Influence of leucine on protein metabolism, phosphokinase expression, and cell proliferation in human duodenum1–4

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ABSTRACT

Background: Although leucine increases protein anabolism through the mammalian target of rapamycin (mTOR) pathway in human muscles, its effects on intestinal protein metabolism remain unknown.

Objective: We aimed to assess the effects of leucine on duodenal protein metabolism in healthy humans and to elucidate the signaling pathways involved.

Design: Eleven healthy volunteers received for 5 h, on 2 occasions in random order, an enteral supply of maltodextrins (0.25 g·kg−1·h−1) or maltodextrins and leucine (0.035 g·kg−1·h−1) simultaneously with a continuous intravenous infusion of [2H5]phenylalanine (9 mol·kg−1·h−1). Endoscopic duodenal biopsy samples were collected and frozen until analyzed. Phenylalanine enrichment was assessed by gas chromatography–mass spectrometry in duodenal protein and in free intracellular amino acid pools used as precursor to proteasome-dependent proteolysis (15).

Results: Leucine supplementation slightly reduced FSR (mean ± SEM: 81.3 ± 6.3%/d) compared with maltodextrins alone (91.7 ± 8.5%/d; P = 0.0537). In addition, total proteasome activity decreased significantly with leucine (236 ± 21 compared with 400 ± 58 relative fluorescence units/µg protein; P < 0.05), with no modification of chymotrypsin-like, trypsin-like, caspase-like, or peptidase activities. Leucine did not affect the mTOR pathway but did increase the phosphorylation states of PI3K, Akt, AMPK, p38 MAPK, JNK, GSK-3α, STAT3, and STAT5 and increased cyclin D1 mRNA concentrations, which suggested that leucine may enhance cell proliferation.

Conclusion: Enteral leucine supplementation decreased proteasome activity in duodenal mucosa and enhanced cell proliferation through the PI3K/Akt/GSK-3α/catenin pathway. This trial was registered at clinicaltrials.gov as NCT01254110. *Am J Clin Nutr* 2011;93: 1255–62.

INTRODUCTION

Gut barrier plays a critical role in the defense of organisms. Stress events, chronic inflammatory diseases, or infections are mainly associated with an increase in intestinal permeability and then to a dysregulation of gut homeostasis (1). Intestinal barrier is regulated by a balance on one hand between cell proliferation and apoptosis and, on the other hand, between protein synthesis and degradation. The protein fractional synthesis rate (FSR) approached 50% per day in the human duodenal mucosa (2, 3)—a value much higher than that of other major tissues, such as liver or muscle. Previous studies reported that nutritional states or interventions can affect gut protein synthesis (3–8). Amino acids have been mainly reported to modulate intestinal protein metabolism. Glutamine, the preferential substrate of enterocytes, enhanced protein metabolism in Caco-2 cells (8) and in human duodenum (3). Arginine also stimulated protein synthesis in the small intestine of piglets with rotavirus-induced enteritis (9) but not in the duodenal mucosa of healthy volunteers (10). Surprisingly, leucine effects have been poorly documented in the intestine (11, 12), whereas leucine improved protein metabolism in muscle by stimulating protein synthesis (13, 14) and inhibiting proteasome-dependent proteolysis (15).

One major pathway involved in the regulation of initiation and elongation of protein synthesis by amino acids is the mammalian target of rapamycin (mTOR) pathway (16, 17). Leucine activated the mTOR pathway in intestinal epithelial cells in vitro (18–20) and cell migration (20). Another pathway, general controller nonderepressible 2 kinase (GCN2), is more sensitive to a limitation of amino acid availability, as we recently reported in vitro (21). To our knowledge, the mechanisms involved in the regulation of intestinal protein metabolism by luminal amino acids or protein supply have been poorly documented in vivo. A recent study showed that leucine supplementation of a low-protein diet stimulated jejunal protein synthesis in neonatal pigs through the mTOR pathway (12). However, the influence of leucine on...
protein metabolism in human intestine and on the involved signaling pathways remains unknown. Thus, the major aim of this experimental study was to assess the effects of an acute enteral leucine supply on duodenal protein metabolism and to elucidate the underlying mechanisms in humans.

