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An enteral leucine supply modulates human duodenal mucosal proteome and decreases the expression of enzymes involved in fatty acid beta-oxidation

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ABSTRACT

Leucine is well known to regulate protein metabolism in muscle. We recently reported that enteral leucine infusion decreased proteasome activity in human duodenal mucosa and enhanced intestinal cell proliferation, but its effects on gut proteome remain unknown. Therefore, we aimed to assess the effects of an enteral leucine infusion on the whole proteome of duodenal mucosa. In this work, 5 healthy volunteers received for 5 h, on 2 occasions and in random order, an enteral supply of maltodextrins (0.25 $g.kg^{-1}.h^{-1}$) or maltodextrins supplemented with leucine (0.035 $g.kg^{-1}.h^{-1}$). At the end of infusion, endoscopic duodenal biopsy samples were collected and analyzed by 2D-PAGE. Eleven protein spots were differentially and significantly ($P < 0.05$) expressed in response to the leucine-supplemented maltodextrins compared with maltodextrins alone. Forty percent of identified proteins by mass spectrometry were located in mitochondria. Four proteins were involved in lipid metabolism: HADHA, ACADVL and CPT2 expressions were reduced, whereas FABP1 expression was increased. In addition, the expression of DHA kinase involved in glycerol metabolism was also downregulated. Finally, leucine supplementation altered the duodenal mucosal proteome by regulating the expression of several enzymes mainly involved in lipid metabolism. These results suggest that leucine supplementation may slowdown fatty acid beta-oxidation in human duodenal mucosa.

KEYWORDS

Enteral nutrition, leucine, duodenum, proteome, fatty acid oxidation.

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INTRODUCTION

Gut barrier plays a major role in the prevention of local and systemic infections as first-line defense against antigens derived from ingested food, xenobiotics, bacteria and viruses. Enhanced intestinal permeability is involved in several chronic inflammatory diseases or in infectious complications [1-3]. Intestinal barrier is partly regulated by a balance between protein synthesis and degradation, and protein fractional synthesis rate (FSR) approached 50% per day in human duodenal mucosa [4]. Previous studies reported that the administration of specific nutrients, i.e. glutamine or arginine, can modulate intestinal protein metabolism [4-9].

Although the effects of leucine are well established in muscle, i.e. stimulation of protein synthesis [10, 11] and reduction of proteasome-mediated proteolysis [12], its effects on intestinal protein metabolism have been poorly documented. In intestinal epithelial cells, leucine has been shown to activate the mammalian target of rapamycin (mTOR) signaling pathway which is involved in the regulation of protein synthesis [13-15]. In neonatal pigs, Murgas Torrazza et al reported that leucine supplementation of a low-protein diets stimulated jejunal mucosal protein synthesis through the activation of the mTOR effectors [16]. A similar study showed that dietary supplementation with 0.27% leucine increased protein synthesis in proximal and distal small intestine but not in the colon of weaning pigs [17]. In contrast, we recently reported that a high dose of leucine affected neither duodenal mucosal protein FSR nor mTOR pathway but decreased proteasome activity in human duodenal mucosa [18]. In addition, leucine enhanced intestinal cell proliferation through the PI3K/Akt/GSK-3α/β-catenin pathway [18]. However, the influence of enteral leucine supplementation on human gut proteome in healthy conditions remains unknown.

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Thus, the aim of this experimental study was to assess the effects of an acute enteral leucine supply on the expression of duodenal mucosal proteins and to elucidate the leucine-modulated metabolic pathways.

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SUBJECTS AND METHODS

Clinical protocol and ethical authorizations

The current study was performed in accordance with the guidelines of the Center for Clinical Investigations, after approval by the local ethics committee (North-west I, France). Five healthy volunteers (3 M, 2 F) gave their written informed consent for this study. The subjects were in good general health and had no hepatic, renal, or cardiac dysfunction or any medical or surgical digestive history. The volunteers had a mean (± SEM) age of 21.9 ± 4.8 y and body mass index (in kg/m²) of 22.2 ± 0.6.

During the three days before the experimental trial, all subjects consumed a controlled diet providing 30 kcal and 0.9 g protein.$kg^{-1}.d^{-1}$. On the morning of the study after an overnight fasting, the subjects received over 5 h, on two 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 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. To avoid a comparison of leucine supply to a fasted status, we compared maltodextrins supplemented with leucine with maltodextrins alone. Thirty minutes after the end of infusion, endoscopic biopsy samples were collected from the duodenal mucosa, immediately snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Protein extraction and 2DE-separation

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Protein extraction and 2DE-separation were performed as previously described [19]. Briefly, endoscopic samples were homogenized in ice-cold lysis buffer containing 7 mol urea/L, 2 mol thiourea/L, 4% (wt:vol) CHAPS, 50 mmol dithiothreitol/L, 25 mmol spermine tetrahydrochloride/L, 0.5% (vol:vol) IPG buffer pH 3-10 NL (GE Healthcare), and a protease inhibitor cocktail (P2714; Sigma Aldrich). Total protein extract (40 μg for silver staining or 400 μg for CBB G-250 staining) was used to rehydrate Immobiline DryStrip gels (pH 3-10 NL 18 cm; GE Healthcare). Proteins were resolved in the first dimension by isoelectric focusing for a total of 50 000 V-h by using the Ettan IPGphor 3 (GE Healthcare). After equilibration of the strips, the second dimension was performed on 8-16% polyacrylamide gradient gels (20 cm x 18 cm x 1mm) and was run on an Ettan Dalt*six* vertical system (GE Healthcare). The 2D gels were silver-stained (PlusOne Silver Staining kit; GE Healthcare) or dyed following the CBB G-250 staining method [19].

