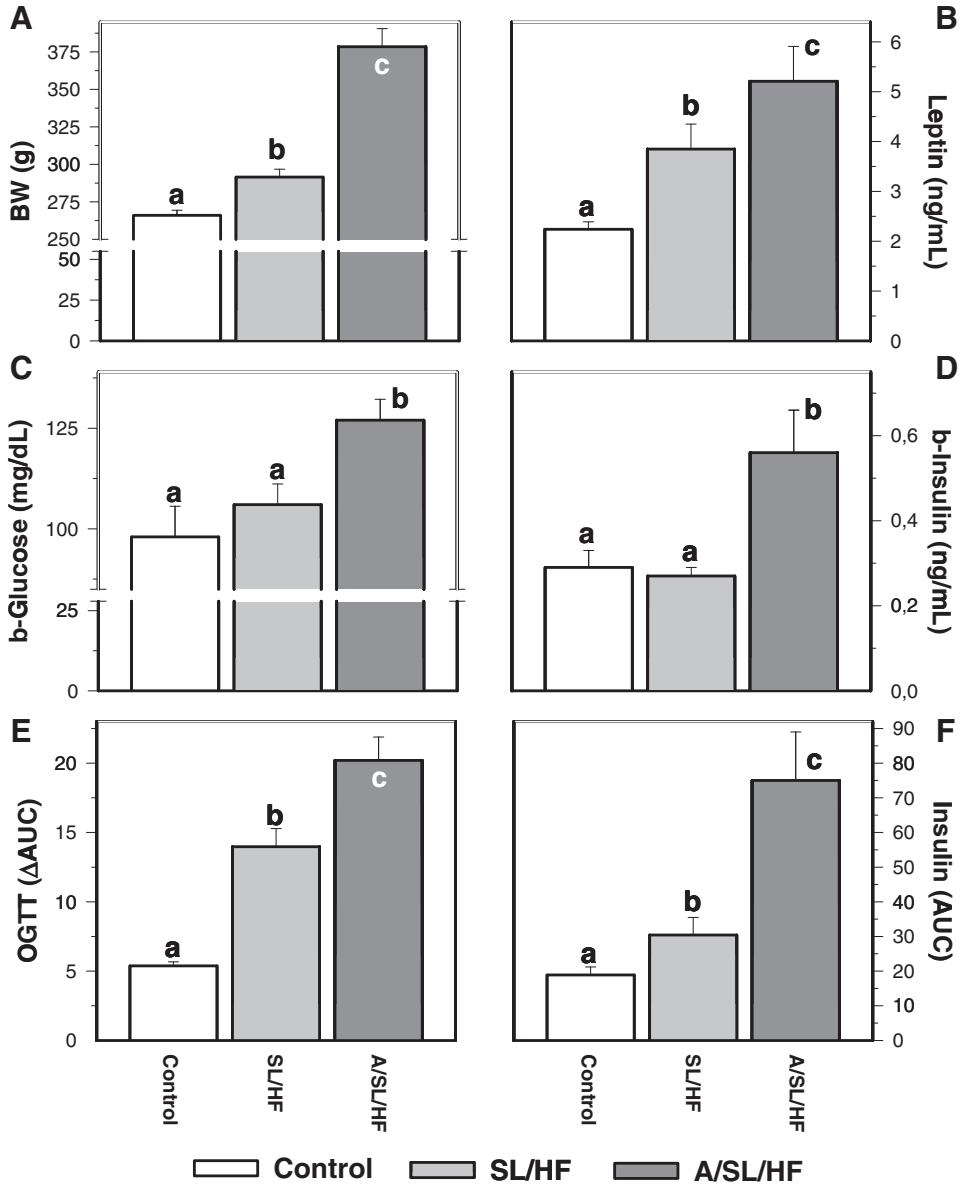

Fig. 3. Compilation of metabolic parameters in the three different experimental groups: (A) control rats raised in litters of 12 pups per dam and fed with low fat diet after weaning (NL/LF); (B) moderately overweighed rats, raised in litters of 4 pups per dam and fed with high fat diet after weaning (SL/HF); and (C) overtly overweighed rats, submitted to neonatal androgenization, and raised in litters of 4 pups per dam with feeding on high fat diet after weaning (A/SL/HF). Body weights (BW) and basal leptin, glucose and insulin levels in the three experimental groups are presented in panels A–D. In addition, results from OGTT tests in the same groups are shown in the lower panels. Integral glycemic responses, calculated as net increases over the basal glucose concentrations, are presented as AUC values (mg/dl·180 min/1000) in panel E. Integral insulin responses during the first 60-min after the oral glucose bolus are depicted as AUC values (ng/ml 60 min) in panel F. Groups with different superscript letters are statistically different ($P<.05$).

In fact, S6K1 phosphorylation status increased in parallel with the reduction of insulin-induced ^{Ser473}AKT phosphorylation in human preadipocytes under high glucose level conditions, similar to in 3T3-L1 [7]. In agreement with rosiglitazone data, previous studies confirmed that rosiglitazone decrease inhibitory serine phosphorylation of IRS-1 and insulin resistance [13,14].