SUBJECTS AND METHODS

Twelve healthy volunteers (7 men and 5 women) were included in the study and were in good general health with no hepatic, renal, or cardiac dysfunction and no medical or surgical history of digestive disease. One volunteer withdrew her consent and thus was excluded from the study. The remaining 11 volunteers had a mean (±SD) age of 28.8 ± 10.4 y and body mass index (in kg/m²) of 22.3 ± 1.2. The study was approved by the local ethics committee (North-west I, France), and the subjects gave written informed consent. Over 3 d, all subjects consumed a controlled diet providing 30 kcal and 0.9 g protein · kg⁻¹ · d⁻¹. The meals were prepared by the hospital dietary unit. The study began at 0800 in the morning of day 4, after the subjects had fasted for 12 h overnight. The subjects received over 5 h, on 2 occasions, and in a random order by a nasogastric feeding tube either maltodextrins (control condition: 0.25 g · kg⁻¹ · h⁻¹; Lactalis Nutrition Santé, Torcé, France) or maltodextrins plus leucine (leucine condition: 0.035 g free L-leucine · kg⁻¹ · h⁻¹; Cooper, Melun, France). The infusion rate was 3.5 mL · kg⁻¹ · h⁻¹. The time elapsed between the 2 occasions ranged from 2 to 4 wk. The dose of leucine supplied was chosen as a compromise between the dose used in former studies on muscle proteins and a higher dose needed to affect the very high protein turnover rate of the intestinal mucosa. The present experimental study was designed to evaluate the effects of leucine on intestinal protein metabolism and, particularly, on signaling pathways. Thus, to avoid a comparison of leucine supply to a fasted status, we compared maltodextrins supplemented with leucine with maltodextrins alone.

After the baseline blood samples were collected, a bolus tracer was infused that provided 9 μmol/kg [1⁰-H²]phenylalanine [90% mole percentage enrichment (MPE); Mass Trace Inc, Woburn, MA]. After the bolus was infused, 9 μmol · kg⁻¹ · h⁻¹ of [l⁰-H²]phenylalanine was intravenously infused until endoscopy. The tracer had been previously tested for sterility and pyrogenicity and was diluted in saline in the hospital pharmacy on the evening before study and kept at 4°C until used.

The upper endoscopy (model XQ20; Olympus, Tokyo, Japan) was performed 30 min after the end of enteral feeding. Sixteen mucosal biopsy samples were collected from the distal duodenum; 2 samples were fixed in formalin for histologic assessment, and 14 samples were immediately frozen in liquid nitrogen and stored at −80°C for analysis of isotopes, proteolytic activity, protein expression, and mRNA concentrations.

Protein FSR

Mucosal tissue samples were processed as previously described (3, 22). Briefly, mucosal tissue samples were quickly rinsed in ice-cold 0.9% NaCl and immediately ground. Proteins were precipitated with 10% trichloroacetic acid, and free amino acids were then taken with the supernatant fluid. The supernatant fluid was prepared as plasma to measure free amino acid enrichment. The protein pellet containing isotopically enriched proteins was dissolved in 1 mol NaOH/L and then hydrolyzed in 6 mol HCl/L at 110°C for 18 h to allow analysis of the enrichment of amino acids released from protein hydrolysis.

The enrichments of [³⁵H]phenylalanine were determined in the mucosal intracellular free amino acid pools and in the mucosal proteins by gas chromatography–mass spectrometry (MSD 5972; Hewlett-Packard, Palo Alto, CA) by using tert-butylmethylysilyl derivatives as previously (3). Appropriate standard curves were run simultaneously for determination of the enrichments.

The FSR of duodenal mucosal protein was calculated as follows:

$$\text{FSR}\left(\% / d\right) = \left[\left(E_t - E_0\right) / E_p\right] \times 1 / t \times 24 \times 100$$

where Et is the enrichment in tissue protein at time t (in %), Eo is the natural abundance of the labeled amino acid in intestinal mucosal protein (in %), Ep is the enrichment of the precursor pool at plateau (in %), and t is the duration of the tracer infusion (in h). Isotopic enrichment at baseline was determined in normal duodenal biopsy samples from ambulatory patients undergoing endoscopy for medical reasons. The precursor pool used was the intracellular free amino acid pool.