Differential analysis of 2D gel images

2D gel images were captured by scanning silver-stained gels with an ImageScanner™ II (GE Healthcare). Differential analysis was performed by using ImageMaster 2D Platinum v5.0 software (GE Healthcare) for spot detection, quantification, matching and comparative analysis. Each tissue sample was subjected to 2DE three times to minimize run-to-run variation, and each set of three gels was compared by using ImageMaster to confirm the nonappearance of statistically differential spots within the set of gels. The most representative gel (gel migration, spot definition, and spot number) of each set was used to test the influence of leucine compared with the control condition. The expression level was determined by the relative volume of each spot in the gel and expressed as %volume, calculated as spot volume/Σvolumes of all spots

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resolved in the gel. Variations in abundance were calculated as the ratio of average values of %volume for a group of spots between the two conditions. Only spots with a %volume variation ratio greater than 1.5 were considered relevant. The corresponding *P* values were determined by using the Student's *t* test (significance level $P < 0.05$) after spot %volume log-transformation.

Protein identification by LC-ESI-MS/MS

The protein spots of interest were excised from CBB-stained 2D gels by using the Ettan Spot Picker (GE Healthcare), and automated in-gel digestion of proteins was performed on the Ettan Digester (GE Healthcare) as previously described [19]. Peptide extracts were then resuspended in 10 μL of 5% (vol:vol) acetonitrile/0.1% (vol:vol) formic acid and then analyzed with a nano-LC1200 system coupled to a 6340 Ion Trap mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies). Briefly, peptides were enriched and desalted on a 40 nL RP-C18 trap column and separated on a Zorbax (30-nm pore size, 5-μm particle size) C18 column (43 mm long x 75 μm inner diameter; Agilent Technologies). A 9-min linear gradient (3%-80% acetonitrile in 0.1% formic acid) at a flow rate of 400 nL/min was used, and the eluent was analyzed with an Ion Trap mass spectrometer as previously described [20].

For protein identification, MS/MS peak lists were extracted and compared with the protein databases by using the MASCOT Daemon version 2.2.2 (Matrix Science) search engine. The searches were performed with the following specific parameters: enzyme specificity, trypsin; one missed cleavage permitted; no fixed modifications; variable modifications, methionine oxidation, cysteine carbamidomethylation, serine, tyrosine and threonine phosphorylation; monoisotopic; peptide charge, 2+ and 3+; mass tolerance for precursor ions, 1.5 Da; mass tolerance for

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fragment ions, 0.6 Da; ESI-TRAP as instrument; taxonomy, human; database, UniProtKB/Swiss-Prot v55.6 (390696 sequences; 140503634 residues). Protein hits were automatically validated if they satisfied one of the following criteria: identification with at least two top ranking peptides (bold and red) each with a MASCOT score of more than 49 ($P < 0.01$), or at least two top ranking peptides (bold and red) each with a MASCOT score of more than 42 ($P < 0.05$). To evaluate false-positive rates, all the initial database searches were performed using the "decoy" option of MASCOT. Results were considered relevant if the false-positive rate never exceeded 1%.

Western blotting analysis

Total protein extracts (20 μg) were resolved in 4-20% SDS-PAGE gels and electrotransferred onto nitrocellulose membrane (GE Healthcare) as previously described [19]. After transfer, membranes were soaked in TBS-T solution [10 mmol Tris-HCl/L pH 7.4, 150 mmol NaCl/L, 0.2% (vol:vol) Tween 20] with 3% (wt:vol) BSA for 2 h at room temperature. Blots were washed three times for 10 min with TBS-T and subsequently incubated overnight at 4°C in TBS-T and 3% (wt:vol) BSA with various specific primary antibodies (Santa Cruz Biotechnology): rabbit polyclonal antibodies anti-CPT2 (sc-130730, 1:200) or anti-OTC (sc-102051, 1:200); goat polyclonal antibodies anti-ACADVL (sc-74900, 1:2000), anti-DHA kinase (sc-161516, 1:2000), anti-FABP1 (sc-16064, 1:1500), anti-NDRG1 (sc-19464, 1:500) or anti-HADHA (sc-82185, 1:1500). Membranes were washed three times for 10 min with TBS-T, incubated with horseradish peroxidase-conjugated donkey anti-goat (sc-2020, 1:5000; Santa Cruz Biotechnology) or swine anti-rabbit (P0399, 1:5000; Dako) IgG for 1 h at room temperature, and then washed four times in TBS-T. Immunocomplexes were revealed by using

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the ECL detection system (GE Healthcare). Protein bands were quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare). The blots were reprobed with a mouse anti-β-actin monoclonal antibody (A5441; 1:5000; Sigma Aldrich) as a loading control.

