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Leucine supplementation in rats induced a delay in muscle IR/PI3K signaling pathway associated with overall impaired glucose tolerance☆

Michèle Balage[a,b,c,*], Joëlle Dupont[d], Isabelle Mothe-Satney[e,f], Sophie Tesseraud[g], Laurent Mosoni[a,b,c], Dominique Dardevet[a,b,c]

[a] Inra, UMR 1019 Nutrition Humaine, F-63122 Saint Genés Champanelle, France
[b] Clermont Université, UFR Médecine, UMR 1019 Nutrition Humaine, F-63000, Clermont-Ferrand, France
[c] CRNH Auvergne, F-63000, Clermont-Ferrand, France
[d] INRA, UMR 85 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France
[e] INSERM U907, Nice F06000
[f] Université de Nice-Sophia-Antipolis, Faculté de Médecine, F-06107 Nice, France
[g] INRA, UR83 Recherches Avicoles, F-37380 Nouzilly, France

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Abstract

Although activation of the mammalian target of rapamycin complex/p70 S6 kinase (S6K1) pathway by leucine is efficient to stimulate muscle protein synthesis, it can also exert inhibition on the early steps of insulin signaling leading to insulin resistance. We investigated the impact of 5-week leucine supplementation on insulin signaling and sensitivity in 4-month old rats fed a 15% protein diet supplemented (LEU) or not (C) with 4.5% leucine. An oral glucose tolerance test was performed in each rat at the end of the supplementation and glucose transport was measured in vitro using isolated epitrochlearis muscles incubated with 2-deoxy-D-[^{3}H]-glucose under increasing insulin concentrations. Insulin signaling was assessed on gastrocnemius at the postabsorptive state or 30 and 60 min after gavage with a nutrient bolus. Tyrosine phosphorylation of IRβ, IRS1 and PI3 kinase activity were reduced in LEU group 30 min after feeding (−36%, −36% and −38% respectively, $P<.05$) whereas S6K1, S6rp and 4EBP1 phosphorylations were similar. Overall glucose tolerance was reduced in leucine-supplemented rats and was associated with accumulation of perirenal adipose tissue (+27%, $P<.05$). Conversely, in vitro insulin-response of muscle glucose transport tended to be improved in leucine-supplemented rats. In conclusion, dietary leucine supplementation in adult rats induced a delay in the postprandial stimulation in the early steps of muscle insulin signaling without muscle resistance on insulin-induced glucose uptake. However, it resulted in overall glucose intolerance linked to increased local adiposity. Further investigations are necessary to clearly define the beneficial and/or deleterious effects of chronic dietary leucine supplementation in healthy subjects.

Keywords: Leucine; Insulin; Signaling; Glucose tolerance; Rat

1. Introduction

While increased carbohydrate and fat intake is considered as the major cause in the development of insulin resistance, it has been recently hypothesized that elevated dietary protein and/or amino acid consumption may also contribute to this syndrome and the development of Type 2 diabetes [1–5]. Among amino acids, branched chain amino acids (BCAA) and especially leucine play a major regulatory role in body metabolism at multiple levels. Indeed, leucine is an essential amino acid that serves not only as a substrate for protein synthesis but is also recognized as a potent signal nutrient that regulates signaling pathways involved in the stimulation of protein synthesis [6,7]. Protein synthesis stimulation by leucine is linked to activation of cell signaling pathways involving the mammalian target of rapamycin complex (mTOR), which activates two key regulatory proteins involved in the regulation of translation initiation such as p70 S6 kinase (S6K1) and 4EBP1 [6]. However, activation of the mTOR/S6K1 pathway by amino acids has been shown to induce a decrease in tyrosine or an increase in serine IRS1 phosphorylations which in turn may lead to impaired PI3K activity, a critical kinase implicated in the mechanism of insulin action on glucose transport and metabolism [8–11]. Thus, it has been hypothesized that amino acid excess used to promote muscle protein metabolism might in turn inhibit the first steps of insulin signaling and decrease glucose utilization in skeletal muscle to finally promote insulin resistance [1–5]. However, these later studies have been performed acutely in vitro on cell cultures or with acute amino acid

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* Corresponding author. UNH Centre de Clermont-Ferrand–Theix, INRA, 63122 Saint Genès Champanelle, France. Tel.: +33 473 62 45 06; fax: +33 473 62 47 55.
E-mail address: balage@clermont.inra.fr (M. Balage).

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infusion. Conversely, other findings are not consistent with these observations and demonstrated that BCAA, including leucine, have beneficial effects on glycemic control and glucose metabolism [12–16]. Manders et al. [17–19] demonstrated that coingestion of protein hydrolysate and amino acids or leucine reduced hyperglycemia and improved glucose tolerance in type 2 diabetic patients. Ten weeks of dietary leucine supplementation has been shown to reduce diet-induced obesity and hyperglycemia and to improve glucose tolerance in mice fed a high-fat diet [20]. Moreover, She et al. [21] demonstrated that disruption of the mitochondrial branched-chain aminotransferase isozyme gene (*BCATm*) in mice which resulted in a dramatic increase in BCAA plasma level was associated with increased glucose tolerance and insulin sensitivity. However, the recent study of Newgard et al. [22] demonstrated that chronic BCAA supplementation contributed to the development of insulin resistance in rats fed a high-fat diet. Thus, it remains unclear whether a chronic dietary leucine supplementation may alter insulin signaling and induce insulin resistance as recorded in acute amino acid excess studies or whether it may improve insulin sensitivity as observed in insulin resistance states.