This increase of ^{pThr389}S6K1 levels in adipose tissue may be mediated through mTOR pathway. In fact, ^{pSer2448}mTOR levels increased in parallel with ^{pThr389}S6K1 in both rats and human adipose tissues. In mice models with uninhibited expansion of adipose tissue and in pharmacological models in which an effective expansion of fat mass can be accomplished, such as in the context of the PPARγ agonist therapies, a healthy metabolic profile is attained despite excess of body fat mass. In fact, rosiglitazone (a well known PPARγ agonist) administration in subcutaneous preadipocytes decreased ^{pThr389}S6K1/S6K1 in parallel to the improvement of insulin signaling in high glucose medium. ^{pThr389}S6K1 levels in subcutaneous adipose tissue were strongly associated with hyperglycemia and hyperinsulinemia suggesting that increased ^{pThr389}S6K1 in adipose tissue could be a useful marker to evaluate impaired glucose tolerance-associated adipose tissue dysfunction. ^{pThr389}S6K1/$_{total}$S6K1 was not significantly increased in visceral adipose tissue, perhaps reflecting its relative metabolic inflexibility. Metabolic inflexibility may be envisioned as the relatively low capacity of visceral adipose tissue in response to different metabolic challenges [15-18]. For example, beneficial effects of PPARγ agonists on metabolic disturbances are mediated through subcutaneous but not visceral adipose tissue [18,19]. Thus, subcutaneous adipose tissue might reflect more efficiently metabolic adaptation compared with visceral adipose tissue. Even though, ^{pThr389}S6K1/$_{total}$S6K1 was significantly associated with fasting glucose and inversely with *IRS1* and *FASN* gene expression in this fat

