

Differential effect of long-term leucine supplementation on skeletal muscle and adipose tissue in old rats: an insulin signaling pathway approach

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Abstract Leucine acts as a signal nutrient in promoting protein synthesis in skeletal muscle and adipose tissue via mTOR pathway activation, and may be of interest in age-related sarcopenia. However, hyper-activation of mTOR/S6K1 has been suggested to inhibit the first steps of insulin signaling and finally promote insulin resistance. The impact of long-term dietary leucine supplementation on insulin signaling and sensitivity was investigated in old rats (18 months old) fed a 15% protein diet supplemented (LEU group) or not (C group) with 4.5% leucine for 6 months. The resulting effects on muscle and fat were examined. mTOR/S6K1 signaling pathway was not significantly altered in muscle from old rats subjected to long-term dietary leucine excess, whereas it was increased in adipose tissue. Overall

glucose tolerance was not changed but insulin-stimulated glucose transport was improved in muscles from leucine-supplemented rats related to improvement in Akt expression and phosphorylation in response to food intake. No change in skeletal muscle mass was observed, whereas perirenal adipose tissue mass accumulated (+45%) in leucine-supplemented rats. A prolonged leucine supplementation in old rats differently modulates mTOR/S6K pathways in muscle and adipose tissue. It does not increase muscle mass but seems to promote hypertrophy and hyperplasia of adipose tissue that did not result in insulin resistance.

Keywords Leucine supplementation · Sarcopenia · Muscle · Adipose tissue · Insulin signaling

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Introduction

Aging is frequently associated with major quantitative changes in body composition characterized by a decrease in lean body mass (i.e., muscle mass) and an increase in body fat mass. The clinical and functional consequences of muscle mass loss (known as sarcopenia) are decreased physical performance and autonomy of elderly people. The consequences of fat mass accumulation are less obvious but may play a major role in the metabolic changes observed with aging, especially the development of insulin resistance.

The origin of age-related sarcopenia is multifactorial (Boirie 2009; Kim et al. 2010), but it has become obvious that muscle protein loss during aging may be partly explained by a decreased ability of muscle to respond appropriately to anabolic stimuli such as food intake (Mosoni et al. 1995; Dardevet et al. 2002) through a decreased response and/or sensitivity of protein synthesis and degradation to physiologic concentrations of amino acids, especially leucine (Dardevet et al. 2000; Combaret et al. 2005; Cuthbertson et al. 2005; Katsanos et al. 2005; Prod'homme et al. 2005; Katsanos et al. 2006). These age-related alterations may be overcome by dietary leucine supplementation. Indeed, postprandial stimulation of muscle protein synthesis and inhibition of protein degradation in old rats can be restored by leucine-supplemented meals (Dardevet et al. 2002; Rieu et al. 2003; Combaret et al. 2005). Similarly, increasing leucine intake has been shown to improve muscle protein synthesis in the elderly human (Rieu et al. 2006), which can be restored to that obtained in younger subjects (Katsanos et al. 2006). Overall, these data have suggested that increasing leucine availability may represent a nutritional strategy to limit muscle protein loss during aging. However, a beneficial effect of leucine supplementation on muscle mass in the elderly has not been clearly demonstrated (Balage and Dardevet 2010). Only Verhoeven et al. (2009) assessed the effect of a mid-term (3 months) leucine supplementation on skeletal muscle mass and strength in healthy elderly men, and reported no beneficial effect of this nutritional intervention.

The anabolic effect of leucine results in its capacity to stimulate protein synthesis through activation of cell signaling pathways involving the mammalian target of rapamycin complex1 (mTORC1), which

activates two key regulatory proteins involved in the regulation of translation initiation: p70 S6 kinase (S6K1) and 4EBP1 (Vary and Lynch 2007). Leucine is also a potent activator of protein synthesis in white adipose tissue via the same pathway (Lynch et al. 2002b) suggesting that leucine supplementation may therefore affect both muscle mass and fat mass.

Hyperactivation of the mTORC1→S6K1 pathway by amino acids has been shown to increase insulin receptor substrate 1 (IRS1) phosphorylation on serine residues which in turn may lead to impaired activity of PI3K/Akt, critical kinases implicated in the mechanism of insulin action on glucose transport and metabolism (Boura-Halfon and Zick 2009). Thus, it has been hypothesized that amino acids used to promote muscle protein metabolism might in turn inhibit the first steps of insulin signaling and finally promote insulin resistance (Um et al. 2006; Tremblay et al. 2007a). Alternatively, it has also been reported that dietary supplements of leucine or branched chain amino acids (BCAA) may improve glucose metabolism and overall glucose tolerance especially in insulin resistant states through multiple mechanisms including increasing energy expenditure, food intake regulation, and increasing glucose–alanine cycle (Layman 2003; Layman and Walker 2006; She et al. 2007; Zhang et al. 2007; Jitmir and Willoughby 2008). Recently, we reported that short-term leucine supplementation (5 weeks) in young adult rats impaired whole body glucose tolerance but improved insulin-stimulated glucose transport in skeletal muscles (Balage et al. 2011). Leucine is also a precursor of fatty acids and sterols from its carboxyskeleton (Odessey and Goldberg 1972; Rosenthal et al. 1974; Frick et al. 1981). Because long-term free leucine supplementation in elderly has not been extensively investigated, it is not possible to draw conclusions on the efficacy of leucine to counteract or prevent muscle protein loss during aging. Moreover, contradictory data do not allow concluding whether chronic leucine supplementation may be beneficial or not on overall glucose tolerance and insulin sensitivity.

This study was thus conducted to examine the impact of a sustained dietary leucine excess (6 months *ad libitum*) on overall glucose tolerance, insulin response on muscle glucose transport *in vitro*, and body composition in old rats. A special focus was put

on studying insulin signaling pathways in skeletal muscle and adipose tissue.

Materials and methods

Animals and diets

This experiment was performed in accordance with the National Research Council's Guidelines for the Care and Use of Laboratory Animals (DSV-63-08). Old Wistar rats (18 months old, $n=120$ at the start of experiment) were maintained in collective cages (three rats per cage) under controlled environmental conditions (temperature 21°C, hygrometry 55%, 12-h dark period starting at 0900 h) with water and food *ad libitum*. They were separated into two groups fed either a control diet (C group) or a leucine-supplemented diet (LEU group) containing 15% protein from herring flour, 6% lipids, and 60% starch/sucrose (Table 1) for 6 months. The leucine-supplemented diet was supplemented with leucine (4.45%) and with appropriate amounts of valine and isoleucine in order to prevent the fall of plasma BCAA concentrations induced by leucine supplementation as previously described (Rieu et al. 2003). The control diet was supplemented with glycine to render the diets isocaloric and to balance the amino acid supplement of the LEU diet. Mean food intake and individual body weight were recorded every 2 weeks.

Oral glucose tolerance test (OGTT)

Conscious rats were subjected to oral glucose administration (1 g/kg body weight) after 18 h fasting, followed by glucose and insulin measurement from blood samples taken in the tail vein as previously described (Balage et al. 2011) at baseline (before starting the experimental period) and at the end of the nutritional experiment.