In our study, only one tissue sampling was performed after the continuous infusion of labeled amino acids to evaluate mucosal protein FSR as previously reported by us (3, 7, 10) and other groups (2, 23, 24). Even though this approach might underestimate modifications of FSR, as suggested by some authors (25, 26), we thought that in healthy volunteers it was not possible for ethical reasons to perform 4 endoscopic procedures over 2 wk.

Proteasome activities

Endoscopic biopsy samples were homogenized in ice-cold lysis buffer containing 30 mmol Tris-HCl/L (pH 7.2), 1 mmol dithiothreitol/L, and 1% Triton X-100; placed on ice for 15 min; and then centrifuged for 15 min at 4°C and 12,000 rpm. Proteasome activities were evaluated as previously described (27–29) by spectrofluorimetry on a microtiter plate fluorometer (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) with the use of fluorogenic proteasome substrates—Suc-LLVY-MCA (PSIII; Calbiochem, San Diego, CA) for chymotrypsin-like activity, Boc-LSTR-MCA (Sigma Aldrich) for trypsin-like activity, and Z-LLE-MCA (Sigma Aldrich) for caspase-like or PGPH activity—in the presence or absence of a proteasome inhibitor, 10 μmol lactacystin/L (Sigma Aldrich). Thus, proteasome activity was calculated as the difference between the activity measured in the absence of lactacystin and the activity measured in the presence of lactacystin.

Macroarrays of phosphokinases

Expression of phosphorylated proteins was assessed by using the Proteome Profiler Human Phospho-Kinase Array kit (R&D Systems, Abingdon, United Kingdom) according to the manufacturer’s instructions; Briefly, after membranes coated with specific antibodies directed against phosphokinases were blocked, 400 μg duodenal mucosal proteins were loaded on these membranes. After incubation with specific detection buffers and wash
steps, phosphokinase expression was determined by using the ECL detection system (GE Health Care, Orsay, France). Each spot was quantified by densitometry with the use of Image-Scanner III and ImageQuant TL software (GE Health Care). Only spots with a %vol variation ratio >1.5 were considered relevant.

**Western blot analysis**

Proteins (25 μg) were separated on 4–12% Tris-Glycine resolving gels (Invitrogen, Cergy-Pontoise, France) and transferred to a nitrocellulose membrane (GE Health Care), which was blocked for 1 h at room temperature with 5% (wt:vol) nonfat dry milk or bovine serum albumin in Tris-buffered saline (TBS) (10 mmol Tris/L, pH 8; 150 mmol NaCl/L) plus 0.05% (wt:vol) Tween 20. Then, the primary antibody was incubated overnight at 4°C: goat polyclonal antibodies [anti-4E-BP1 (sc-6025) and anti-phosphorylated-p70S6 kinase (Thr389, sc-11759); SantaCruz Biotechnology, Tebu-bio, Le Perray en Yvelines, France], rabbit polyclonal antibodies [anti-eiF2α (sc-11386), SantaCruz Biotechnology; anti-phosphorylated-4E-BP1 (Ser65, sc-18091-R), anti-p38 mitogen-activated protein kinase (MAPK) (sc-535), and anti-phosphorylated-p38 MAPK (Tyr182, sc-7975-R), SantaCruz Biotechnology; anti-phosphorylated-eiF2α (Ser51, no. 9722) and anti-phosphorylated-p70S6 kinase (Thr389, sc-11386), SantaCruz Biotechnology; and anti-phosphorylated-Pi3K (p85 (Tyr458)/p55 (sc-11386), no. 4228S, Cell Signaling Technology)], and mouse polyclonal antibodies [anti-p70S6 kinase (sc-8418), Santa Cruz Biotechnology; and anti-β-actin, Sigma Aldrich]. After 3 washes in a blocking solution of 5% (wt:vol) nonfat dry milk or bovine serum albumin in TBS/0.05% Tween 20, a 1-h incubation with peroxidas-conjugated goat anti-goat, anti-rabbit, or anti-mouse IgG (1:5000; SantaCruz Biotechnology) was performed. After 3 additional washes, immunocomplexes were revealed by using the ECL detection system. Protein bands were quantified by densitometry with the use of ImageScanner III and ImageQuant TL software.

