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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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1 **ABSTRACT**

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

18

19

20 **KEYWORDS**

21

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

23

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24 INTRODUCTION

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

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

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

49

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50 **SUBJECTS AND METHODS**

51

52 *Clinical protocol and ethical authorizations*

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

59 During the three days before the experimental trial, all subjects consumed a controlled
60 diet providing 30 kcal and 0.9 g protein. $\text{kg}^{-1}.\text{d}^{-1}$. On the morning of the study after an overnight
61 fasting, the subjects received over 5 h, on two occasions, and in a random order by a nasogastric
62 feeding tube either maltodextrins (control condition: $0.25 \text{ g}.\text{kg}^{-1}.\text{h}^{-1}$; Lactalis Nutrition Santé,
63 Torcé, France) or maltodextrins plus leucine (leucine condition: $0.035 \text{ g free L-leucine}.\text{kg}^{-1}.\text{h}^{-1}$;
64 Cooper, Melun, France). The infusion rate was $3.5 \text{ mL}.\text{kg}^{-1}.\text{h}^{-1}$. The dose of leucine supplied was
65 chosen as a compromise between the dose used in former studies on muscle proteins and a higher
66 dose needed to affect the very high protein turnover rate of the intestinal mucosa. To avoid a
67 comparison of leucine supply to a fasted status, we compared maltodextrins supplemented with
68 leucine with maltodextrins alone. Thirty minutes after the end of infusion, endoscopic biopsy
69 samples were collected from the duodenal mucosa, immediately snap-frozen in liquid nitrogen,
70 and stored at -80°C until analysis.

71

72 *Protein extraction and 2DE-separation*

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

85

86 *Differential analysis of 2D gel images*

87 2D gel images were captured by scanning silver-stained gels with an ImageScannerTM II
88 (GE Healthcare). Differential analysis was performed by using ImageMaster 2D Platinum v5.0
89 software (GE Healthcare) for spot detection, quantification, matching and comparative analysis.
90 Each tissue sample was subjected to 2DE three times to minimize run-to-run variation, and each
91 set of three gels was compared by using ImageMaster to confirm the nonappearance of
92 statistically differential spots within the set of gels. The most representative gel (gel migration,
93 spot definition, and spot number) of each set was used to test the influence of leucine compared
94 with the control condition. The expression level was determined by the relative volume of each
95 spot in the gel and expressed as %volume, calculated as spot volume/ Σ volumes of all spots

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96 resolved in the gel. Variations in abundance were calculated as the ratio of average values of
97 % volume for a group of spots between the two conditions. Only spots with a % volume variation
98 ratio greater than 1.5 were considered relevant. The corresponding *P* values were determined by
99 using the Student's *t* test (significance level $P < 0.05$) after spot % volume log-transformation.

100

101 ***Protein identification by LC-ESI-MS/MS***

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

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

119 fragment ions, 0.6 Da; ESI-TRAP as instrument; taxonomy, human; database, UniProtKB/Swiss-
120 Prot v55.6 (390696 sequences; 140503634 residues). Protein hits were automatically validated if
121 they satisfied one of the following criteria: identification with at least two top ranking peptides
122 (bold and red) each with a MASCOT score of more than 49 ($P < 0.01$), or at least two top
123 ranking peptides (bold and red) each with a MASCOT score of more than 42 ($P < 0.05$). To
124 evaluate false-positive rates, all the initial database searches were performed using the “decoy”
125 option of MASCOT. Results were considered relevant if the false-positive rate never exceeded
126 1%.