Experimental design at the end of nutritional period

After the 6-month period of supplementation, food was removed on the evening before sacrifice. On the next morning, each group of rats (C and LEU) was separated into two groups: one group that received a bolus of water and constituted the post-absorptive

Table 1 Composition of the diets

Ingredients	Control	LEU
	g/kg dry matter	
Herring flour	206	206
Tryptophan ^a	2	2
Cystine ^a	3.3	3.3
Glycine	60	0
Leucine	0	44.5
Isoleucine	0	9.8
Valine	0	5.7
Peanut oil	20	20
Sunflower oil	20	20
Sucrose	100	100
Cellulose	50	50
Mineral mixture ^b	35	35
Vitamin mixture ^b	10	10
Choline bitartrate (41.1% choline)	2.5	2.5
Wheat starch	491.4	491.4

The amino acid mixture of the nutrient bolus is composed in grams/100 g mixture: histidine 2.34, leucine 7.39, isoleucine 4.58, valine 5.34, lysine 9.59, methionine 2.92, phenylalanine 4.08, tryptophan 0.98, threonine 4.28, arginine 6.58, glutamine 3.69, proline 3.90, cysteine 1.07, tyrosine 3.11, glycine 5.92, alanine 6.31, glutamic acid 12.64, aspartic acid 9.12, asparagine 2.20, and serine 3.99

^aThe diets were supplemented with tryptophan and cystine in order to reach the recommended dietary allowances of these two amino acids in old rats according to AIN93M (Reeves et al. 1993)

^bComposition based on AIN-93M-MX and AIN-93-VX composition (Reeves et al. 1993)

state (PA groups), whereas the other group received a nutrient bolus containing 1 g of glucose, 1.2 g of sucrose, and 1 g of an amino acid mixture (Table 1 legend) and constituted the postprandial state (PP groups). The objective of the nutrient bolus was to stimulate acute insulin secretion without increasing acute plasma leucine level (i.e., the leucine content was similar in the nutrient bolus given in C and LEU group) in order to detect the effect of long-term leucine supplementation on postprandial stimulation of insulin signaling pathway, independently of an acute effect of leucine. Rats were then anaesthetized 30 or 60 min after gavage with sodium pentobarbital (6 mg/100 g body weight) and epitrochlearis muscles from PA groups were rapidly dissected intact for *in vitro* skeletal muscle glucose transport assays. Rats were then killed by exsanguination through the

abdominal aorta. Blood was collected, centrifuged, and frozen in liquid nitrogen. Liver, gastrocnemius muscles, and perirenal adipose tissue were rapidly removed, weighed, freeze-clamped, and frozen in liquid nitrogen. Other hindlimb skeletal muscles [tibialis anterior, extensor digitorum longus (EDL), and soleus], heart, spleen, and kidneys were quickly excised and weighed. All tissues were stored at -80°C until analysis.

Effect of insulin on skeletal muscle glucose transport *in vitro*

Epitrochlearis muscles were dissected intact for the *in vitro* measurement of skeletal muscle glucose transport as previously described (Dardevet et al. 1994) with 5.0 mmol/l of 2-deoxy-D- ^3H glucose (DOG; 0.5 $\mu\text{Ci/ml}$) in the absence or presence of various concentrations of insulin.

Analytical procedures

Plasma glucose, insulin, leptin, triglycerides, cholesterol, and HDL; and plasma amino acid concentrations were determined as previously described (Balage et al. 2011).

Western blots

Muscle analysis An aliquot of frozen gastrocnemius powder (0.3 g) was homogenized in 10 vol of buffer as previously described (Balage et al. 2011). The homogenate was centrifuged at $10,000\times g$ at 4°C for 10 min. Aliquots of supernatants were diluted in sample buffer, boiled for 5 min, and stored at -20°C until protein immunoblot analyses. Equal amounts of proteins were separated by SDS–PAGE and transferred to PVDF membranes. Immunoblotting was performed using appropriate antibodies: Akt, p-Akt (T308), p-Akt (S473), mTOR, p-mTOR (S2448), S6K1, p-S6K1 (T389), p-S6K1 (T421/S424), S6, p-S6 (S240/244), p-S6 (S235/236), eEF2 and p-eEF2 (T56) (Cell Signaling, Beverly, MA, USA), and 4EBP1 (Bethyl Laboratories, Montgomery, TX, USA). IR β , IRS1, and PI3K were determined after immunoprecipitation as previously described (Balage et al. 2011). Briefly, aliquots of frozen gastrocnemius powder were homogenized and homogenates were centrifuged at $12,000\times g$ for 20 min at 4°C . After

normalization for protein concentration, 1 mg of total protein was used to immunoprecipitate various proteins using 5 μg of appropriate antibodies [IR β (Biosciences, Le Pont de Claix, France) or IRS1 (Upstate Biotechnology Inc., Lake Placid) at 4°C overnight. The immunocomplexes were precipitated with 40 μl of protein A-agarose for 1 h at 4°C . After two sequential washes, the resulting pellets were boiled for 4 min in reducing Laemmli buffer containing 80 mM dithiothreitol. Proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were then incubated overnight with appropriate antibodies: anti-phosphotyrosine antibodies (PY20; Biosciences) or anti-phosphoserine antibody [p-IRS1 (S636/639)] and then with total IR β or IRS1 antibodies. PI3K activity was measured in p85 immunoprecipitates from muscle homogenates (2 mg protein) using an anti-rat p85 subunit antibody (Upstate Biotechnology Inc., Euromedex, France) and protein G agarose beads (GE Healthcare Europe GmbH).

The blots were revealed using an enhanced chemiluminescence (ECL) western blotting kit (GE Healthcare). Films were then scanned and identified bands were quantified by densitometry using Image J 1.41o (NIH, USA). Total signaling proteins were expressed in arbitrary units and phosphorylated proteins were corrected for total proteins.

Adipose tissue analysis White adipose tissue submerged in liquid nitrogen was crushed using pestle and mortar. Four hundred milligrams of tissue was collected in tubes containing ceramic beads (1.4 mm diameter) with 1 ml of lysis buffer [10 mM potassium phosphate, 50 mM β -glycerophosphate, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, pH 7.4 supplemented with 1 mM DTT, 1 M sodium orthovanadate, leupeptin (5 $\mu\text{g/ml}$), aprotinin (150 $\mu\text{g/ml}$), and 4-(2-aminomethyl)-benzenesulfonyl-fluoride (AEBSF, 250 $\mu\text{g/ml}$)] and homogenized in a tissue/cell grinder (Precellys; Bertin Technologies, Montigny-le-Bretonneux, France) at 6,500 rpm for 5 s. After a 1-min 2,500-rpm centrifugation at 4°C , the upper lipid phase was removed and the tissue extract transferred into a new tube. One percent (v/v) NP-40 was added and lysis was performed for 30 min at 4°C , then lysates were centrifuged for 20 min at 4°C at 13,000 rpm. The supernatant was then removed and total protein concentrations determined by using Bradford assay and

spectrophotometer (BioRad, 500-0006). Seventy micrograms of proteins per sample was loaded in reducing conditions (Laemmli buffer) on SDS–PAGE (5% to 12% polyacrylamide gel depending on the size of the proteins analyzed). Following electrophoresis, proteins were transferred to a PVDF membrane and immunoblotting was performed using different antibodies according to the aim of experiments. When phosphoproteins were analyzed, the membranes were stripped [for 30 min at 50°C in buffer containing 2-mercaptoethanol 100 mM, SDS 2% (v/v), and Tris–HCl 62.5 mM, pH 6.7], and reprobed with antibodies recognizing the native protein. Antibody binding was revealed by enhanced chemiluminescence and analyzed with the Kodak Image Station 2000R (Rochester, NY, USA).