**Quantitative reverse transcriptase–polymerase chain reaction**

After reverse transcription of 1.5 μg total RNA into cDNA by using 200 units of SuperScript II Reverse Transcriptase (Invitrogen) as previously described (27), quantitative polymerase chain reaction (qPCR) was performed by SYBR Green technology on a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Marnes la Coquette, France) in duplicate for each sample as previously described (27). GAPDH was used as the endogenous reference gene. The specific primers for cyclin D1 were 5'-TGATGTCGCCCTTCCATGTC-3' and 5'-TCC- AATCATCCGGAATGAGTGC-3' and for GAPDH were 5'- TGCCATCAATGACCCCTTCA-3' and 5'-TGACCTTGCCCA CAGCCTTG-3'.

**TABLE 1**
Biological characteristics of healthy volunteers before and after 5 h of enteral maltodexins alone (control) or with leucine supplementation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leucine</th>
<th>Nutrient</th>
<th>Time</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycemia (mmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
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<tr>
<td>5 h</td>
<td>5.9 ± 0.3</td>
<td>5.1 ± 0.2</td>
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<tr>
<td><strong>Plasma insulin (pmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>42.4 ± 3.8</td>
<td>42.0 ± 2.9</td>
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<tr>
<td>5 h</td>
<td>116.1 ± 14.1</td>
<td>128.2 ± 13.7</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>NS</td>
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<tr>
<td><strong>Plasma isoleucine (μmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>63.9 ± 3.0</td>
<td>59.6 ± 4.1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
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<tr>
<td>5 h</td>
<td>32.2 ± 2.2*</td>
<td>6.6 ± 0.5*</td>
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<tr>
<td><strong>Plasma leucine (μmol/L)</strong></td>
<td></td>
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<tr>
<td>0 h</td>
<td>134 ± 6</td>
<td>127 ± 6</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>5 h</td>
<td>107 ± 4*</td>
<td>50.7 ± 20*</td>
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<td><strong>Plasma methionine (μmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>27.9 ± 1.3</td>
<td>25.8 ± 1.5</td>
<td>&lt;0.05</td>
<td>&lt;0.0001</td>
<td>NS</td>
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<tr>
<td>5 h</td>
<td>19.8 ± 0.9</td>
<td>14.5 ± 1.0</td>
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<tr>
<td><strong>Plasma tyrosine (μmol/L)</strong></td>
<td></td>
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<tr>
<td>0 h</td>
<td>55.0 ± 2.3</td>
<td>50.8 ± 3.7</td>
<td>&lt;0.05</td>
<td>&lt;0.0001</td>
<td>NS</td>
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<tr>
<td>5 h</td>
<td>42.2 ± 1.6</td>
<td>31.6 ± 1.5</td>
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<tr>
<td><strong>Plasma valine (μmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>230 ± 8</td>
<td>211 ± 10</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>5 h</td>
<td>166 ± 6*</td>
<td>69 ± 4*</td>
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<tr>
<td><strong>Plasma EAA (μmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>700 ± 23</td>
<td>660 ± 23</td>
<td>&lt;0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
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<tr>
<td>5 h</td>
<td>565 ± 16*</td>
<td>421 ± 14*</td>
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<tr>
<td><strong>Plasma NEAA (μmol/L)</strong></td>
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<tr>
<td>0 h</td>
<td>1825 ± 69</td>
<td>1814 ± 78</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>5 h</td>
<td>1718 ± 59</td>
<td>1776 ± 77</td>
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</table>

1 All values are means ± SEMs; n = 11. EAA, essential amino acids; NEAA, nonessential amino acids. The results were compared by using 2-factor ANOVA with Bonferroni post hoc tests. *Significantly different from 0 h, P < 0.05. †Significantly different from control, P < 0.05.