127

128 ***Western blotting analysis***

129 Total protein extracts (20 μg) were resolved in 4-20% SDS-PAGE gels and
130 electrotransferred onto nitrocellulose membrane (GE Healthcare) as previously described [19].
131 After transfer, membranes were soaked in TBS-T solution [10 mmol Tris-HCl/L pH 7.4, 150
132 mmol NaCl/L, 0.2% (vol:vol) Tween 20] with 3% (wt:vol) **BSA** for 2 h at room temperature.
133 Blots were washed three times for 10 min with TBS-T and subsequently incubated overnight at
134 4°C in TBS-T and 3% (wt:vol) **BSA** with various specific primary antibodies (Santa Cruz
135 Biotechnology): rabbit polyclonal antibodies anti-CPT2 (sc-130730, 1:200) or anti-OTC (sc-
136 102051, 1:200); goat polyclonal antibodies anti-ACADVL (sc-74900, 1:2000), anti-DHA kinase
137 (sc-161516, 1:2000), anti-FABP1 (sc-16064, 1:1500), anti-NDRG1 (sc-19464, 1:500) or anti-
138 HADHA (sc-82185, 1:1500). Membranes were washed three times for 10 min with TBS-T,
139 incubated with horseradish peroxidase-conjugated donkey anti-goat (sc-2020, 1:5000; Santa
140 Cruz Biotechnology) or swine anti-rabbit (P0399, 1:5000; Dako) IgG for 1 h at room
141 temperature, and then washed four times in TBS-T. Immunocomplexes were revealed by using

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

145

146 *Gene Ontology*

147 eGOnv2.0 software was used to annotate proteins with Gene Ontology terms. eGOn used
148 a two-sided Fisher's exact test to determine whether any GO categories were significantly
149 enriched in proteins that had been identified as being altered by leucine supplementation ($p <$
150 0.05).

151

152 *Statistical analysis*

153 The results are expressed as mean \pm SEMs and were compared by using GraphPad Prism
154 5.0 (GraphPad Software Inc). To evaluate the effects of leucine, statistical analysis were assessed
155 using non parametric Wilcoxon test or Student's t test for paired data. For all, $P < 0.05$ was
156 considered significant.

157

158 **RESULTS**

159

160 *Leucine infusion modified human duodenal mucosal proteome*

161 In both groups, approximately 2250 protein spots per gel were detected within a *pI* range
162 of 3 to 10 and a relative molecular mass range of 10-180 kDa (**Figure 1**). Eleven protein spots
163 were differentially (*i.e.* at least ± 1.5 fold modulated) and significantly (Student's *t* test, $P < 0.05$)
164 regulated in response to enteral leucine supplementation (Figure 1): the expression of 7 protein
165 spots was reduced, whereas the expression of 4 other protein spots was increased. These protein
166 spots were analyzed by using liquid chromatography coupled with electron spray ionization
167 MS/MS, and 10 spots were identified (**Table 1**), whereas spot numbered 10 remained
168 undetermined. Among the identified proteins, 7 proteins were downregulated after enteral
169 leucine supplementation (Table 1): Trifunctional enzyme subunit alpha (HADHA, spot 1, **Figure**
170 **2**), Carnitine O-palmitoyltransferase 2 (CPT2, spot 2, Figure 2), Acyl-Coenzyme A
171 dehydrogenase very long-chain (ACADVL, spot 3, Figure 2), ATP-dependant dihydroxyacetone
172 kinase (DHA kinase, spot 4, Figure 2), N-myc downstream-regulated gene 1 protein (NDRG1,
173 spot 5), Ornithine carbamoyltransferase (OTC, spot 7), and Sulfotransferase 1A1 (SULT1A1,
174 spot 8). Three proteins were upregulated after enteral leucine supplementation (Table 1):
175 gastricsin precursor (PGC, spot 6), carbonic anhydrase 1 (CA1, spot 9), and Fatty acid-binding
176 protein liver (FABP1, spot 11).