Analysis of adipokines

White adipose tissue was crushed using pestle and mortar submerging in liquid nitrogen. Four hundred milligrams of pulverized tissues was then homogenized in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 1.7 mM KH₂PO₄, pH 7.4) using tubes containing ceramic beads (1.4 mm diameter) and a tissue/cell grinder (Precellys) at 6,500 rpm for 5 s. Samples were centrifuged for 1 min at 4°C and 2,500 rpm. The intermediary phase was then removed and submitted twice to thermal shock (freezing in liquid nitrogen followed by thawing at 37°C). Samples were then centrifuged for 5 min at 4°C and 5,000×g, and total protein concentrations were determined by using Bradford assay. TNF- α and IL-6 were quantified with an Elisa kit (Diaclone, Besançon, France) according to a protocol furnished by the manufacturer. Leptin in adipose tissue ($n=18$ – 19 per group) was quantified by using an Elisa kits from Linco Research, Inc. (St Charles, MO, USA).

Real-time RT–PCR

Adipose tissue samples were homogenized in Trizol (Invitrogen Life Technologies Inc., Gaithersburg, MD, USA), total RNAs were extracted, and 1 μ g from each sample was reverse-transcribed into cDNAs using Quantitect RT kit (Qiagen, Hilden, Germany). RNA concentration was determined with a spectrophotometer (MC2, SAFAS, Monte Carlo) and

quality of RNA by the OD260:280 ratio and on agarose gels. Real-time PCR was performed by using the ABI prism 7000 sequence detector system (Applied Biosystems). The following forward (F) and reverse (R) primers, respectively, were used for amplifications: 36B4 (used for normalization of the results), 5'-CCACTAAAATCTCCAGAGGTAC-3', 5'-ATGATCAGCCCCGAAGGAGAAGG-3'; PKB β , 5'-CTCAGCATCAACTG-GCAGGAT-3', 5'-GAAGGGTGGCAGGAGCTTCT-3'; RICTOR, 5'-GCATGTGC-CTCCACCAGAT-3', 5'-GGCCTCCTCCGTCATGG-3'; ACC α , 5'-TTCGCCAGCA-GAATTTGTTA-3', 5'-AGATCGCATGCATTTCACTG-3'; FASN, 5'-GAACTGAACG-GCATTACTCGG-3', 5'-AAATCCAACATGGGACAC; SREBP1c, 5'-AAGGATGCTC-GTGCCAGTG-3', 5'-ACTCAGTGCCAGGTTAGAAGC-3'; peroxisome proliferator-activated receptor γ (PPAR γ), 5'-TCTCACAAATG-CCATCAGGTTTG-3', 5'-GCTGGTCGATATCACTG-GAGATC-3'; and adiponectin, 5'-CCCCTGGCAGGAAAGGA-3', 5'-TCCAGCCCTAC-GCTGAATG-3'.

Statistical analysis

Data are presented as means \pm SEM. Data were analyzed by one-way ANOVA to analyze the supplementation effect (C vs. LEU) or two-way ANOVA to analyze the supplementation and the nutritional state (PA vs. PP states) effects. When a significant overall effect was detected, significant differences among individual means were assessed with Fisher test. When measurements could be repeated over time (body weight, food intake), two-way repeated measure variance analysis were performed. All tests were performed using XLStat (Addinsoft NY, USA, version 7.5.2). Differences were considered as significant for $P < 0.05$.

Results

Animal characteristics

Leucine supplementation dramatically increased plasma leucine concentration in the fed state (Fig. 1a) (+300%, $P < 0.0001$) and moderately in the post-absorptive (PA) state (Fig. 1b) (+20%, $P < 0.05$).

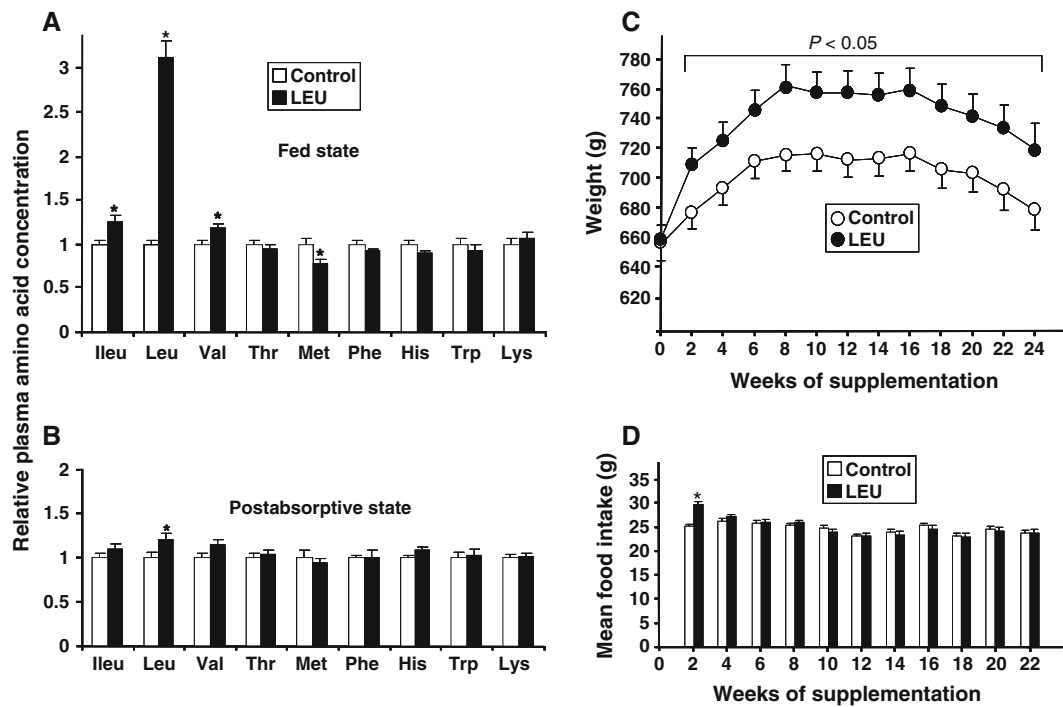


Fig. 1 Effects of a 6-month leucine supplementation in old rats on plasma concentration of essential amino acids in the fed state (a), in the post-absorptive state (measured after an overnight food deprivation) (b) (values are expressed as relative level to control

values and are means \pm SEM, $n=15$ – 16 in each group), on body weight (c), and food intake (d) (values are expressed as means \pm SEM, $n=35$ and 36). * $P<0.05$ vs. control group

It is important to note that other plasma BCAA levels (i.e., isoleucine and valine) were not changed by leucine supplementation in the PA state and were slightly increased in the fed state (+25% for isoleucine, +17% for valine, $P<0.05$). Except for plasma methionine in the fed state, other essential amino acid concentrations were similar in C and LEU groups.

During the 6-month nutritional period, 31 rats died due to aging or were sacrificed because of health problems: no difference was observed between control and leucine-supplemented groups. Body weights of old rats were similar between groups at the beginning of the experiment. Rats supplemented with leucine gained significantly more weight than those on control diet did (Fig. 1c), but this weight gain only occurred during the first 2 weeks of the diet ($+47\pm 3$ vs. $+20\pm 3$ g in LEU and C groups, respectively; $P<0.0001$) and was concomitant to an increase in food intake in this group (29.7 ± 0.6 g/day vs. 25.1 ± 0.5 for LEU and C groups, respectively; $P<0.0001$) (Fig. 1d). The weight difference was maintained until the end of the nutritional

period even though food intake in LEU group returned to values in C group.