This study was then designed to examine the effect of a 5-week chronic leucine supplementation in adult healthy rats on insulin signaling pathways in the postabsorptive state and in response to postprandial elevation of plasma insulin induced by a nutrient bolus. In addition, we have examined the effect of leucine supplementation on whole body insulin sensitivity using an oral glucose tolerance test (OGTT) and insulin response on muscle glucose transport in vitro. The main results of the study showed that chronic leucine supplementation induced a delay in postprandial stimulation of the early steps of insulin signaling that did not result in insulin resistance on muscle glucose transport. However, we observed an impairment of overall glucose tolerance associated with an increase in perirenal adipose tissue in leucine-supplemented rats.

2. Methods and materials

2.1. 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). Forty-eight 4-month old male Wistar rats produced and bred locally (Unité Expérimentale de Nutrition Comparée, INRA, Theix, France) 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 fed either a control diet (C group, $n=24$) or a leucine-supplemented diet (LEU group, $n=24$) containing 15% protein from herring flour, 6% lipids and 60% starch/sucrose. The leucine-supplemented diet was supplemented with leucine (4.45%) according to previous experiments [23,24]. It was also supplemented with appropriate amounts of valine and isoleucine in order to prevent the fall of plasma BCAA concentrations induced by leucine supplementation [24]. The control diet was supplemented with glycine to render the diets isonitrogenous and isocaloric (Table 1). Diets were prepared as pellets and were given for 5 weeks. Food intake and rat weights were monitored once a week during the 5 weeks of the experimental period.

2.2. Oral glucose tolerance test

An OGTT was performed in all rats at the end of the nutritional intervention period. Rats were fasted for 18 h before the test and 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. Samples were analyzed for glucose by the glucose oxidase method using a Cobas Mira analyzer (ABX Diagnostics, Horiba ABX France). Plasma insulin was measured using a sensitive rat insulin RIA kit (SRI-13K from Linco, Labodia France). Plasma insulin and glucose responses to the glucose load were estimated by the 135-min integrated area under the curves (AUC) using the trapezoidal method. The overall insulin sensitivity was estimated by the insulin–glucose index (IG index) determined by the product of the AUCs for glucose and insulin as previously used [25].

Table 1
Composition of the diets

	Control	LEU
	g / kg dry matter	
Ingredients		
Herring flour	205	205
Tryptophane[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	492.2	492.2

[a] The diets were supplemented with tryptophane and cystine in order to reach the recommended dietary allowances of these two amino acids in adult rats according to AIN93M [44].
[b] Composition based on AIN-93M-MX and AIN-93-VX composition [44].

2.3. Experimental design

After the 5-week period of supplementation, food was removed on the evening before the 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 (5 ml) and constituted the postabsorptive state (PA) groups whereas the other group received a nutrient bolus containing in 5 ml water: 1 g of glucose, 1.5 g of sucrose and 1 g of an amino acid mixture (in g/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) and constituted the postprandial state (PP) groups. It is important to note that C and LEU groups received the same amount of leucine through the nutrient bolus which corresponded to the leucine content in the protein from herring flour used as the protein source in the diets. Indeed, the objective of this nutrient bolus was to greatly stimulate acute insulin secretion in order to detect the effect of the chronic leucine supplementation on postprandial stimulation of insulin signaling pathway independently of acute leucine activation on mTOR/S6K1 signaling pathway. Thirty or 60 min after the nutrient bolus, rats were then anaesthetized with sodium pentobarbital (6 mg/100g body weight) and epithrochlearis 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 and gastrocnemius muscles were rapidly removed, weighed, freeze-clamped and frozen in liquid nitrogen. Other posterior leg skeletal muscles [tibialis anterior, extensor digitorum longus (EDL) and soleus], heart, spleen, kidneys and perirenal adipose tissue were quickly excised, weighed and frozen in liquid nitrogen. All tissues were stored at −80° until analysis.

2.4. 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 [26]. Muscles were incubated in the absence or presence of various concentrations of insulin (1, 7 and 75 nM) and with 5.0 mM of 2-deoxy-D-[^{3}H] glucose (DOG: 0.5 μCi/ml). They were then rinsed, blotted, weighed and digested in 1 M NaOH for 20 min at 80°C for radioactivity determination [26]. 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 nanomoles of DOG per milligram of muscle per 15 min.

2.5. Plasma hormone and substrate levels

Plasma insulin, glucagon and leptin were measured using specific rat RIA kit from Linco (Labodia France). Plasma triglycerides, cholesterol and high-density lipoprotein (HDL) were determined by enzymatic method on a Cobas Mira analyzer (ABX Diagnostics, Horiba ABX France). Total muscle lipids from pooled tibialis anterior muscles from each group were measured according to the Folch method [27]. Plasma concentrations of amino acids were measured after deproteinization with sulfosalicylic acid as previously described [28] by ion-exchange chromatography (Bio-Tek Instruments ARL, St Quentin Yvelines, France).

2.6. Measurement of skeletal muscle signaling pathways

2.6.1. Down-stream mTOR signaling pathways

An aliquot of frozen gastrocnemius powder (0.3g) was homogenized in 10 volumes of a buffer A as previously described [28,29]. The homogenate was centrifuged at 10,000 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. For determination of total eEF2 and total 4EBP1, S6K1, S6 and their phosphorylation state, samples containing equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated overnight with appropriate antibodies: eEF2, S6K1, p-S6K1 (T389), p-S6K1 (T421/S424), S6, p-S6 (S240/244), p-S6 (S235/236) (Cell Signaling, Beverly, MA, USA) and 4EBP1 (Bethyl Laboratories).