2 Excludes leucine.
Plasma amino acid concentrations

Plasma amino acid concentrations were measured by using an amino acid analyzer (Biotronik LC3000-Eppendorf, Maintal, Germany). Plasma concentrations of insulin and insulin-like growth factor-I were analyzed by routine radioimmunoassay.

Statistical analysis

The results are expressed as means ± SEMs and were compared by using GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, CA). To evaluate the effects of leucine over time (plasma data), statistical analysis consisted of a 2-factor analysis of variance for repeated measures and post hoc Bonferroni tests. A nonparametric paired Wilcoxon’s test was used to evaluate the effects of leucine at 5 h (data from biopsy samples). P values < 0.05 were considered significant.

RESULTS

Histologic examination of duodenal biopsy samples showed no signs of mucosal lesions in any subjects. Plasma measures in healthy volunteers before and after enteral supplementation are shown in Table 1. Plasma leucine was markedly increased after leucine supplementation compared with after the control. In contrast, essential amino acids (except leucine) decreased, particularly other branched-chain amino acids, valine, and isoleucine.

Leucine supplementation did not significantly affect duodenal FSR compared with the control (Figure 1A) but did decrease total proteasome activity (P < 0.05; Figure 1B). However, a trend for a decrease in FSR was observed, approximately 12% (P = 0.0537). In addition, leucine supplementation did not affect a specific proteasome activity. We only observed a trend for a decrease in chymotrypsin-like and peptidase activities (Figure 1). Total ubiquitinated proteins were not modified (data not shown).

To assess the signaling pathways affected by leucine supplementation, we used macroarrays directed against 46 phosphoproteins. In these 46 proteins, we observed that leucine affected 11 proteins (Figure 2). Interestingly, leucine supplementation increased the phosphorylation of the MAPK proteins p38 (T180/Y182) and c-Jun N-terminal kinase (JNK) (T183/Y185, T221, and Y223) and of the signal transducer and activator of transcription (STAT) proteins STAT3 (Y705), 5a (Y689), 5b (Y699), and 6 (Y641). Leucine also increased the phosphorylation of Akt (S473), glycogen-synthase kinase (GSK)-3α/β (S21/S9), AMP-activated protein kinase z1 (AMPKz1) (T174), and fgr (Y412). Total β-catenin expression was increased.

FIGURE 1. Effects of leucine supplementation on duodenal protein metabolism. Mean ± SEM protein fractional synthesis rate (FSR) based on L-[2H5]phenylalanine incorporation (A) and total (B), chymotrypsin-like (C), trypsin-like (D), caspase-like (E), and peptidase (F) proteasome activities in the duodenal mucosa of healthy volunteers after 5 h of enteral maltodextrins alone (control) or with leucine supplementation. n = 11. P values were derived by using Wilcoxon’s test. AU, arbitrary units.

FIGURE 2. Effects of leucine supplementation on phosphorylated signaling proteins in duodenal mucosa. Representative macroarray results (A) and densitometric analysis (B) of phosphorylated (p-) signaling proteins in the duodenal mucosa of healthy volunteers after 5 h of enteral maltodextrins alone (control) or with leucine supplementation. Results (means ± SEMs; n = 6) are expressed as CVs [leucine – control/control (Leu-Contr/Contr)] and were compared by using Wilcoxon’s test. Significant changes (>50% of increase or decrease and P < 0.05) are shown by black bars. eNOS, endothelial nitric oxide synthase; STAT, signal transducer and activator of transcription; FAK, focal adhesion kinase; RSK, ribosomal s6 kinase; PLC-γ1, phospholipase C-γ1; AMPK, AMP-activated protein kinase; CREB, cAMP response element-binding; mTOR, mammalian target of rapamycin; MSK, mitogen- and stress-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; GSK, glycogen-synthase kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated protein kinase.
by leucine supply (Figure 2). In contrast, leucine did not affect ERK 1/2 phosphorylation or mTOR or p70S6 kinase phosphorylation, in agreement with the absence of an effect on FSR. To confirm some results by Western blot, we assessed phosphorylated (p-) p-Akt, p38, and p-p38 expressions. As shown in Figure 3, the expression of both p-p38 and p38 increased after leucine supplementation. In contrast, we confirmed that p-p70S6 kinase and p-4EBP-1 expression, another component of the mTOR pathway, was not modified by leucine.