As shown in Table 2, muscle mass was not changed by leucine supplementation, whereas perirenal adipose

Table 2 Skeletal muscle and organ weight in control and leucine-supplemented rats

	Control	LEU
Muscles (g)		
Gastrocnemius	5.31 \pm 0.1	5.15 \pm 0.11
Tibialis anterior	1.83 \pm 0.04	1.80 \pm 0.04
EDL	0.470 \pm 0.009	0.452 \pm 0.008
Soleus	0.392 \pm 0.009	0.390 \pm 0.009
Perirenal adipose tissue (g)	17.63 \pm 1.07	25.53 \pm 1.51*
Heart (g)	1.71 \pm 0.04	1.69 \pm 0.03
Liver (g)	16.19 \pm 0.44	16.90 \pm 0.34
Kidneys (g)	6.06 \pm 0.40	5.78 \pm 0.35
Spleen (g)	1.453 \pm 0.051	1.383 \pm 0.047

Values are means \pm SEM, $n=35$ and 36

* $P<0.0001$

tissue weight was dramatically increased (+45%, $P < 0.0001$). Leucine supplementation did not affect heart, liver, kidneys, or spleen weights.

Overall insulin sensitivity (OGTT)

Because no difference was observed between control and leucine-supplemented rats before starting the nutritional experiment, OGTT data were combined and presented as baseline results. Basal (post-absorptive state) plasma glucose concentrations were similar in C and LEU groups at the end of the nutritional period (5.56 ± 0.13 and 5.48 ± 0.20 mmol/l in C and LEU groups, respectively) but were significantly higher than baseline values (4.95 ± 0.09 mmol/l, $P < 0.05$ vs. C and

LEU values). Basal insulin concentrations were not significantly different in C and LEU groups at the end of supplementation (1.19 ± 0.12 vs. 1.51 ± 0.14 ng/ml in C and LEU groups, respectively; $P = 0.09$), but baseline values were higher than basal C group values (1.52 ± 0.08 vs. 1.19 ± 0.12 ng/ml, $P < 0.05$). Glucose and insulin plasma concentrations and areas under the curve (AUCs) in response to the oral glucose bolus were similar in both groups at the end of the nutritional period (Fig. 2a, b). We note that glucose AUCs increased, whereas insulin AUCs decreased at the end of supplemental period compared to baseline suggesting a time effect of insulin response to glucose challenge. However, the insulin–glucose indexes (overall insulin sensitivity index) estimated by the product

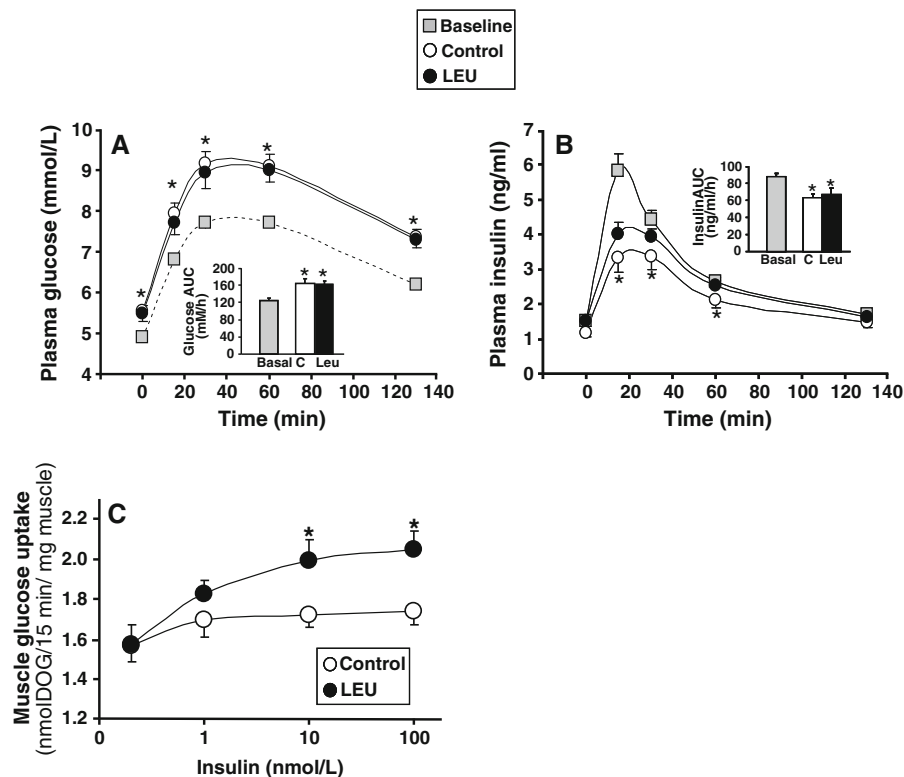


Fig. 2 Effects of a 6-month leucine supplementation in old rats on insulin sensitivity. Oral glucose tolerance test (OGTT) at baseline (before experimental period) and in control and leucine-supplemented rats (a, b). Blood samples were taken from the tail vein for the determination of fasting glucose and insulin levels. Then, an oral glucose challenge (1 g/kg body weight) was given by gavage and plasma samples were taken at 15, 30, 60, and 135 min after glucose administration. Time-course changes and area under the curve (AUC) in plasma glucose (a) and plasma insulin levels (b). Values are means \pm SEM ($n = 34$ and 35). $*P < 0.05$ vs. baseline values. Dose–response curves of insulin-

stimulated glucose transport in isolated epitrochlearis muscles from control and leucine-supplemented rats after an overnight food deprivation (c). Epitrochlearis muscles were incubated in the absence or presence of various concentrations of insulin (1, 10, and 100 nmol/l) and with 5.0 mmol/l of 2-deoxy-D- $[^3\text{H}]$ glucose (DOG). Muscle glucose transport was calculated by dividing the radioactivity within the epitrochlearis muscles by the specific activity of the DOG into the incubation medium; it was expressed as nmoles DOG per milligram of muscle per 15 min. Values are means \pm SEM ($n = 13$ – 16 determinations per insulin concentration). $*P < 0.05$ vs. control group

of the AUCs for glucose and insulin (Cortez et al. 1991) were not changed during time and were similar in C and LEU groups ($10,873 \pm 690$, $10,236 \pm 1,013$, and $11,034 \pm 1,593$ relative units at baseline and in C and LEU groups, respectively), demonstrating that overall glucose tolerance was not affected by long-term leucine supplementation in old rats.

Glucose transport in response to insulin in isolated epitrochlearis muscles

In order to determine the effect of leucine supplementation on muscle insulin sensitivity, we assessed the *in vitro* insulin response of glucose transport in skeletal muscles isolated from fasted control and leucine-supplemented rats. Basal glucose transport was similar in muscles from C and LEU groups (Fig. 2c). Increasing insulin concentration in the incubation medium significantly stimulated glucose transport in epitrochlearis muscles from LEU group ($P < 0.005$), whereas it did not in muscles from C group ($P = 0.4$). The maximum insulin response was thus significantly increased in leucine-supplemented rats compared to controls (Fig. 2c).

Skeletal muscle signaling pathways

Amino acid and insulin signaling pathway analysis were performed on gastrocnemius muscles taken at the end of the experimental period in the post-absorptive state (PA), 30 min (PP30) and 60 min (PP60) after oral administration of the nutrient bolus. The nutrient bolus induced a similar increase in plasma insulin in C and LEU groups (Fig. 3a) with a 3.5-fold increase 30 min after the nutrient bolus in both groups. As wanted, plasma leucine concentration was similar in C and LEU groups whatever the time after the bolus ingestion (Fig. 3b). Thus, any significant differences observed between C and LEU groups in signaling pathway activation will be due to chronic leucine exposure.