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

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

182

183 ***Biological significance of the leucine-modulated proteins***

184 Using the gene ontology tool eGOn v2.0 [21] and UniProt, all the proteins identified by
185 mass spectrometry were assigned to different categories. Each protein was categorized based on
186 molecular function, biological process and cellular component. When grouped according to
187 molecular function, most of the identified proteins (8 proteins) were involved in catalytic
188 processes, and 7 proteins were binding proteins. Classification based on biological process
189 revealed that 4 leucine-modulated proteins were implicated in lipid metabolic process: HADHA,
190 CPT2 and ACADVL occur notably in the fatty acid beta-oxidation whereas FABP1 plays a role
191 in intracellular lipid transport. Other identified proteins were involved in glycerol and
192 xenobiotics metabolism, cell growth and differentiation, proteolysis, urea cycle, and CO₂
193 transport (Table 1). Classification based on cellular component revealed that 4 proteins were
194 located in mitochondria: HADHA, CPT2, ACADVL and OTC.

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195 **DISCUSSION**

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

199
200 Leucine supplementation significantly decreased CPT2, ACADVL and HADHA
201 expression in human duodenal mucosa suggesting that leucine may impair intestinal fatty acid
202 oxidation (FAO). Intestinal FAO is a key metabolic pathway that contributes to energy
203 homeostasis for intestinal cells but also to organism metabolic homeostasis. Indeed, even if fatty
204 acids may be a source of energy for human enterocytes [22-24], the regulation of intestinal FAO
205 participates to organism response to food ingestion. Interestingly, the capacity of lipid catabolism
206 in the small intestine was higher in obesity-resistant mice [25] and the stimulation of intestinal
207 FAO after ingestion of either α -linolenic acid rich diacylglycerols [26] or fish oil [27] was
208 associated with body weight loss.

209 We speculate that the regulation of intestinal FAO by leucine may be a key issue to better
210 understand the effects of leucine on whole body. By limiting intestinal FAO, leucine may
211 contribute to increase postprandial lipidemia and thus fatty acid disposal for peripheral tissues.
212 Leucine supplementation has been proposed as a potential therapeutic strategy to prevent obesity
213 [28] or sarcopenia of aging [29, 30]. Nevertheless, contradictory data were reported concerning
214 the effect of long-term supplementation with leucine on body composition. Indeed, long-term
215 leucine supplementation decreased fat mass in high fat diet-induced obese mice [28, 31] and in
216 aging rats [32]. Nairizi et al. showed that leucine supplementation of drinking water did not alter
217 body composition or adiposity of mice fed with a high-fat diet [33]. Similarly, 3 months of

218 leucine supplementation affected neither fat mass nor skeletal muscle mass in healthy elderly
219 men [34]. More recently, Zeanandin et al reported that long-term dietary leucine excess
220 promoted hypertrophy and hyperplasia of adipose tissue in old rats, whereas skeletal muscle
221 mass was not changed [35]. In this latter study, perirenal adipose tissue weight was increased by
222 45%.

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

236
237 In the present study, leucine supplementation also reduced the expression of NDRG1 in
238 human duodenal mucosa (Figure 3). NDRG1, which is strongly expressed in the human small
239 intestine and epithelial cells [40, 41], is involved in stress responses [42, 43], apoptosis [44],
240 allergic responses [45], cell growth and differentiation [46]. In mouse embryonic fibroblast cells,
241 mRNA level of NDRG1 was up-regulated in response to leucine starvation [47]. We recently

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

256

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

262

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264

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271 interpretation of the data. None of the authors declared a conflict of interest.

272

273 **ABBREVIATIONS**

274

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

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TABLE:**Table 1. Human duodenal mucosal proteome changes in response to leucine infusion¹**

Spot number	Swiss-Prot accession number	Protein name	Biological process	Theoretical		Fold change ²	Score on MASCOT	Sequence coverage (%)	Peptide hit
				<i>pI</i>	<i>Mr</i> (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; *pI*, 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 \pm 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 \pm 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.