2.6.2. Early steps in insulin signaling pathways

An aliquot of frozen gastrocnemius powder was homogenized in a buffer B as previously described [30]. Homogenates were centrifuged at 12,000×*g* for 20 min at 4°C. After normalization for protein concentration (1 mg), various proteins were immunoprecipitated from the supernatants using 5 μg of appropriate antibodies [IRβ (Biosciences, Le Pont de Claix, France) or IRS1 (Upstate Biotechnology, Lake Placid, NY, USA] 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, Le Pont de Claix, France) or anti-phosphoserine antibody [p-IRS1 (S636/639)] and then with total IRβ or IRS1 antibodies.

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

PI3K activity was measured in p85 immuno-precipitates from muscle homogenates prepared in buffer B (2 mg protein) using an anti-rat p85 subunit antibody (Upstate Biotechnology, Euromedex, France) and protein G agarose beads (GE Healthcare Europe) as described previously [31]. Protein kinase B (PKB) activity was measured in PKB immuno-precipitates from muscle homogenates prepared in buffer B (1 mg protein) according to the non radioactive Akt kinase assay kit (Cell Signaling, Beverly, MA, USA). PKB activity was normalized to total PKB using specific PKB antibody (Cell Signaling, Beverly, MA, USA) recovered after immunoprecipitation.

2.7. Statistical analysis

Data are presented as means±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) to analyze the supplementation effect (C vs. LEU) or two ways to analyze the supplementation and the nutritional state (PA vs. PP state) 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. A linear regression analysis was performed to correlate IG index and adipose tissue weight individual values, and slopes were compared using a t test. All tests were performed using XLStat (Addinsoft, New York, NY, USA, version 7.5.2). Differences were considered as significant at $P<.05$.

3. Results

3.1. Animal characteristics

Over the experimental period, mean food intake was similar in both groups (24.2±0.5 and 24.3±0.4 g dry matter ingested per day in C and LEU groups, respectively). Total body weight was similar before the experimental period (366±4 and 364±5 g in each group), it increased significantly in both groups during the experimental period ($P<.0001$) and weight gain over the 5 weeks of supplementation was not significantly different between the two groups (73±3 and 81±5 g in C and LEU groups).

Leucine supplementation had no effect on muscle mass expressed in weight (Table 2) or in percentage of body weight (data not shown). Protein content in gastrocnemius muscle was also similar in both groups (16.76±0.17 and 17.02±0.13 mg/100 mg tissue in C and LEU groups, respectively). Leucine supplementation did not change the weight of heart, liver, spleen and kidneys expressed in total weight (Table 2) or in percentage of body weight (data not shown). By contrast, perirenal adipose tissue weight was significantly increased expressed in g (+27%, $P < .05$) (Table 2) or in percentage of body weight (1.513±0.085 vs. 1.204±0.087%, respectively, $P<.02$) in leucine-supplemented rats compared to control rats, but total lipid muscle concentration was not changed (2.12±0.30 and 1.98±0.13 mg/100 mg tissue in C and LEU groups, respectively).

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

	Control	LEU
Body weight (g)	441±6.7	445±7.6
Gastrocnemius (g)	2.41±0.04	2.39±0.05
Tibialis anterior (g) (×2)	1.54±0.02	1.56±0.02
EDL (mg) (×2)	368±5	369±7
Soleus (mg) (×2)	307±6	293±7
Heart (g)	1.46±0.06	1.35±0.04
Liver (g)	11.36±0.27	11.10±0.27
Spleen (g)	0.859±0.028	0.863±0.029
Kidneys (g) (×2)	2.81±0.08	2.69±0.09
Perirenal adipose tissue (g)	5.30±0.45	6.74±0.44*

Values are means±S.E.M., $n=24$. *$P<.05$.

At the end of the supplementation period, plasma amino acids were measured in C and LEU groups after overnight food deprivation (postabsorptive state) or during the fed state (Table 3). Feeding the leucine-supplemented diet significantly increased plasma leucine concentration (2.8 times) compared to C diet during the fed period whereas it was not changed in the PA state. It is important to note that other plasma BCAA (i.e., isoleucine and valine) were not modified in the LEU group and were maintained at the level observed in the C group. Total essential amino acid concentration was increased in the fed state in both groups ($P<.0001$); the increase was more marked in LEU group ($P<.005$) and mainly resulted from elevated plasma leucine induced by supplementation. Leucine supplementation did not change basal plasma metabolites and hormones measured at the end of the supplementation period (triglycerides: 0.357±0.032 vs. 0.353±0.029 mM; cholesterol: 1.05±0.04 vs. 1.04±0.06 mM; HDL: 0.226±0.012 vs. 0.225±0.015 mM; glucagon: 56.3±2.0 vs. 58.2±3.1 pg/ml and leptin: 2.04±0.27 vs. 2.64±0.30 ng/ml in C and LEU groups respectively). It should be noted that basal plasma leptin was positively related to perirenal adipose tissue weight ($y=0.39x-0.18$; $R^2=0.5$; $P<.005$; not shown).

3.2. Oral glucose tolerance tests

Overall insulin sensitivity has been examined using an OGTT at the end of the nutritional intervention (Fig. 1). Basal plasma glucose concentration (5.33±0.17 and 5.38±0.13 mM in C and LEU groups,

Table 3
Plasma amino acid concentration in control and leucine-supplemented rats in the postabsorptive or the fed state

State	Control		LEU	
	Postabsorptive	Fed	Postabsorptive	Fed
Leucine	119±4	124±5	122±4	346±19*,†
Isoleucine	72±2	76±3	69±3	79±3*
Valine	168±7	176±8	168±7	190±10
Histidine	40±2	52±3*	44±3	47±2
Lysine	465±10	581±13*	500±12	661±15*,†
Methionine	45±1	77±3*	37±2†	64±3*,†
Phenylalanine	79±2	83±3	83±3	85±3
Threonine	229±9	277±14*	196±6†	234±10*,†
Total	1216±28	1448±31*	1219±28	1706±45*,†

Values, expressed in μM, are means±S.E.M.