Muscle mTOR signaling pathway As shown in Fig. 4, chronic leucine supplementation had minor effect on the mTOR/S6K1 pathway in skeletal muscle. The protein levels of mTOR, S6K, S6rp, and eEF2 were similar in C and LEU groups (Fig. 4a); similarly, total amount of 4EBP1 represented by the sum of the various phosphorylated forms were similar in both groups (not shown).

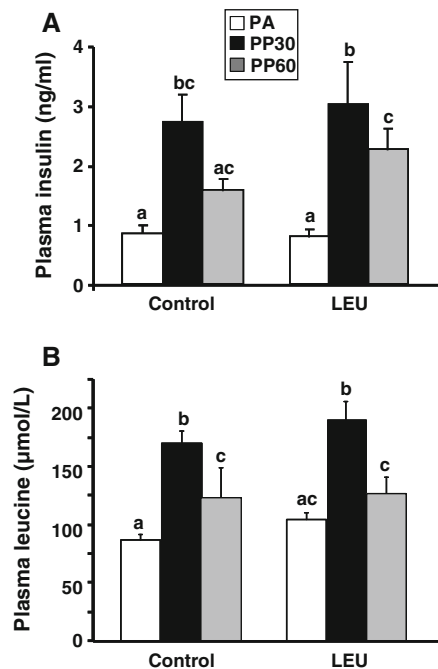


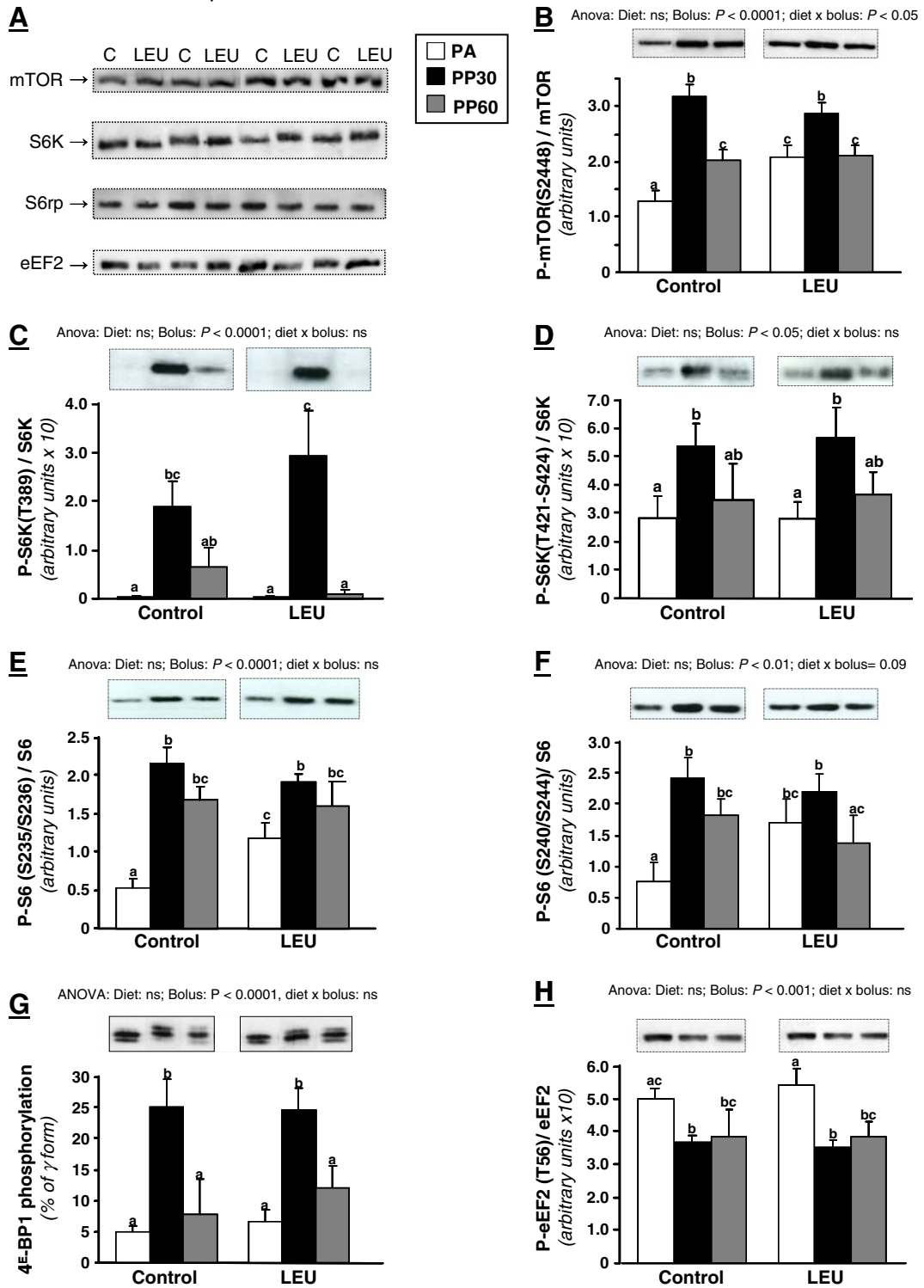
Fig. 3 Plasma insulin (a) and leucine (b) concentrations in control and leucine-supplemented old rats after an overnight food deprivation (PA), 30 min (PP30), or 60 min (PP60) after gavage with a nutrient bolus. Values are means \pm SEM ($n = 10$ – 12). Values with different letters are significantly different at least at $P < 0.05$

Before the nutrient bolus (i.e., in the PA state), long-term leucine supplementation only induced a significant increase in the phosphorylation state of mTOR and S6rp (Fig. 4b, e, and f) without alteration of other signaling proteins. The stimulation of mTOR, S6K1, S6rp, and 4EBP1 phosphorylation induced by nutrient intake was not different in leucine-supplemented rats than in controls (Fig. 6b–g). Phosphorylation state of eEF2 was similarly decreased by the nutrient bolus in control and LEU groups (Fig. 6h).

Early steps of insulin signaling Total protein content of IR β and IRS1 were similar in control and leucine-

Fig. 4 Muscle mTOR pathway analysis. Total proteins (a) and phosphorylation status of b mTOR (S2448), c S6K1 (T389), and d (T421/S424); e S6 (S235/236) and f (S240/244); g 4EBP1 and h eEF2 (T56) in gastrocnemius muscles from control and leucine-supplemented old rats after an overnight food deprivation (PA), 30 min (PP30), or 60 min (PP60) after gavage with a nutrient bolus. Values are means \pm SEM ($n = 9$ – 12 in each nutritional condition). Values with different letters are significantly different at least at $P < 0.05$

Total proteins



supplemented rats (not shown). In the basal state, long-term leucine supplementation did not induce alteration of tyrosine phosphorylation of IR β (1.31 ± 0.10 vs. 1.37 ± 0.22 arbitrary units in C and LEU groups, respectively) and IRS1 (1.31 ± 0.21 vs. 1.13 ± 0.24 arbitrary units in C and LEU groups, respectively). Similarly, it did not alter serine phosphorylation of IRS1 at S636/639 (0.96 ± 0.06 vs. 0.98 ± 0.07 arbitrary units in C and LEU groups, respectively).