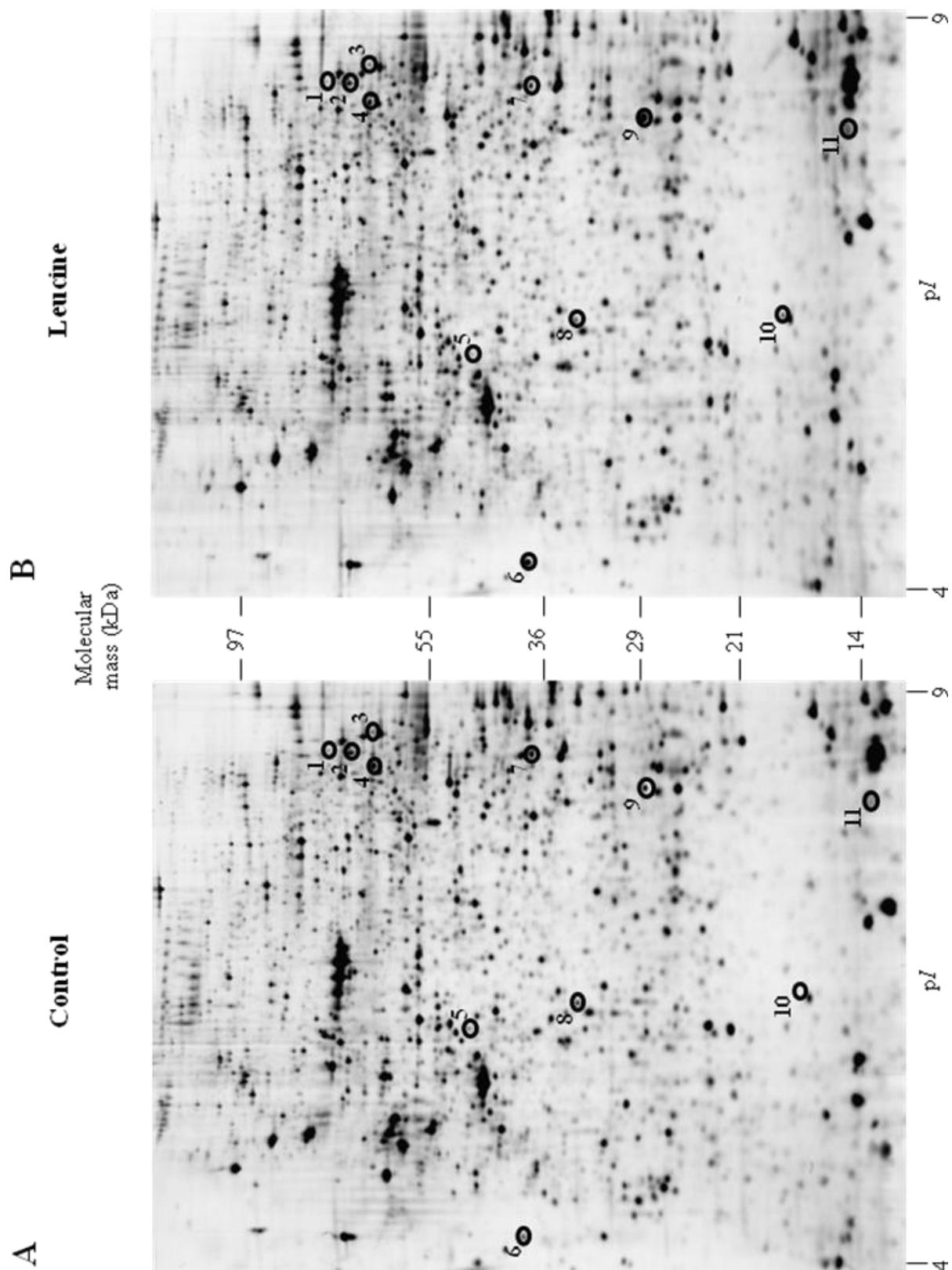
DHA Kinase plays a critical role in glycerol metabolism by converting glycerol to glycerol 3-phosphate in an ATP dependent reaction. HADHA, CPT2 and ACADVL are involved in the fatty acid beta-oxidation process whereas FABP1 plays a role in intracellular fatty acid transport. Upregulated and downregulated 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