* $P<.05$ vs. postabsorptive value in the same group.

† $P<.05$ vs. the corresponding value in the control group.

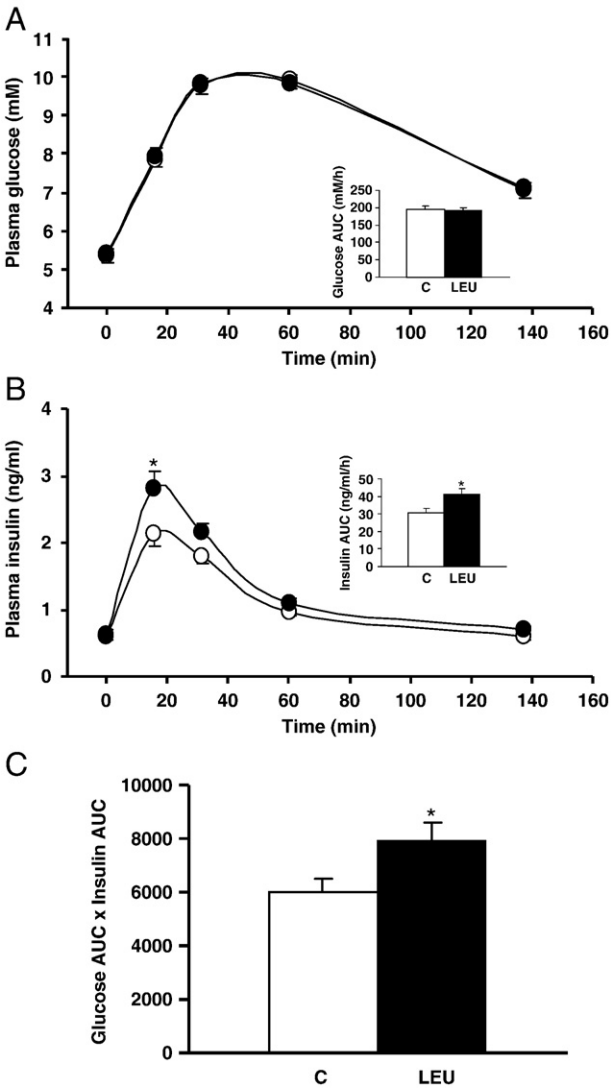

Fig. 1. OGTT in control (white circles and bars) and leucine-supplemented (black circles and bars) rats after an overnight food deprivation. Time-course changes in plasma glucose (A), plasma insulin (B) levels after orally administration of a glucose bolus (1g/kg) and IG index (AUC glucose×AUC insulin) (C). Values are means±S.E.M. (n=21 and 22). *P<.05.

respectively) and basal plasma insulin level (0.596±0.04 and 0.621±0.06 ng/ml, respectively) were similar at the end of the experimental period in both groups. Whereas the glucose AUC in response to the oral glucose bolus was similar in both groups (Fig. 1A), the insulin AUC was higher in LEU group (+32%, P<.05) (Fig. 1B). The IG index estimated by the product of the AUCs for glucose and insulin was greater in leucine supplemented rats than in control (+29%, P<.05) (Fig. 1C), suggesting that leucine supplementation had induced whole body insulin resistance.

3.3. Skeletal muscle signaling pathways

Amino acid and insulin signaling pathway analysis was performed by Western blot on gastrocnemius muscles taken at the end of the experimental period in the postabsorptive state (PA) and 30 (PP30) or 60 (PP60) min after oral administration of a nutrient bolus containing amino acids and carbohydrates. As expected, the nutrient bolus increased plasma insulin level in both groups 30 min after ingestion whereas it returned to basal

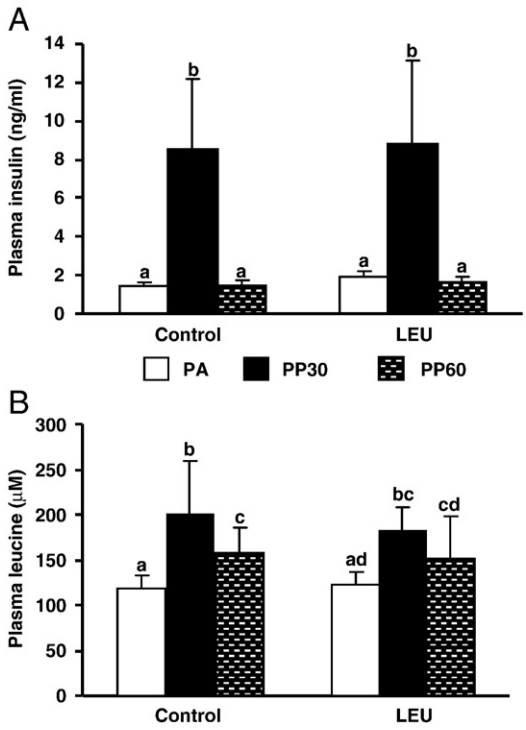

Fig. 2. Plasma insulin (A) and leucine (B) concentrations in control and leucine-supplemented rats after an overnight food deprivation (PA), 30 (PP30) or 60 min (PP60) after gavage with a nutrient bolus. Values are means±S.E.M. (n=6–12). Values with different letters are significantly different at least at P<.05.

level after 60 min (Fig. 2A). Basal plasma leucine concentration was similar in C and LEU groups, it increased significantly after ingestion of the nutrient bolus without significant differences between C and LEU groups (Fig. 2B). The same data were obtained with BCAA (data not shown).