We next examined the activation of key signaling proteins involved in insulin-stimulated glucose transport such as PI3K activity and Akt phosphorylation on T308 and S473 (Fig. 5). PI3 kinase activity was faintly but significantly increased after the nutrient bolus ($P < 0.05$) in C and LEU groups (Fig. 5a). Long-term leucine supplementation induced a moderate but significant increase in total Akt expression in gastrocnemius muscle (6.40 ± 0.21 vs. 5.53 ± 0.27 arbitrary units in LEU and C groups, respectively; $P < 0.05$) (Fig. 5b). Moreover, Akt phosphorylation at T308 and S473 was increased in leucine-supplemented rats 30 min after the nutrient bolus, whereas it was not significantly changed by nutrient intake in the control group.

Adipose tissue analysis

Leucine supplementation did not change basal plasma lipids measured at the end of the supplementation period (triglycerides— 1.38 ± 0.16 vs. 1.22 ± 0.12 mmol/l; cholesterol— 2.31 ± 0.16 vs. 2.39 ± 0.18 mmol/l; HDL— 0.68 ± 0.05 vs. 0.61 ± 0.04 mmol/l in C and LEU groups, respectively).

Lipogenesis is dependent upon two key enzymes: fatty acid synthase (FAS) and acetylcoA carboxylase (ACC). FAS expression is regulated by a transcription factor belonging to the Sterol Regulatory Element Binding family, SREBP1c. The process of differentiation of preadipocytes into mature fat-loaded adipocytes involves major transcriptional regulators, such as peroxisome proliferator-activated receptors (PPARs). mRNA levels of FAS, ACC α , and SREBP1c were not significantly different between groups (Fig. 6a–c). In contrast, the leucine group showed a significant increase in PPAR γ mRNA levels (Fig. 6d). Adipose tissue is an endocrine organ that synthesizes and excretes different adipokines such as leptin, adiponectin, and cytokines (Wang et al. 2008). When produced

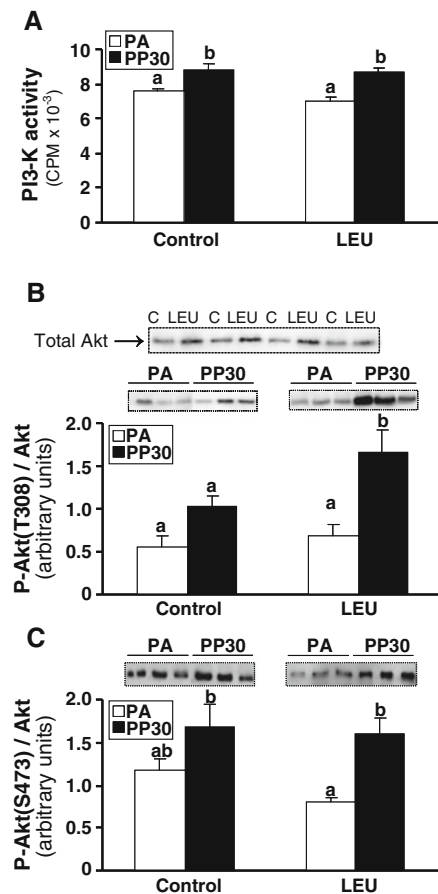
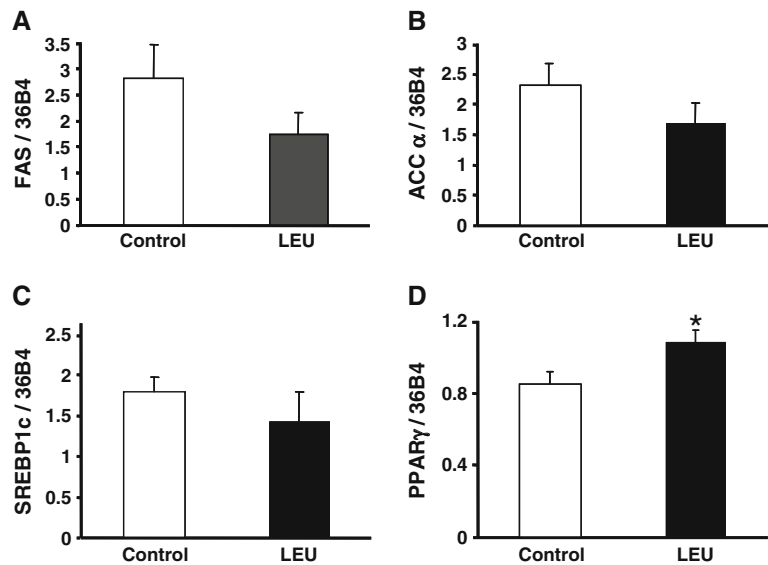


Fig. 5 PI3 kinase activity (a) and phosphorylation state of Akt (T308) (b) and (S473) (c) in gastrocnemius muscles from control and leucine-supplemented old rats after an overnight food deprivation (PA) or 30 min (PP30) after gavage with a nutrient bolus. Values are means \pm SEM ($n=7-10$ in each nutritional condition). Values with different letters are significantly different at least at $P < 0.05$

in excess, cytokines such as TNF α or IL-6 induce muscle damages by activating proteolysis, which could counteract the anabolic effect of leucine and promote muscle insulin resistance. On the opposite, adiponectin and leptin improve insulin sensitivity in muscle (Yaspelkis et al. 2004). In the white adipose tissue, protein levels of TNF- α and IL-6 (not shown) were not different between groups. There was a significantly higher protein level of leptin in the white adipose tissue of the leucine-supplemented rats compared to control ones (0.58 ± 0.06 vs. 0.45 ± 0.05 ng/ μ g protein, respectively; $P < 0.05$), which correlated with a significant increase in basal plasma leptin in leucine-supplemented rats (39.1 ± 3.9 vs. 22.6 ± 3.3 ng/ml,

Fig. 6 mRNA levels of FAS (a), ACC α (b), SREBP1c (c), and PPAR γ (d) in adipose tissue from control and leucine-supplemented old rats. Results are expressed relative to the reference gene, 36B4 (values are means \pm SEM; $n=7-9$ for SREBP1c, FAS, and ACC α ; $n=12$ for PPAR γ). * $P<0.05$



respectively; $P<0.005$). Finally, mRNA levels of adiponectin were not modified by the prolonged leucine supplementation compared to the control group (not shown).

Since leucine has been shown to be able to stimulate protein synthesis in adipose tissue through mTOR signaling (Lynch et al. 2002b), we assessed the activation of some signaling proteins involved in mTORC signaling pathway in adipose tissue in the basal state and 30 min after the nutrient bolus. Leucine supplementation did not affect mTOR protein expression. Postprandial mTOR phosphorylation on S2448 was markedly increased in the leucine group indicating a stimulatory effect of the nutritive bolus that was absent in the control group (Fig. 7a). A similar result was obtained for the phosphorylation of S6K1, i.e., the stimulatory effect in the PP state was only observed in the leucine group (Fig. 7b).

Unlike in muscle, IRS-1 phosphorylation on Ser636/Ser639 (Fig. 8a) was increased in adipose tissue from leucine-supplemented rats. RICTOR protein was markedly decreased (by about 60%) in leucine-supplemented rats (Fig. 8b), with a 30% decrease in its mRNA level (not shown). Total Akt protein was slightly ($P=0.117$) decreased in the leucine group (Fig. 8c) and mRNA level of the β isoform was significantly decreased (about 20%, data not shown). Interestingly, Akt phosphorylation at S473 was not significantly stimulated in the C group after the nutrient bolus, whereas it tended to be

decreased ($P=0.09$) in adipose tissue from leucine-supplemented rats compared to skeletal muscle.