Gene Ontology

eGOnv2.0 software was used to annotate proteins with Gene Ontology terms. eGOn used a two-sided Fisher's exact test to determine whether any GO categories were significantly enriched in proteins that had been identified as being altered by leucine supplementation ($p < 0.05$).

Statistical analysis

The results are expressed as mean ± SEMs and were compared by using GraphPad Prism 5.0 (GraphPad Software Inc). To evaluate the effects of leucine, statistical analysis were assessed using non parametric Wilcoxon test or Student's *t* test for paired data. For all, $P < 0.05$ was considered significant.

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RESULTS

Leucine infusion modified human duodenal mucosal proteome

In both groups, approximately 2250 protein spots per gel were detected within a p*I* range of 3 to10 and a relative molecular mass range of 10-180 kDa (**Figure 1)**. Eleven protein spots were differentially (*i.e.* at least ± 1.5 fold modulated) and significantly (Student's *t* test, $P < 0.05$) regulated in response to enteral leucine supplementation (Figure 1): the expression of 7 protein spots was reduced, whereas the expression of 4 other protein spots was increased. These protein spots were analyzed by using liquid chromatography coupled with electron spray ionization MS/MS, and 10 spots were identified (**Table 1**), whereas spot numbered 10 remained undetermined. Among the identified proteins, 7 proteins were downregulated after enteral leucine supplementation (Table 1): Trifunctional enzyme subunit alpha (HADHA, spot 1, **Figure 2**), Carnitine O-palmitoyltransferase 2 (CPT2, spot 2, Figure 2), Acyl-Coenzyme A dehydrogenase very long-chain (ACADVL, spot 3, Figure 2), ATP-dependant dihydroxyacetone kinase (DHA kinase, spot 4, Figure 2), N-myc downstream-regulated gene 1 protein (NDRG1, spot 5), Ornithine carbamoyltransferase (OTC, spot 7), and Sulfotransferase 1A1 (SULT1A1, spot 8). Three proteins were upregulated after enteral leucine supplementation (Table 1): gastricsin precursor (PGC, spot 6), carbonic anhydrase 1 (CA1, spot 9), and Fatty acid-binding protein liver (FABP1, spot 11).

To further support protein identification and substantiate their semiquantitative expression levels under control and leucine conditions, antibodies against ACADVL, CPT2, DHA kinase, FABP1, HADHA, NDRG1 and OTC were used for western blotting after

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separation of duodenal mucosal biopsy extracts by SDS-PAGE. These data confirmed the results given by 2D gels and liquid chromatography-tandem mass spectrometry (**Figure 3**).

Biological significance of the leucine-modulated proteins

Using the gene ontology tool eGOn v2.0 [21] and UniProt, all the proteins identified by mass spectrometry were assigned to different categories. Each protein was categorized based on molecular function, biological process and cellular component. When grouped according to molecular function, most of the identified proteins (8 proteins) were involved in catalytic processes, and 7 proteins were binding proteins. Classification based on biological process revealed that 4 leucine-modulated proteins were implicated in lipid metabolic process: HADHA, CPT2 and ACADVL occur notably in the fatty acid beta-oxidation whereas FABP1 plays a role in intracellular lipid transport. Other identified proteins were involved in glycerol and xenobiotics metabolism, cell growth and differentiation, proteolysis, urea cycle, and CO_2 transport (Table 1). Classification based on cellular component revealed that 4 proteins were located in mitochondria: HADHA, CPT2, ACADVL and OTC.

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DISCUSSION

In the current study, we showed that supplementation of maltodextrins with leucine altered the duodenal mucosal proteome by regulating the expression of several enzymes mainly involved in the metabolism of lipids and glycerol (**Figure 4**).

Leucine supplementation significantly decreased CPT2, ACADVL and HADHA expression in human duodenal mucosa suggesting that leucine may impair intestinal fatty acid oxidation (FAO). Intestinal FAO is a key metabolic pathway that contributes to energy homeostasis for intestinal cells but also to organism metabolic homeostasis. Indeed, even if fatty acids may be a source of energy for human enterocytes [22-24], the regulation of intestinal FAO participates to organism response to food ingestion. Interestingly, the capacity of lipid catabolism in the small intestine was higher in obesity-resistant mice [25] and the stimulation of intestinal FAO after ingestion of either α-linolenic acid rich diacylglycerols [26] or fish oil [27] was associated with body weight loss.