To better understand how leucine may affect proteasome activities or Akt and other proteins, we studied inhibitor of nuclear factor κB (IkB) and class I PI3K. IkB was not modified (data not shown), whereas PI3K was more activated after leucine than after the control (Figure 3). However, PI3K activation was not dependent on insulin, because the plasma insulin concentration increased similarly in both arms (Table 1).

It was previously reported that p38 and Akt were able to phosphorylate GSK-3β (30), which is inactive in its phosphorylated form (31). Then, unphosphorylated GSK-3β phosphorylates β-catenin (31). Using macroarrays, we observed an increase in total β-catenin (Figure 2). We thus assessed active β-catenin (unphosphorylated form) by Western blot and we found that leucine supplementation increased active β-catenin (Figure 4).

All of these data suggest that leucine might enhance cell proliferation. We evaluated the mRNA concentration for cyclin D1 by qPCR. Leucine supplementation increased cyclin D1 mRNA in duodenal mucosa samples (Figure 4), which suggests that leucine enhanced cell proliferation. In addition, similar results were observed for c-myc expression (Figure 4), but the difference was not significant.

**DISCUSSION**

In the current study, we showed that supplementation of maltodextrins with leucine did not stimulate protein synthesis, but did decrease proteasome activity in human duodenal mucosa. In contrast, we observed a trend for a slight decrease in duodenal mucosal protein synthesis (12%; \( P = 0.0537 \)). In addition, leucine supplementation may enhance intestinal cell proliferation, through the PI3K/Akt/GSK-3/β-catenin pathway.

Gut homeostasis is crucial in the defense of the organism, and gut tissues have a high turnover; \( \sim 50\% \) of mucosal proteins are renewed every day (2, 3). Consequently, gut barrier function and gut homeostasis could be altered in several pathologic conditions. Nutritional strategies have been proposed to counteract gut dysregulation or to improve gut adaptation in some clinical conditions (32), but new approaches are still needed.

The effects of leucine, a branched-chain amino acid, on muscle protein synthesis is well documented in vitro (13), in vivo in animals (33, 34), and in humans (14). However, the influence of leucine on the intestinal tract has been poorly documented. In vitro studies emphasize that leucine activated the mTOR pathway (18–20) and then cell migration (20). Recently, supplementation of a low-protein diet with leucine induced an increase in jejunal protein synthesis in growing pigs (12). In addition, this increase was related to activation of the mTOR pathway (12). To our knowledge, no data have evaluated the effects of leucine on intestinal mucosa in humans. In our study, leucine supplementation slightly reduced the protein synthesis rate in human duodenal mucosa, even if the difference was not significant, and significantly decreased total proteasome activity. In addition, the mTOR pathway was not modified by leucine supplementation at 5 h. These results are not in accordance with those of previous studies showing that leucine supplementation increased p70S6K phosphorylation and activity in intestinal cells in vitro (18, 20) and in vivo in growing pigs (12). However, previous data about leucine supplementation alone did not observe an anabolic response in the liver (35). In the in vivo studies showing an anabolic effect of leucine, low amounts of valine and isoleucine or of proteins were also supplied to avoid the phenomenon of branched-chain amino acid antagonism (12, 14, 34). In our study, the subjects did not receive other branched-chain amino acids, and consequently to leucine supply, a marked decrease in plasma valine and isoleucine concentrations was observed. This latter point, and the decrease in plasma essential amino acid concentrations, may explain the observed effect of leucine on the protein synthesis rate. In addition, in the current study we compared maltodextrins supplying 5 kcal/kg to maltodextrins supplemented with leucine. We previously reported that a nutritional supplement (11.7 kcal/kg) markedly increased duodenal FSR compared with saline (7). Even if the fed status was not associated with an increased FSR in a previous study (4), our data suggest that maltodextrin supply per se increased duodenal protein synthesis, because the FSR was \( \sim 90\% \)/d in control conditions. Insulin may be responsible for the effect of maltodextrins, because insulinemia was similarly increased in both arms in the current study. However, previous studies reported that insulin did not influence the intestinal protein synthesis rate in healthy conditions (25, 36), in contrast with observations made in surgical patients (37).