Chronic leucine supplementation did not modify the total protein amount of insulin/amino acid signaling factors measured in gastrocnemius muscles (Table 4).

3.3.1. Downstream mTOR signaling pathways

Phosphorylation of S6K1 (T421/S424) was greatly increased 30 min after food intake whereas it returned to the basal level after 60 min. No difference was observed between C and LEU groups (Fig. 3A, left). Phosphorylation of S6K1 (T389) was also greatly increased 30 min after the nutrient bolus intake but it remained elevated at 60 min compared to basal level, it was significantly less at 60 min compared to 30 min in LEU group (Fig. 3A, right). However, S6K1 (T389) phosphorylation was similar in C and LEU groups in both 30 and 60

Table 4
Total protein content of insulin signaling pathway in control and leucine-supplemented rats

	Control	LEU
IRS1	51.8±1.6	52.6±1.7
PKB	23.3±1.1	24.1±1.1
S6K1	6.5±0.6	7.6±0.6
S6	13.3±2.0	14.4±1.8
4E-BP1*	26.5±1.4	27.1±0.9
eEF2	8.4±0.5	8.9±0.4

Values are expressed in arbitrary units and are means±S.E.M. (n=18–24 in each group). Because no effect of diet and nutritional state was observed between groups by two-way ANOVA, values (PA, PP30 and PP60) were pooled in each group. *Total 4E-BP1 was the sum of α, β and γ phosphorylated forms.

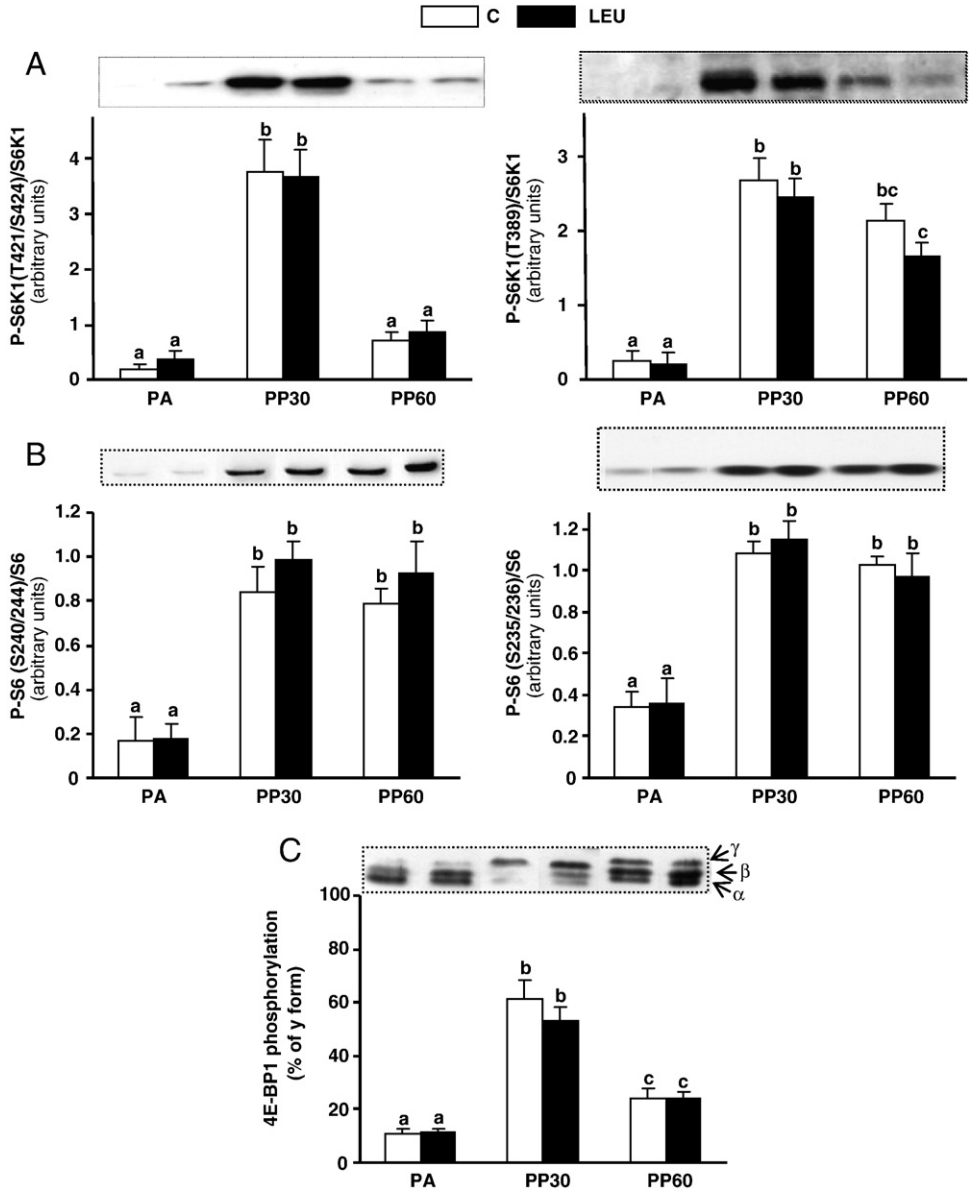

Fig. 3. Phosphorylation of S6K1 at Thr^{421}-Ser^{424} and Thr^{389} (A); rpS6 at $Ser^{240/244}$ and $Ser^{235/236}$ (B) and 4E-BP1 (C) in skeletal muscles from control (white bars) and leucine-supplemented (black bars) rats after an overnight food deprivation (PA), 30 (PP30) or 60 min (PP60) after gavage with a nutrient bolus. Values are means±S.E.M. (n=6–12). Values with different letters are significantly different at least at P<.05.

min after the nutrient bolus intake. S6 phosphorylation (S240/244 and S235/236) was increased 30 min after food intake and remained elevated after 60 min. No difference was observed between C and LEU groups (Fig. 3B). Hyperphosphorylation of 4E-BP1, represented by the percentage of the γ-form, was greatly stimulated 30 min after the nutritional bolus (Fig. 3C), it remains stimulated at 60 min but to a lesser extent. Again, no difference was observed between C and LEU groups.