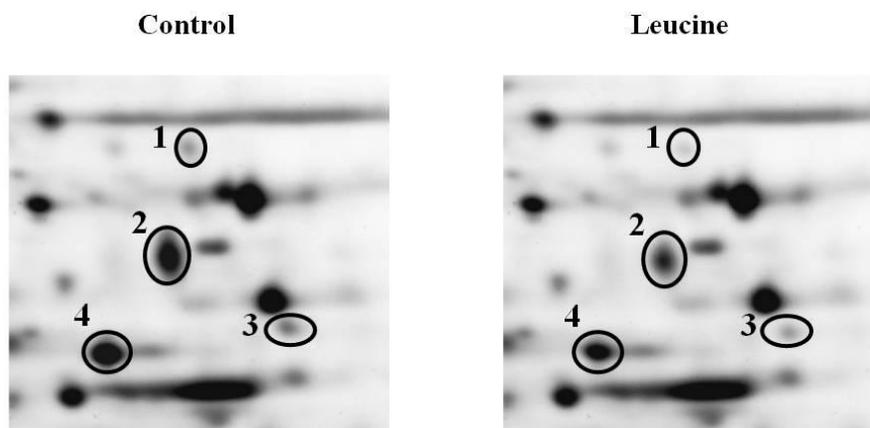


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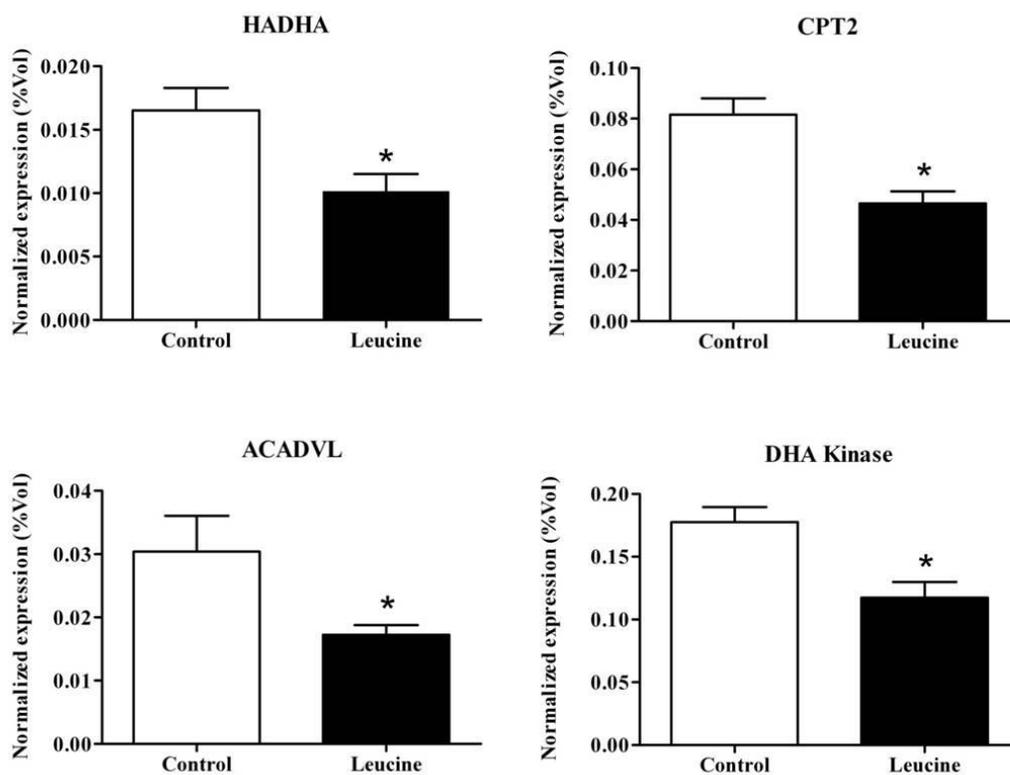
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Figure 2