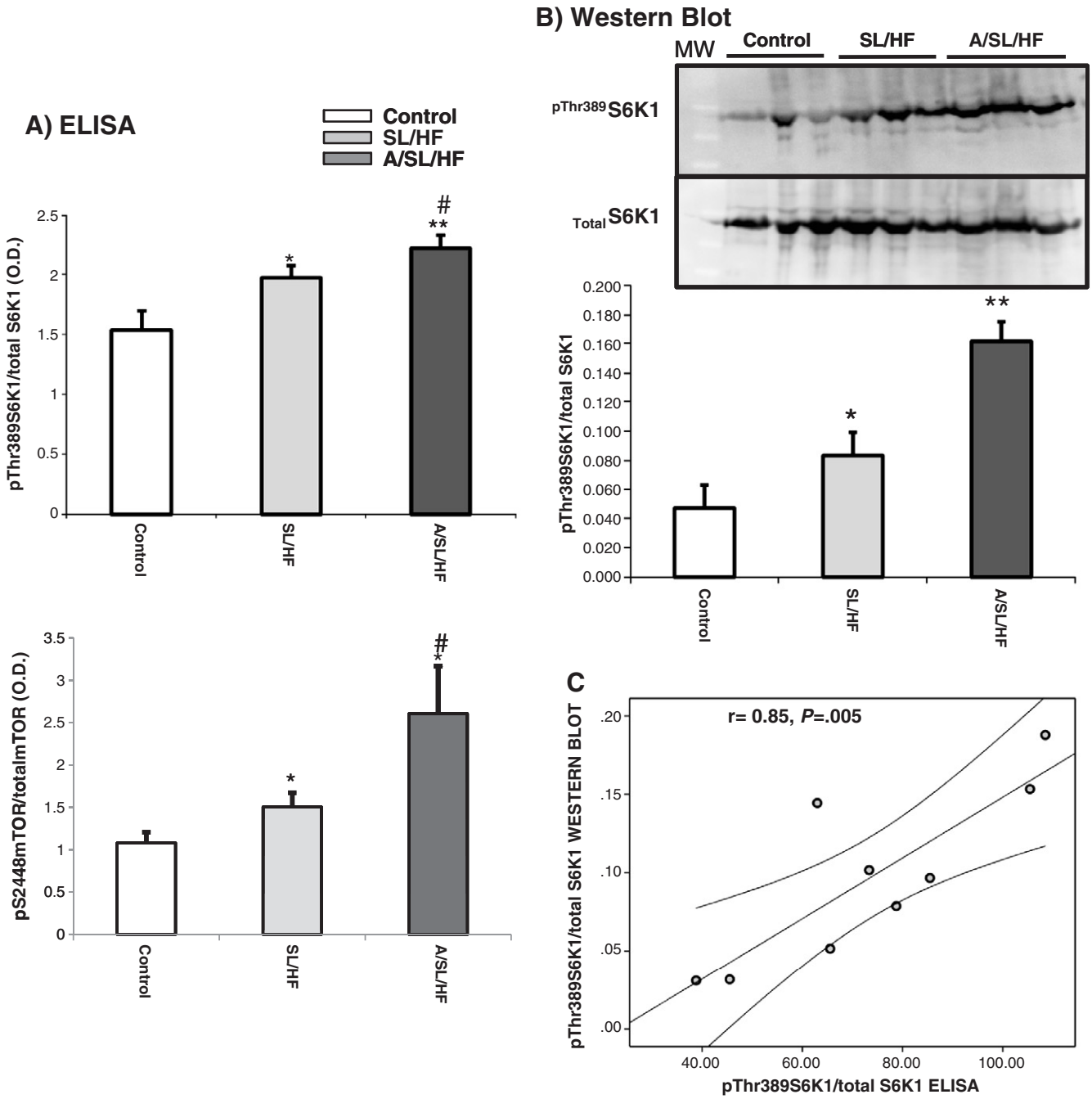

Fig. 4. Comparison of ^{pThr389}S6K1/total S6K1 levels in the three different experimental groups: (A) control rats raised in litters of 12 pups per dam and fed with low fat diet after weaning (NL/LF); (B) moderately overweighed rats, raised in litters of 4 pups per dam and fed with high fat diet after weaning (SL/HF); and (C) overtly overweighed rats, submitted to neonatal androgenization, and raised in litters of 4 pups per dam with feeding on high fat diet after weaning (A/SL/HF). (A) ^{pThr389}S6K1/total S6K1 and ^{pSer2448}mTOR/ Total mTOR measured by ELISA. (B) ^{pThr389}S6K1/total S6K1 measured using western blot assay. *P<.05 in comparison with NL/LF or control rats; **P<.005 in comparison with NL/LF or control rats; #P<.05 in comparison with SL/HF rats; ##P<.005 in comparison with SL/HF rats. (C) Correlation between ^{pThr389}S6K1/total S6K1 measured by ELISA and ^{pThr389}S6K1/total S6K1measured using Western blot.

depot. This inverse association with lipogenic (*FASN*) gene in visceral adipose tissue was in line with the decreased lipogenic gene expression found in adipose tissue of patients with insulin resistance and Type 2 diabetes [20,21]. In addition, the inverse association between ^{pThr389}S6K1/$_{total}$S6K1 and IRS1 gene expression, an insulin pathway-related gene associated with insulin sensitivity in adipose tissue, was another evidence about the importance of ^{pThr389}S6K1/$_{total}$S6K1 as a marker of metabolic derangement in adipose tissue.