We speculate that the regulation of intestinal FAO by leucine may be a key issue to better understand the effects of leucine on whole body. By limiting intestinal FAO, leucine may contribute to increase postprandial lipidemia and thus fatty acid disposal for peripheral tissues. Leucine supplementation has been proposed as a potential therapeutic strategy to prevent obesity [28] or sarcopenia of aging [29, 30]. Nevertheless, contradictory data were reported concerning the effect of long-term supplementation with leucine on body composition. Indeed, long-term leucine supplementation decreased fat mass in high fat diet-induced obese mice [28, 31] and in aging rats [32]. Nairizi et al. showed that leucine supplementation of drinking water did not alter body composition or adiposity of mice fed with a high-fat diet [33]. Similarly, 3 months of

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leucine supplementation affected neither fat mass nor skeletal muscle mass in healthy elderly men [34]. More recently, Zeanandin et al reported that long-term dietary leucine excess promoted hypertrophy and hyperplasia of adipose tissue in old rats, whereas skeletal muscle mass was not changed [35]. In this latter study, perirenal adipose tissue weight was increased by 45%.

To our knowledge, our study shows for the first time that leucine may limit intestinal FAO in humans and suggests that leucine may differentially affect fatty acid metabolism in different tissues. Indeed, in NCI-H716 intestinal cells, leucine downregulated the expression of genes involved in the absorption or synthesis of fatty acids or cholesterol [36]. In contrast, long-term leucine supplementation stimulated the expression of key genes involved in fatty acid metabolism in skeletal muscle of obese mice associated with increased energy expenditure [37]. In the same way, leucine enhanced FAO in C2C12 muscle cells while it reduced fatty acid synthase gene expression in 3T3-L1 adipocytes [38]. It was also reported that lipolysis and expression of β-oxidation genes were significantly increased in white adipose tissue of C57BL/6J mice fed for 7 days with leucine-deficient diet [39]. However, in the present study, we only reported the effects of leucine on the expression of enzymes involved in FAO but we did not assess their activities. Further studies should evaluate intestinal FAO in leucine-supplemented subjects.

In the present study, leucine supplementation also reduced the expression of NDRG1 in human duodenal mucosa (Figure 3). NDRG1, which is strongly expressed in the human small intestine and epithelial cells [40, 41], is involved in stress responses [42, 43], apoptosis [44], allergic responses [45], cell growth and differentiation [46]. In mouse embryonic fibroblast cells, mRNA level of NDRG1 was up-regulated in response to leucine starvation [47]. We recently

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showed that enteral leucine supplementation enhanced intestinal cell proliferation through the PI3K/Akt/GSK-3α/β-catenin pathway in healthy humans [18]. The decrease of NDRG1 observed in the present study may also contribute to the enhancement of cell proliferation. Zhang et al reported that polyamine depletion increased the expression of NDRG1 and finally inhibited intestinal epithelial cell proliferation [48]. In our study, we can speculate that polyamine synthesis in intestinal cells may be increased after leucine supplementation since we observed a reduction of OTC expression (Figure 3), ornithine carbamoyltransferase that catalyzes the conversion of ornithine into citrulline. Consequently to the decrease of OTC, ornithine may be either used for polyamine synthesis or exported to portal vein. Accordingly, Mouillé et al previously showed a decrease of ornithine utilization for citrulline synthesis in rat colonocytes after hyperproteic diet [49]. It is well established that polyamines regulate intestinal cell proliferation [50]. All these data suggest that the decreased expressions of NDRG1 and OTC induced by leucine in the present study may contribute to the enhancement of intestinal cell proliferation, previously reported after leucine supplementation [18].

In conclusion, leucine supplementation altered the expression of several duodenal mucosal proteins, which are mainly involved in lipid metabolism. Leucine supplementation may slowdown fatty acid β-oxidation in duodenal mucosa. Further investigations are needed to evaluate whether reduction of intestinal fatty acid β-oxidation by leucine may contribute to regulate whole body metabolic homeostasis and body composition.

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The funders of this study did not interfere in the design, implementation, analysis, or interpretation of the data. None of the authors declared a conflict of interest.

ABBREVIATIONS

ACADVL, Acyl-Coenzyme A dehydrogenase very long-chain; CA1, Carbonic anhydrase 1; CPT2, Carnitine O-palmitoyltransferase 2; DHA kinase, ATP-dependant dihydroxyacetone kinase; FABP1, Fatty acid-binding protein liver; FAO, fatty acid oxidation; HADHA, Trifunctional enzyme subunit alpha; mTOR, mammalian target of rapamycin; NDRG1, N-myc downstream-regulated gene 1 protein; OTC, Ornithine carbamoyltransferase; PGC, Gastricsin precursor; SULT1A1, Sulfotransferase 1A1.