3.3.2. Early steps in insulin signaling

To assess if chronic leucine supplementation affected the early steps of insulin signaling, we next examined the activation of key signaling proteins involved in insulin action such as tyrosine phosphorylation of IRβ and IRS1, and PI3K and PKB activities which play a major role in insulin-stimulated glucose metabolism in skeletal muscle. Tyrosine phosphorylation of IRβ, IRS1 as well as PI3K activity were significantly reduced (−36%, −36% and −38%, P < .05) in leucine-supplemented rats compared to control 30 min after the nutrient bolus (Fig. 4A–C). This decrease was not maintained at 60 min. In the postabsorptive state no difference was observed between the two groups. Surprisingly, the reduced activity of PI3K observed in leucine-supplemented rats did not induce a reduction in PKB activity (Fig. 4D). In fact, PKB activity was slightly increased by nutrient intake and it was significantly stimulated only in LEU group 30 min after the nutrient bolus intake. Because phosphorylation of IRS1 on serine residue have been implicated in insulin resistance [11,32], we examined the phosphorylation of IRS1 (S636/639). It was not modified in rats supplemented with leucine in both PA and PP states (1.00±0.06, 1.04±0.11 and 0.93±0.05 vs. 1.03±0.07, 1.02±0.09 and 0.87±0.07 arbitrary units of p-ser IRS1/IRS1 for PA, PP30 and PP60 in C and LEU groups, respectively).

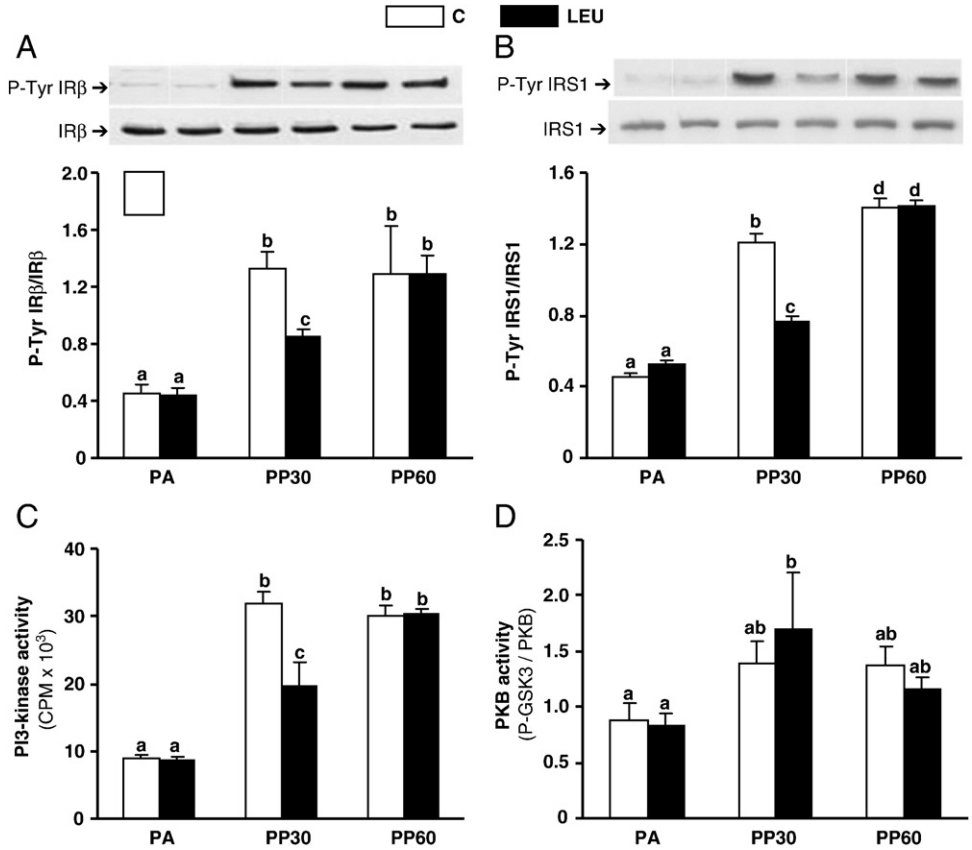

Fig. 4. Phosphorylation of IRβ (A) and IRS1 (B) on tyrosine residues and activities of PI3 kinase (C) and PKB (D) in skeletal muscles from control (white bars) and leucine-supplemented (black bars) rats after an overnight food deprivation (PA), 30 (PP30) or 60 min (PP60) after gavage with a nutrient bolus. Values are means±S.E.M. (n=6-12). Values with different letters are significantly different at least at P<.05.

3.4. 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 control and leucine supplemented rats. In the absence of insulin (basal state), glucose uptake was similar in muscles from C and LEU groups. Increasing insulin concentration in the incubation medium significantly increased the rate of glucose transport in epitrochlearis muscles with half-maximum response around 2–4 nM in both groups.