Similar to our data in human adipose tissue, it has been recently reported that women with gestational diabetes mellitus show impaired IRS1 signaling associated with increased S6K1 activation in skeletal muscle in vivo [22]. Otherwise, antisense S6K1 inhibition (>90% reduction) in the liver and epididymal fat from male rats for up to 4 weeks induce insulin sensitization and attenuate weight gain with low risk for serious toxicity [23]. Finally, the possible contribution of another nutritional components, as amino acids

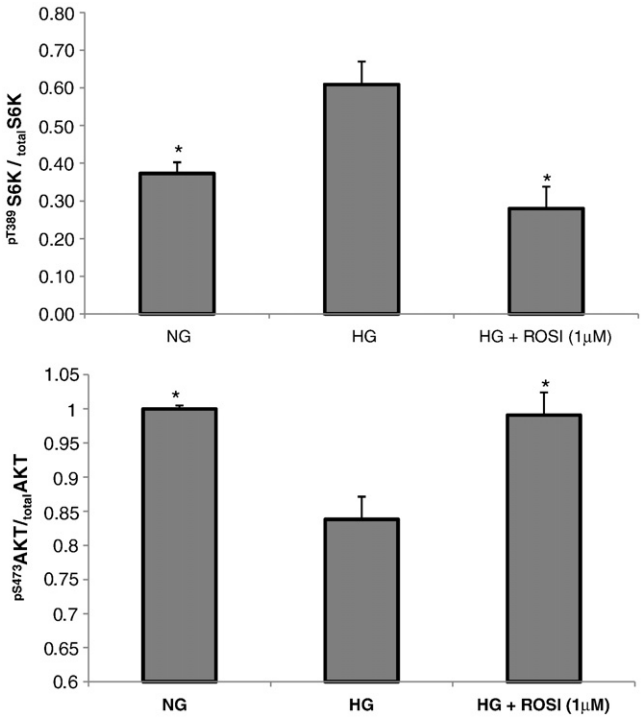

Fig. 5. Effects of high glucose medium and rosiglitazone on $^{pThr389}S6K1/_{total}S6K1$ and $^{pSer473}AKT/_{total}AKT$ levels in subcutaneous human preadipocytes. *P<.05 in comparison with high glucose (HG) conditions.

(which are increased in association with obesity [24]) should be considerate in further observational studies.

Both ELISA and western blotting are two excellent techniques for determining the phosphorylation level of S6K1. ELISA has a higher sensitivity than western blot and uses two specific antibodies in the same procedure. A high number of samples can be analyzed by ELISA (40 samples in 3 hour by ELISA against 10 samples in 2 days using Western blotting). We here validated the use of ELISA for $^{pThr389}S6K1$ determination, performing western blotting in a representative group of samples.

While similar analyses have not been conducted in male rodents as yet, our experimental analyses in female rats are endowed with a particular mechanistic dimension, as they allow monitoring the impact on adipose S6K1 phosphorylation of different obesogenic/diabetogenic manipulations, in well-controlled hormonal conditions in the adult female. Further experiments in our animal models addressing the metabolic effects of targeted pharmacological manipulation of the S6K1 pathway in the adipose will help to further delineate the functional roles of such signaling cascade in this tissue.

In conclusion, higher $^{pThr389}S6K1$ in subcutaneous adipose tissue reflects impaired glucose tolerance-induced adipose tissue dysfunction.

Acknowledgments

We acknowledge the clinical help of Oscar Rovira, the technical assistance of Gerard Pardo and the administrative help of Roser Rodríguez.

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