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TABLE:

Table 1. Human duodenal mucosal proteome changes in response to leucine infusion[1]

Spot number	Swiss-Prot accession number	Protein name	Biological process	Theoretical		Fold change[2]	Score on MASCOT	Sequence coverage (%)	Peptide hit
				p*I*	*M*r (kDa)				
1	P40939	Trifunctional enzyme subunit alpha (HADHA)	Lipid metabolism	9.16	82.9	−1.63	82	4	3
2	P23786	Carnitine O-palmitoyltransferase 2 (CPT2)	Lipid metabolism	8.38	73.7	−1.75	166	8	4
3	P49748	Acyl-Coenzyme A dehydrogenase very long-chain (ACADVL)	Lipid metabolism	8.92	70.3	−1.76	132	5	3
4	Q3LXA3	ATP-dependant dihydroxyacetone kinase (DHA Kinase)	Glycerol metabolism	7.12	58.9	−1.51	321	25	10
5	Q92597	Protein NDRG1	Cell growth	5.49	42.8	−1.51	159	11	3
6	P20142	Gastricsin precursor (PGC)	Proteolysis	4.36	42.4	+1.98	103	3	2
7	P00480	Ornithine carbamoyltransferase (OTC)	Urea cycle	8.75	39.9	−1.52	282	24	10
8	P50225	Sulfotransferase 1A1 (SULT1A1)	Xenobiotics metabolism	6.16	34.2	−1.57	66	9	3
9	P00915	Carbonic anhydrase 1 (CA1)	CO_2 transport	6.59	28.9	+1.98	195	24	6
10	-	Undetermined	-	-	-	+1.56	-	-	-
11	P07148	Fatty acid-binding protein liver (FABP1)	Lipid metabolism	6.60	14.2	+1.88	86	25	2

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[1] MASCOT, algorithm that uses mass spectrometric data to identify proteins from primary sequence databases; *Mr*, molar mass; p*I*, isoelectric point.

[2] +, upregulated; ⁻, downregulated.

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FIGURE LEGENDS:

Figure 1. Silver-stained 2-dimensional gel images representing total proteins extracted from human duodenal mucosal biopsy samples after maltodextrins (A) and leucine-supplemented maltodextrins (B) enteral perfusion. Differentially expressed proteins (ie, at least ±1.5 fold modulated; circled spots with a number) were determined by statistical analysis (Paired Student's *t* test, $P < 0.05$) and correspond to the samples analyzed by liquid chromatography-tandem mass spectrometry. Protein identification results are depicted in Table 1.

Figure 2. Effects of leucine supplementation on the expression of four duodenal mucosal proteins involved in fatty acid or glycerol metabolism. A: Representative enlargements of silver-stained 2-dimensional gels issued from healthy subjects infused enterally with maltodextrins (Control) or leucine-supplemented maltodextrins (Leucine) showing 4 differentially expressed proteins. The circles represent the expression of HADHA (spot 1), CPT2 (spot 2), ACADVL (spot 3) and DHA Kinase (spot 4) in control and leucine conditions, respectively. These proteins were downregulated after 5 h of enteral leucine supplementation. B: HADHA, CPT2, ACADVL and DHA kinase expression levels are shown for the control and leucine groups. The display values for graphs are means ± SEMs; $n = 5$. *Significantly different from control group, $P < 0.05$ (Paired Student's *t* test).

Figure 3. Effects of leucine supplementation on ACADVL, CPT2, DHA kinase, FABP1, HADHA, NDRG1 and OTC **expression in human duodenal mucosa.** Representative

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immunoblots (A) and densitometric analysis of ACADVL, CPT2, DHA kinase, FABP1, HADHA, NDRG1 and OTC expression (B) in duodenal mucosa of healthy volunteers after 5 h of enteral maltodextrins alone (control) or with leucine supplementation. Values are means ± SEMs; (n = 5). *Significantly different from control group, $P < 0.05$ (Paired Student's t test). AU, arbitrary units.

Figure 4. Role of five relevant leucine-modulated proteins in lipid and glycerol metabolism. DHA Kinase plays a critical role in glycerol metabolism by converting glycerol to glycerol 3-phosphate in an ATP dependent reaction. HADHA, CPT2 and ACADVL are involved in the fatty acid beta-oxidation process whereas FABP1 plays a role in intracellular fatty acid transport. Upregulated and downregulated proteins are indicated with + and –, respectively. Their intervention levels in lipid or glycerol metabolism are indicated with arrows. ACADVL, Acyl-Coenzyme A dehydrogenase very long-chain; CPT2, Carnitine O-palmitoyltransferase 2; DHA kinase, ATP-dependant dihydroxyacetone kinase; FABP1, Fatty acid-binding protein liver; HADHA, Trifunctional enzyme subunit alpha.