Discussion

In the present study, 6-month dietary leucine supplementation did not potentiate mTORC/S6K1 pathway in muscle and did not induce insulin resistance in old rats as determined by OGTT, muscle glucose uptake, or insulin signaling. Rats receiving the leucine-supplemented diet increased their body weight during the first 2 weeks of supplementation, but this weight gain did not translate into muscle mass increase. On the contrary, leucine supplementation induced a dramatic increase in fat mass as assessed by a marked (44%) increase in perirenal adipose tissue weight associated with increased mTOR/S6K1 and a trend to a negative feedback on early steps of insulin signaling.

A defect in postprandial anabolism with age has been proposed to be one of the mechanisms responsible for the loss of muscle mass during aging. This anabolic resistance has been attributed to a decrease in leucine sensitivity and may be counteracted by increasing leucine proportion in the diet in both rodents and humans (Dardevet et al. 2002; Rieu et al. 2003, 2006, 2007; Combaret et al. 2005; Katsanos et al. 2006). Consequently, it has been suggested that long-term dietary leucine supplementation may be necessary to counteract or prevent muscle loss during

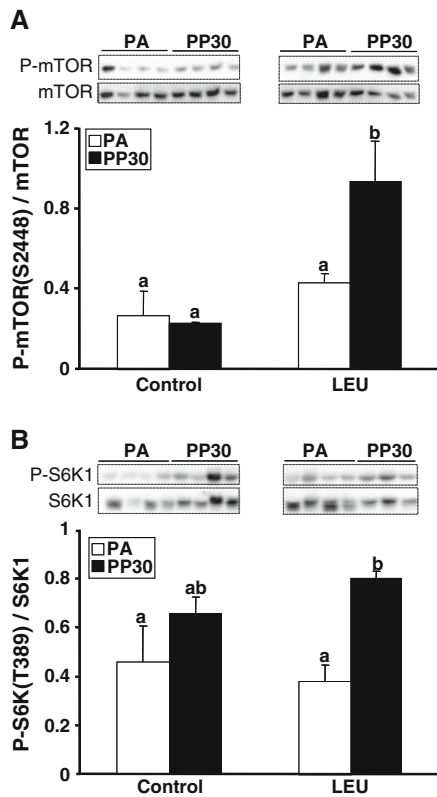


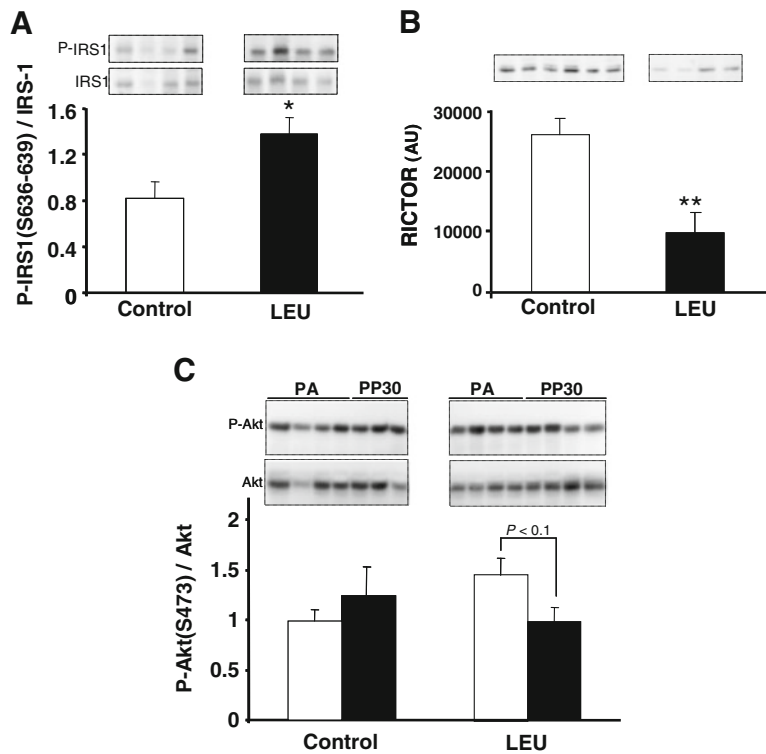
Fig. 7 Phosphorylation of mTOR (S2448) (a) and S6K1 (T389) (b) in white adipose tissue from control and leucine-supplemented old rats after an overnight food deprivation (PA) or 30 min (PP30) after gavage with a nutrient bolus. Values are means \pm SEM ($n=4-6$). Values with different letters are significantly different at least at $P<0.05$

aging. Recently, Verhoeven et al. (2009) did not obtain any effect of a 3-month leucine supplementation on skeletal muscle mass and strength in healthy elderly men. However, two confounding effects might explain their data: they did not report a significant elevation of plasma leucine concentration in leucine-supplemented subjects and plasma valine was lower than in placebo group suggesting that a defect in valine availability may have counteracted the anabolic effect of leucine. In the present study, we did not observe any significant increase in skeletal muscle mass in old rats supplemented *ad libitum* with free leucine for 6 months despite a significant increase in plasma leucine. This absence of change cannot result from imbalance of BCAA concentrations because plasma valine and isoleucine levels were similar to that of the control group in the post-absorptive state and were slightly increased in the fed state. Lack of

muscle mass gain after chronic leucine supplementation could be explained by a loss of leucine efficiency to stimulate postprandial muscle protein synthesis when given *ad libitum* after a long-term period compared to short-term supplementations through a leucine-enriched meal, as demonstrated previously (Rieu et al. 2003, 2007). The lack of major activation of the mTOR pathway observed in skeletal muscle from leucine-supplemented rats is consistent with such a hypothesis. Alternatively, fat mass excess in the leucine group might have contributed to induce a low-grade inflammation in rats that should have attenuated leucine's effect on muscle tissue. Indeed, we previously showed that development of low-grade inflammation partly contributes to the postprandial anabolic resistance in old rats (Balage et al. 2010). We also demonstrated that antioxidants are able to improve leucine's ability to stimulate protein synthesis in muscles of old rats (Marzani et al. 2008), suggesting that a combination of leucine and antioxidant supplementation may be more efficient to counteract age-related sarcopenia than leucine alone. The optimal nutritional strategies based on leucine supplementation to prevent aging-related muscle loss remain to be defined.

Several reasons might have contributed to the increase in adipose tissue mass in leucine-supplemented rats. First, rats in the leucine group showed an increased food intake the first 2 weeks, concomitant to their weight gain. After this period, both groups had comparable food intakes. It is of interest to mention that, as we did not assess body composition throughout the study, we can only assume that fat mass gain was a continuous process, but probably more important in the first 2 weeks of the study due to increased food intake. The mechanism by which leucine supplementation may induce increased food intake is not clear. Indeed, Cota et al. (2006) reported an anorexigenic effect of leucine in rats but when directly injected in the intracerebroventricular area. However, Zhang et al. (2007) reported orexigenic effects of an oral leucine supplementation in mice; Krauss and Mayer (1965) showed that this effect lasted only for 2 weeks, as observed in our experiment. Leucine may also increase food intake through an increase in gastric emptying or by competing with tryptophan, a precursor of serotonin, for crossing the blood-brain barrier (Laviano et al. 2005). Alternatively, adipose tissue accumulation