Interestingly, we showed a decrease in proteasome activity with leucine supplementation, as previously reported in muscle (15, 38), which seems to not be related to nuclear transcription.
factor κB. Indeed, IκBα was not affected by leucine. Thus, supplementation of maltodextrins with leucine was able to decrease intestinal protein synthesis and total proteasome activity, which suggested a decrease in intestinal protein turnover, which could be an energy- and amino acid–saving adaptation.

In the current study, we studied for the first time in humans signaling proteins affected by enteral leucine in the intestinal mucosa. We observed that leucine supplementation increased the phosphorylation status of STAT5, JNK, or AMPKζ. However most interestingly, leucine supplementation was associated with activation of PI3K, p38 MAPK, and Akt and inhibition of GSK-3α/β. Leucine was also able to increase active β-catenin. All of these results suggest that leucine supplementation enhanced cell proliferation, as confirmed by the increase in cyclin D1 mRNA concentrations. Indeed, we can hypothesize that leucine activates the PI3K/Akt pathway, as previously reported in L6 myotubes (39). Both Akt and p38 MAPK are able to phosphorylate GSK-3α/β, which is inactive in its phosphorylated form (30, 31). In addition, PI3K activation was not related to insulin, because we observed no differences in plasma insulin between the 2 conditions. In a previous study in L6 myotubes, leucine also activated PI3K independently of insulin receptor (39). GSK-3α/β plays a key role in the regulation of both protein translation and cell proliferation. Because we did not observe differences in the protein synthesis rate, we hypothesize that GSK-3 inactivation may regulate cell proliferation through β-catenin. In our study, we found that both total β-catenin and active β-catenin accumulated after leucine supplementation.

Interestingly, during total parenteral nutrition in mice, active β-catenin decreased consequently to an increase in p-Akt and p-GSK-3, which was associated with the down-regulation of intestinal epithelial cell proliferation (40). In the same model, glutamine-supplemented total parenteral nutrition restored cell proliferation and signaling cascade (40). GLP-2 was also able to enhance cell proliferation and protein synthesis through the Akt/GSK-3 pathway in total parenteral nutrition–fed neonatal piglets (41). In other studies, the use of GSK-3 inhibitors was able to have beneficial or protective effects. For instance, GSK-3 inhibitor limited the development of ischemia/reperfusion injury of the gut (42) and of experimental colitis (43). It was recently reported that the reduction in intestinal epithelial cell proliferation observed during necrotizing enterocolitis may be related to the following cascade: TLR-4/Akt/GSK-3/β-catenin (44). In addition, GSK-3 inhibition induced a reduction in tight junction proteins and an increase in intestinal permeability in vitro (45). All of these data suggest that GSK-3 plays a pivotal role in cell response, and GSK-3 was recently proposed as a promising therapeutic target for selectively reducing exaggerated intestinal immune reactions toward the luminal flora in inflammatory bowel disease (46). The effects of leucine on GSK-3 are thus interesting and should be further assessed under pathophysiologic conditions. Although our data focused only on the duodenal mucosa, because of tissue accessibility in healthy conditions, evaluation of the effects of leucine on the jejunal or ileal mucosa is of interest.

In the current study, we were not able to determine how leucine activates the PI3K/Akt pathway. We hypothesize that β-hydroxy-β-methylbutyrate, a metabolite of leucine, mediates these effects, because β-hydroxy-β-methylbutyrate was previously reported to improve muscle protein anabolism through limitation of proteasome activity (47–49) and to enhance myoblast proliferation through the MAPK and PI3K/Akt pathways (50).

In conclusion, the supplementation of maltodextrins with leucine decreases proteasome activity and possibly intestinal protein synthesis. In addition, leucine may increase intestinal cell proliferation through PI3K/Akt–mediated GSK-3 inactivation. Leucine’s effects should be further evaluated during intestinal diseases.

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