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FIGURE LEGENDS:

Figure 1

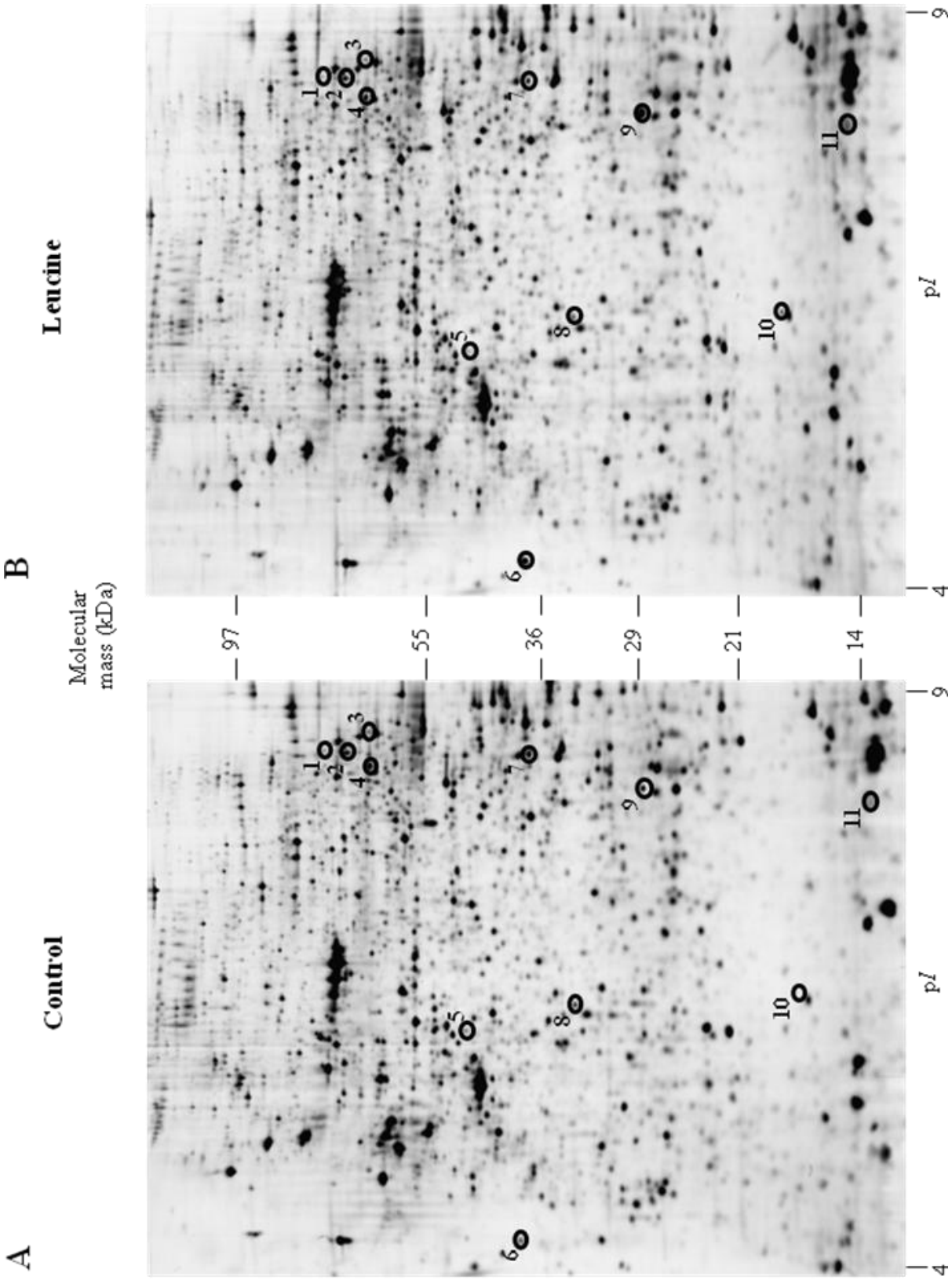

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