Fig. 8 Phosphorylation of IRS1 (S636/639) (a), RICTOR expression (b), and phosphorylation of Akt (S473) (c) in white adipose tissue from control and leucine-supplemented old rats. Akt phosphorylation (S473) was determined after an overnight food deprivation (PA) or 30 min after gavage with a nutrient bolus (PP30). Values are means \pm SEM ($n=4-6$). * $P<0.05$, ** $P<0.005$



might result from leucine supplementation *per se*. Indeed, we previously showed a 25% increase in adipose tissue weight in young rats fed a leucine-supplemented diet, whereas food intake was not increased (Balage et al. 2011). Adipose tissue gain in leucine group could also be due to decreased energy expenditure despite contradictory results were described. Indeed, Cheng et al. (2010) reported that young mice maintained on a 7-day leucine-deficient diet underwent a rapid loss of abdominal fat and showed an increase in total energy expenditure. Opposite findings were found by Zhang et al. (2007) who reported that leucine supplementation increased energy expenditure and decreased adiposity. Elevated levels of leptin in both adipose tissue and plasma in the leucine group compared to the control group are consistent with white adipose tissue hypertrophy (Lonnqvist et al. 1997). Moreover, increased activation of the mTOR pathway in adipose tissue from leucine-supplemented rats, as assessed by increased phosphorylation of both mTOR and S6K1, is consistent with a protein synthesis-induced hypertrophy. Previously, Lynch et al. (2002a, b) showed that an acute or a 12-day leucine administration significantly increased

adipose tissue protein synthesis in young rats. Leucine seems to have also a direct positive effect on adipocyte differentiation, as suggested by a higher PPAR γ gene expression in the leucine group. Amino acids influence adipogenesis, as reported by *in vitro* experiments in 3T3L1 adipocytes; a recent study has shown that leucine acts as regulator of adipocyte differentiation factors such as PPAR γ and C/EBP α , and is a promoter of PPAR γ activity via the mTOR pathway (Kim and Chen 2004). The increased PPAR γ gene expression after a 6-month leucine supplementation is consistent with these findings and may explain the persistence of a body weight difference between groups, 5 months after their food intake became similar. Even though no microscopic examination was performed, we can hypothesize that leucine supplementation induced both hypertrophy and hyperplasia of white adipose tissue.

A main objective of this work was to assess the effect of long-term leucine supplementation on insulin sensitivity. Indeed, BCAA or leucine alone has been implicated in the regulation of insulin sensitivity with conflicting results. For example, Zhang et al. (2007) showed that long-term leucine supplementation reduced diet-induced obesity and hyperglycemia,

and improved glucose tolerance in mice fed a high-fat diet. In addition, She et al. (2007) demonstrated that disruption of the mitochondrial branched-chain aminotransferase isozyme gene (BCATm) in mice which resulted in a dramatic increase in BCAA plasma levels was associated with increased glucose tolerance and insulin sensitivity. Finally, co-ingestion of AA mixture or leucine with protein hydrolysate improves glycemic control in type 2 diabetic patients (Manders et al. 2005, 2006). However, it has also been reported that leucine or BCAA could impair insulin sensitivity or glucose tolerance. Newgard et al. (2009) reported that chronic BCAA supplementation decreased glucose tolerance and contributed to the development of insulin resistance in rats fed a high-fat diet. We recently showed that short-term (5 weeks) leucine supplementation impaired whole-body glucose tolerance in young adult rats that correlated with perirenal adipose tissue accumulation (Balage et al. 2011). In the present study, 6-month leucine supplementation did not change overall glucose tolerance in old rats despite a significant increase in perirenal adipose tissue content suggesting an age-related effect of leucine supplementation: decreased overall glucose tolerance in young vs. no effect in old rats. In agreement, Verhoeven et al. (2009) did not observe any changes in glycemic control and/or whole-body glucose tolerance in healthy elderly men subjected to a long-term leucine supplementation. Alternatively, it could be speculated that whole-body insulin resistance was partly present in old rats (18 months old) before leucine supplementation compared to young ones (4 months old), limiting the leucine effect on glucose tolerance. Leucine has been shown to promote glucose uptake in isolated soleus muscle from normal rats (Nishitani et al. 2002) or in rats with liver cirrhosis (Nishitani et al. 2005). In contrast, Doi et al. (2003) found that leucine did not increase glucose transport in C2C12 myotubes or *in vivo* in gastrocnemius from young rats (Doi et al. 2005, 2007). In the present study, basal glucose transport was unchanged in isolated muscle from long-term leucine-supplemented old rats, but the insulin-stimulated glucose transport was improved as previously observed in young rats (Balage et al. 2011). This improvement was sustained by the elevated phosphorylation of Akt at T308 and S473 observed in muscles from leucine-supplemented rats; Akt playing a key role in insulin-stimulated glucose transport. The improved Akt phos-

phorylation in leucine-supplemented rats was not linked to increased PI3K activity, which was similar in control and LEU groups. Besides being a downstream effector of PI 3-kinases, Akt may also be activated in a PI 3-kinase-independent manner (Bhaskar and Hay 2007), but this was not investigated in the present study. It may be surprising that increased insulin-stimulated glucose uptake did not translate into overall glucose tolerance increase in leucine-supplemented rats (see above). However, Kleemann et al. (2010) reported that development of insulin resistance is a time- and tissue-dependent process. They clearly showed that high-fat-diet-induced insulin resistance in mice produced overall glucose intolerance at week 1, decreased insulin sensitivity only at week 3 in liver, at week 12 in white adipose tissue, without change in skeletal muscle using hyperinsulinemic clamp technique. Interestingly, phosphorylation of IRS1 on S636/639 was increased in adipose tissue from leucine-supplemented rats, whereas mRNA and protein levels of Akt as well as mRNA and protein levels of RICTOR (an activator of Akt phosphorylation) were decreased. These data were in favor of decreased insulin sensitivity on glucose metabolism in adipose tissue compared to muscle despite no measurements were done on glucose metabolism.

It has been suggested that acute overactivation of the mTORC1→S6K1 pathway by amino acids may alter early steps of insulin signaling and promote insulin resistance (Tremblay and Marette 2001; Tremblay et al. 2007a, b). The present study showed that chronic leucine supplementation did not induce a similar effect in skeletal muscle. Indeed, S6K1 phosphorylation was similar in control and leucine-supplemented rats both in the basal state and after administration of the nutrient bolus, suggesting that chronic exposure to leucine excess has not induced sustained activation of S6K1. This correlated with similar (1) tyrosine phosphorylation of IR β and IRS1, (2) serine phosphorylation of IRS1, and (3) PI3K activity in muscle from control and leucine-supplemented rats. Recently, Newgard et al. (2009) reported that chronic BCAA supplementation in young rats induced an elevation in mTOR/S6K1 phosphorylation in response to feeding but only when rats were fed a high-fat diet. Unlike in muscle, sustained leucine excess tended to induce activation of mTOR/S6K1 pathway in adipose tissue and inhibition of IRS/Akt pathway. Further studies are needed for a better comprehension of the

cross-talk between muscle and adipose tissue in a critical situation such as aging and to determine the optimum nutritional design to prevent age-related sarcopenia using dietary leucine (i.e., combination with exercise, other nutrients, or discontinuous supplementation periods) without fat mass gain.

In summary, this study shows that long-term leucine supplementation in old rats does not improve skeletal muscle mass but induces a gain in fat mass, probably through initial orexigenic effects, stimulation of the mTOR pathway, and increased PPAR γ expression in adipose tissue. Chronic leucine supplementation does not induce a sustained activation of the mTOR pathway in skeletal muscle, consistent with an absence of insulin signaling impairment and overall glucose tolerance. On the contrary, Akt phosphorylation of muscles from leucine-supplemented rats is more responsive to nutrient intake than in control rats and correlates with improved insulin-stimulated glucose transport.

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