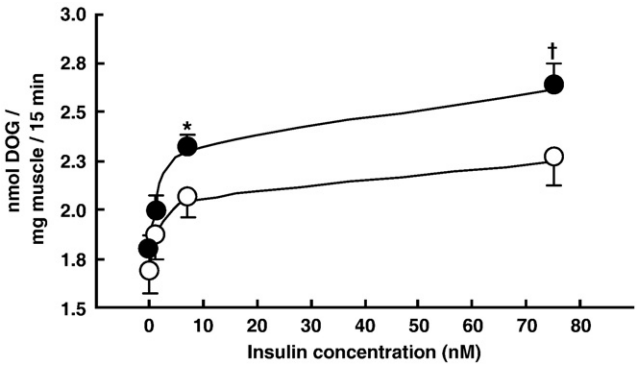

Fig. 5. Dose-response curves of insulin-stimulated glucose transport in isolated epitrochlearis muscles from control (white circles) and leucine-supplemented (black circles) rats after an overnight food deprivation. Values are means±SEM (n=10-12). *P < .05; † P=.07 vs. C group.

However, the maximum response was increased in muscles from LEU group compared to C group (Fig. 5).

4. Discussion

Leucine is recognized as an anabolic factor that regulates muscle protein synthesis through activation of the mTOR/S6K1 pathway, a downstream signal of the insulin PI3 kinase/PKB signaling pathway. Although activation of the mTOR/S6K1 pathway by leucine is efficient to stimulate muscle protein synthesis, a number of studies reported that it can also exert inhibition on the early steps of insulin action leading to insulin resistance [1–3,5]. However, the studies have generally been performed in vitro or in vivo with acute amino acid or leucine increase. In the present study, we observed that dietary chronic leucine supplementation for 5 weeks in adult rats induced a delay in the postprandial stimulation of the early steps of muscle insulin signaling that did not result in insulin resistance on muscle glucose transport measured in vitro. However, perirenal adiposity mass was significant increased (+27%) in leucine-supplemented rats and was associated with a decrease in overall oral glucose tolerance assessed with OGTT.

Studies performed in vitro with cell cultures reported that activation of S6K1 by insulin and amino acids inhibited early events of insulin signaling, such as PI3 kinase activity, linked to a decreased insulin-stimulation of tyrosine phosphorylation of IRβ, IRS1 and IRS2 or an increase in serine residue phosphorylation of IRS1 [8,9,11]. In vivo, stimulation of S6K1 phosphorylation by acute amino acid infusion in human has also been shown to increase the phosphorylation state of IRS1 on serine residues (S312, 636/639 or 1101) which

completely blunted PI3K activity [10,11,32]. Moreover, Baum et al [33] demonstrated that acute oral administration of leucine in rats reduced the duration of the insulin-induced PI3K activity. These data indicated that acute amino acid activation of S6K1 induced serine phosphorylation of IRS substrates that lead to decreased insulin-dependent PI3K activation. However, rare are the studies in which the effect of amino acids on the insulin pathway regulation and sensitivity of muscle glucose metabolism to insulin have been tested at the same time in the same experiment.

In the present study, we showed that chronic dietary leucine supplementation has induced a transient reduction in the postprandial tyrosine phosphorylation of IRβ, IRS1 and PI3K activity. However, these defects were observed whereas phosphorylations of S6, 4EBP1 and especially S6K1 in the basal state or in response to the nutrient bolus were similar in control and leucine-supplemented rats. It suggested that chronic leucine supplementation did not induce sustained alteration of S6K1 phosphorylation. The recent study of Newgard et al. [22] demonstrated that long-term (12–16 weeks) BCAA supplementation in young rats was responsible for a sustained elevation of phosphorylation state of mTOR and S6K1 in response to feeding but only when rats were fed a high-fat diet. Consistent with our results, no change was observed when rats were fed a normal control diet. Whereas prior studies with acute amino acid administration have lead to an increased phosphorylation of IRS1 on serine residues in human [10], the defect in IRβ and IRS1 tyrosine phosphorylation and PI3K activation that we observed in leucine-supplemented rats were not related to an increase in IRS1 phosphorylation on serine$^{636/639}$ residues. However, we cannot exclude that other IRS1-serine residues were phosphorylated in leucine-supplemented rats (i.e., ser^{302}, ser^{1101}) as observed in BCAA-supplemented rodents [22] or in amino acid-infused humans [11].

Because PKB is admitted to be a PI3K downstream effector, it was surprising that PKB was normally activated after food intake in leucine-supplemented rats despite PI3K activity was reduced for the first 30 min. In agreement, Kim et al. [34] and Tremblay et al. [10] found that a decrease in PI3 kinase activity during amino acid infusion in healthy or in diabetic subjects was not associated with impaired PKB activity suggesting that PKB requires only partial PI3 kinase activation to get fully activated. Although the early steps of skeletal muscle insulin signaling pathway were transiently inhibited in leucine-supplemented rats, we did not find a decrease in the insulin-response of muscle glucose transport compared to control rats. These data may be surprising because glucose transport stimulation has been shown to be positively related to the insulin/PI3 kinase signaling pathway activation. However, two methodological limitations of our study must be acknowledged: (1) muscle glucose transport has been measured in epithrochlearis muscles whereas insulin signaling has been investigated in gastrocnemius muscles; (2) glucose transport was measured after muscles were incubated in presence of insulin for 45 min, a time that was beyond the 30 min for which PI3K activity was not maximally stimulated in the leucine supplemented group. Moreover, the plasma insulin levels achieved by the nutrient bolus for insulin signaling measurements (8 ng/ml, i.e.1.4 nM) is below the insulin concentration (>5 nM) that induced an improvement of glucose transport in muscles from leucine-supplemented rats suggesting that high insulin level was necessary to improve insulin effect. Finally, PKB activity in leucine-supplemented rats tended to exhibit a higher postprandial stimulation compared to control rats and may explain the insulin-induced glucose uptake in skeletal muscle we observed in vitro. Consistently, previous studies reported that acute BCAA (isoleucine or leucine) were able to increase glucose uptake either in vitro [14,35] or in vivo in rats [15,16]. To our knowledge, recent studies reporting the effect of in vivo chronic amino acids or BCAA supplementation did not investigate insulin-stimulated glucose uptake in muscle [20,22,36,37].