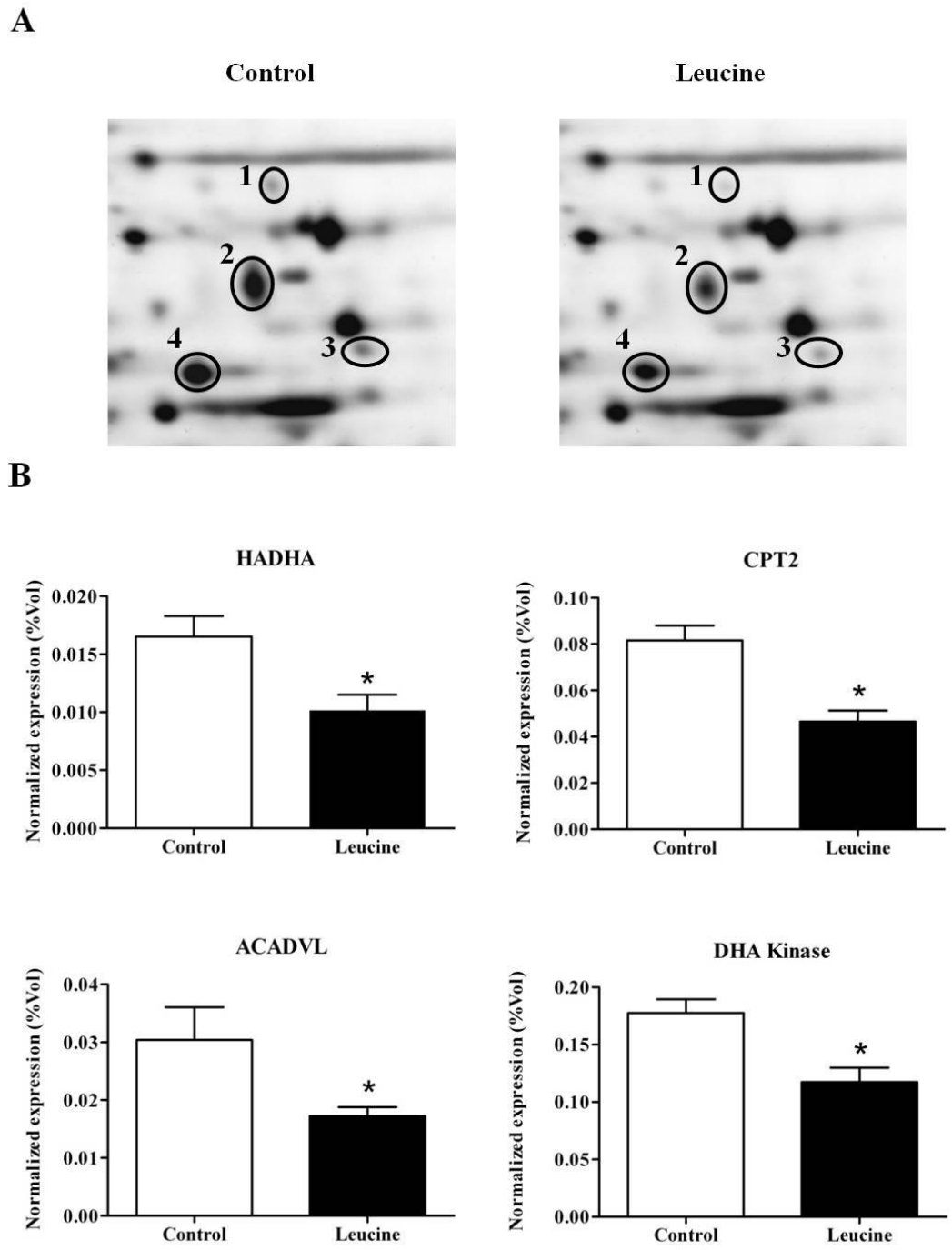
Figure 2



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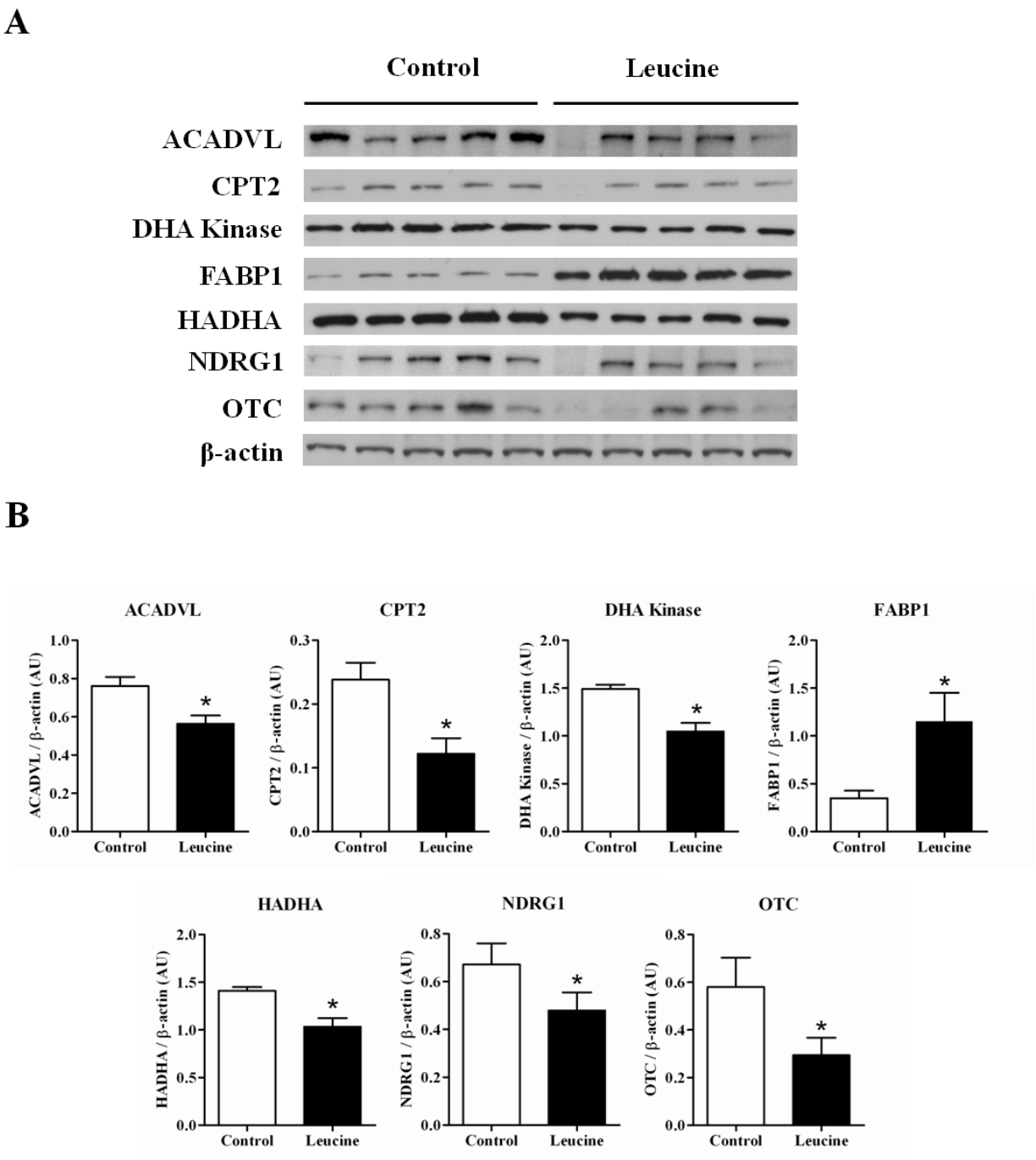