A



B

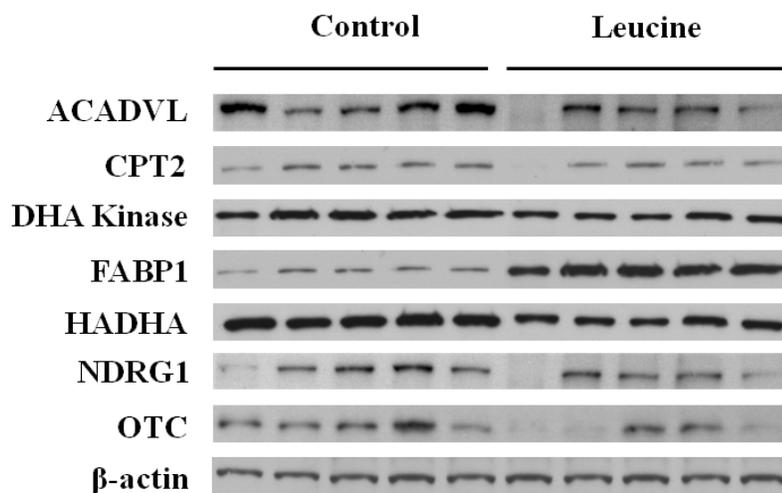


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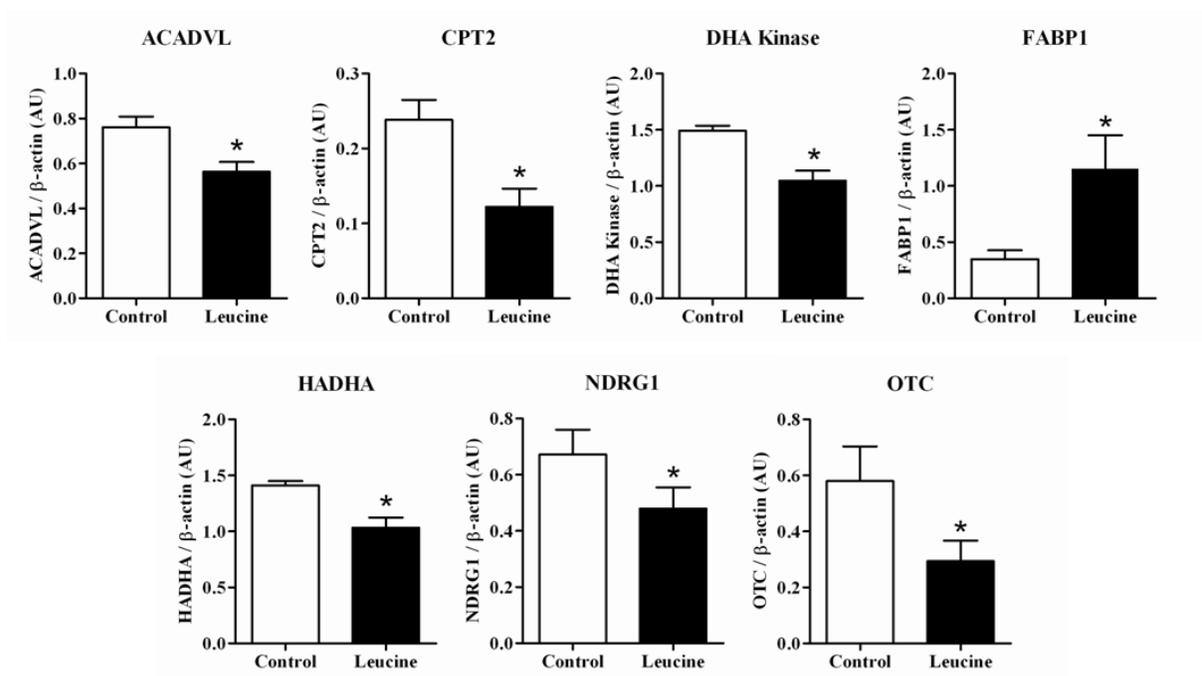
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Figure 3