Although insulin-stimulated glucose transport was improved in muscle from leucine-supplemented rats, we surprisingly observed an overall insulin resistance in leucine supplemented rats characterized by a normoglycemic profile associated with a hyperinsulinemic response (+32% for insulin AUC, $P<.05$) to the oral glucose bolus and a significant increase in the insulin-glucose index (IG index) (+29%, $P<.05$). However, OGTT represented an in vivo estimation of whole body insulin sensitivity which concerned muscle but also adipose tissue. The apparent inconsistency between these two data suggests that it will be necessary to better analyze the effect of leucine excess on insulin sensitivity, not only in skeletal muscle but also in other potential target tissue such as adipose tissue. Despite that a decrease in glucose tolerance was consistent with the results obtained in human on whole body glucose disposal and endogenous glucose production during short-term elevation of plasma amino acid in human [10,38], they were not clearly strengthened by long-term BCAA or leucine supplementation previously reported in insulin resistant rodent models. Indeed, increasing leucine intake for 10 weeks significantly improved glucose tolerance and insulin sensitivity in mice fed a high-fat diet [20], whereas it had no effect on insulin tolerance in mice subjected to the same protocol design during 14 weeks [37]. The inconsistency of these two data was not clearly explained. In their study, Newgard et al [22] shown that BCAA supplementation for 12-16 weeks in rats fed a high-fat diet maintained the glucose intolerance and overall insulin resistance induced by high-fat feeding despite a decrease in food intake and body weight. Moreover, using pair-feeding, they also demonstrated that in high-fat fed rats, BCAA partly contributed to the development of insulin resistance and was mediated by chronic activation of mTOR signaling pathway [22]. Finally, unchanged glucose tolerance and insulin sensitivity after leucine supplementation (using HOMA index) has been described in food-restricted rat [39] or in healthy elderly men [36]. Together, these data revealed that the role of chronic BCAA or leucine supplementation on overall glucose tolerance was not clearly defined and may probably depend on the duration of the supplementation and the degree of insulin resistance state present at the time of supplementation.

Interestingly, we observed that leucine-supplemented rats that displayed an impaired glucose tolerance also exhibited a significant increase (+27%) in visceral (i.e., perirenal) adipose tissue that is commonly correlated to the risk of insulin resistance. However, the total body weight was not yet significantly modified suggesting that this increased adiposity was still localized and/or was just beginning. We noted that individual IG index values (index of insulin sensitivity) and adipose tissue weights, from either C and LEU groups, were significantly correlated (Fig. 6), but the fit slope

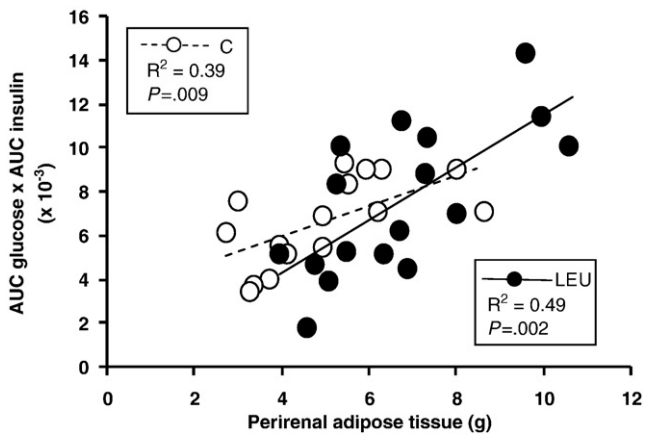

Fig. 6. Relationship between perirenal tissue mass and insulin sensitivity measured by the IG index calculated from OGTT data in control (white circles) and leucine-supplemented (black circles) rats ($y=0.72\,x+3.00$, $R^2=0.39$, $P=.009$ in control group; $y=1.20\times-0.56$, $R^2=.50$, $P=.002$ in leucine-supplemented group).

for the LEU group (1.202±0.320) tended to be higher than for the C group (0.718±0.238) (P=.1) suggesting that chronic leucine supplementation tended to favor the development of obesity and insulin resistance in young adult rats. In the present study, the increase in adiposity did not result from an increase of energy intake since no difference in food intake was observed between control and leucine-supplemented rats along the experimental period as previously shown [22,36,37,40]. However, because leucine supplementation was performed in a normal caloric diet, we can hypothesize that leucine in excess may have been used as energetic substrate, sparing glucose that may have been utilized and stocked as triglycerides by adipose cells (i.e., increasing adiposity). This mechanism associated with the potential anabolic and/or trophic effects of leucine on adipose cells [41–43] may have contributed to adipose tissue enhancement in the supplemented rats.

In conclusion, our results indicate that a dietary leucine supplementation for 5 weeks in young adult rats induced a delay in the postprandial stimulation in the early steps of muscle insulin signaling that did not impaired insulin response of muscle glucose transport measured in vitro. However, it induced an accumulation of perirenal adipose tissue associated with a decrease in overall glucose tolerance. Further investigations are necessary to clearly define whether chronic dietary leucine supplementation is at risk in promoting adipose tissue in healthy subjects.

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