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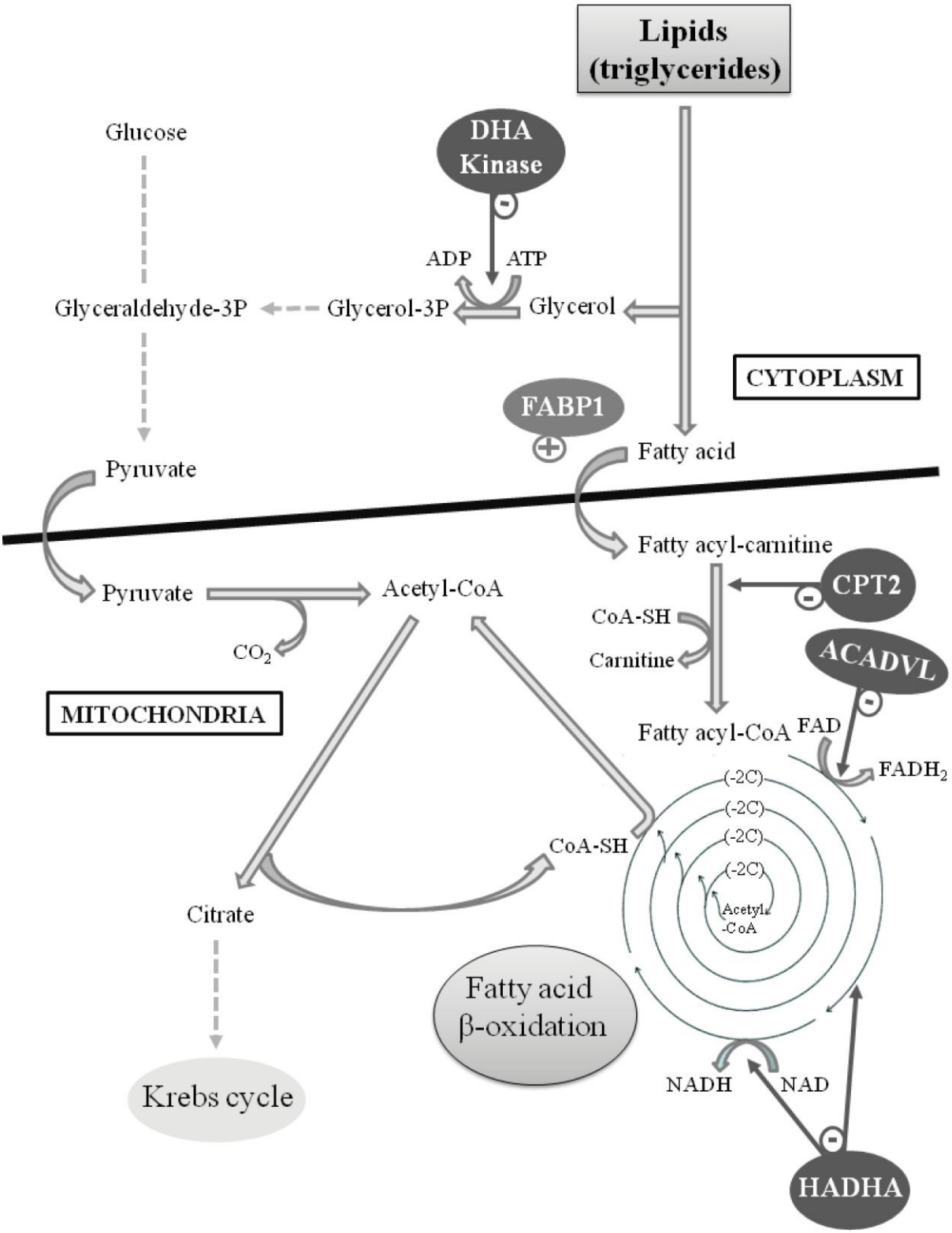
Figure 4



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