A



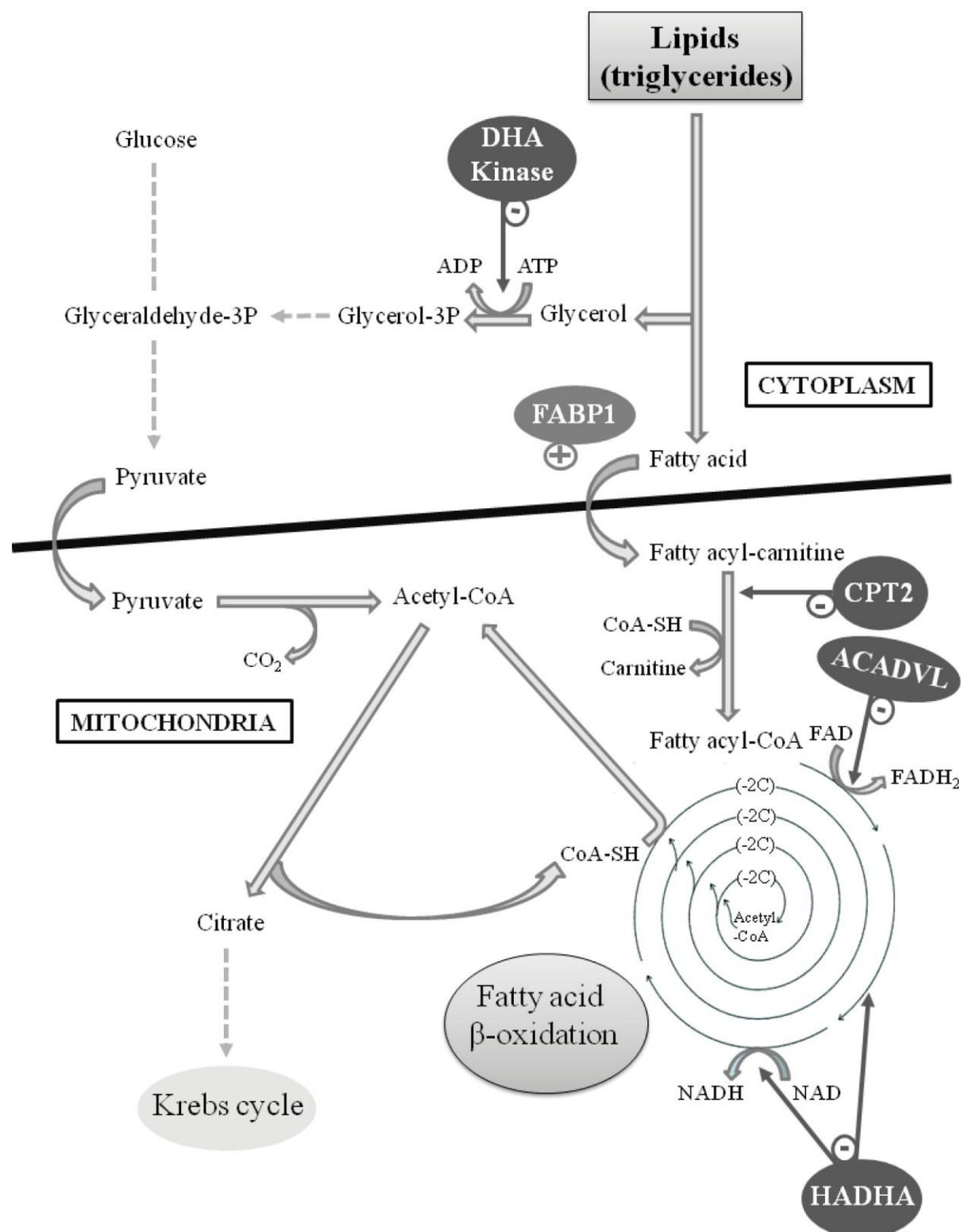
B



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Figure 4



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