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3 **Role of fatty acid transport protein 4 in oleic acid-induced glucagon-like peptide-1**
4 **secretion from murine intestinal L cells**
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Abstract

The anti-diabetic intestinal L cell hormone, glucagon-like peptide-1 (GLP-1), enhances glucose-dependent insulin secretion and inhibits gastric emptying. GLP-1 secretion is stimulated by luminal oleic acid (OA), which crosses the cell membrane by an unknown mechanism. We hypothesized that L cell fatty acid transport proteins (FATPs) are essential for OA-induced GLP-1 release. The murine GLUTag L cell model was therefore used for immunoblotting, ^{3}H-OA uptake assay and GLP-1 secretion assay as determined by radioimmunoassay following treatment with OA plus/minus phloretin, sulfo-N-succinimidyl oleate or siRNA against FATP4. FATP4$^{-/-}$ and Cluster-of-Differentiation (CD36)$^{-/-}$ mice received intraileal OA and plasma GLP-1 was measured by sandwich-immunoassay. GLUTag cells were found to express CD36, FATP1, FATP3 and FATP4. The cells demonstrated specific ^{3}H-OA uptake which was dose-dependently inhibited by 500 and 1000 μM unlabeled OA ($P<0.001$). Cell viability was not altered by treatment with OA. Phloretin and sulfo-N-succinimidyl oleate, inhibitors of protein-mediated transport and CD36, respectively, also decreased ^{3}H-OA uptake, as did knockdown of FATP4 by siRNA transfection ($P<0.05$-0.001). OA dose-dependently increased GLP-1 secretion at 500 and 1000 μM ($P<0.001$), while phloretin, sulfo-N-succinimidyl oleate and FATP4 knockdown decreased this response ($P<0.05$-0.01). FATP4$^{-/-}$ mice displayed lower plasma GLP-1 at 60 min in response to intra-ileal OA ($P<0.05$) while, unexpectedly, CD36$^{-/-}$ mice displayed higher basal GLP-1 levels ($P<0.01$) but a normal response to intra-ileal OA. Together, these findings demonstrate a key role for FATP4 in OA-induced GLP-1 secretion from the murine L cell in vitro and in vivo, while the precise role of CD36 remains unclear.

Key words: carrier-mediated transport, CD36, fatty acid, FATP4, GLP-1, oleic acid, secretion

Abbreviations: CD36, Cluster-of-Differentiation 36; FATP, fatty acid transport protein; GLP-1, glucagon-like peptide-1; GPR, G protein-coupled receptor; MUFA, monounsaturated fatty acid; OA, oleic acid; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering ribonucleic acid; SSO, sulfo-N-succinimidyl oleate

69 **Introduction**

70 The intestinal L cell hormone, glucagon-like peptide-1 (GLP-1), is secreted following
71 nutrient ingestion, leading to glucose-dependent insulin release as well as inhibition of gastric
72 emptying, glucagon secretion and food intake (1; 39; 46; 48). The anti-diabetic properties of this
73 hormone have led to the use of both GLP-1 mimetics and GLP-1 degradation inhibitors in the
74 clinic to treat patients with type 2 diabetes (33). Although nutrients such as sugars and peptones
75 are known to stimulate L cell secretion (15; 20; 35), fats in particular are potent GLP-1
76 secretagogues (10; 18; 21; 23; 24). Furthermore, only fats appear to transit the intestine to the
77 ileum (3; 24; 28), which has the highest density of L cells in the intestine (11).

78 Monounsaturated fatty acids (MUFAs), such as oleic acid (OA) are known to induce
79 beneficial metabolic effects, and arguments have therefore been made in favor of the
80 Mediterranean diet, which is enriched in OA-containing olive oil (14). Indeed, insulin-resistant
81 patients placed on a diet enriched in MUFAs display increased plasma GLP-1 levels and
82 improved glycaemic control (34). A study in rats has also linked dietary OA to increased GLP-1
83 secretion and GLP-1-dependent improvements in glycemic tolerance (37). Furthermore, OA has
84 been demonstrated to directly increase GLP-1 secretion from the intestinal L cell, as observed in
85 the murine GLUTag (23; 37), human NCI-H716 (35), and primary foetal rat intestinal culture
86 (23) L cell models.

87 Several G protein-coupled receptors (GPRs), such as GPR40, GRP120 and GPR119,
88 have been implicated as fatty acid receptors on the L cell, responding to saturated fatty acids,
89 polyunsaturated fatty acids and the fatty acid derivative, oleoylethanolamide, respectively (10;
90 21; 26). In contrast, OA is known to increase GLP-1 secretion through a mechanism that is
91 dependent on the atypical isozyme, protein kinase C (PKC) ζ (23; 24). While OA can directly

activate this enzyme *in vitro* (31), whether and, if so, how it crosses the plasma membrane to permit direct interaction with PKCζ in the intestinal L cell is currently unknown.

Although the topic of fatty acid transport has remained controversial, it is generally believed that the predominant mechanisms underlying fatty acid uptake consist of passive diffusion and a saturable, protein-mediated process (17; 41; 43) Candidates for L cell fatty acid transport proteins include the class B scavenger receptor Cluster-of-Differentiation 36/fatty acid translocase (CD36), as well as isoforms of the fatty acid transport protein (FATP) family. CD36 is widely expressed in the body but is also involved in intestinal absorption of fatty acids, including OA in the proximal gut (8; 32; 40). The isoforms of the FATP family also demonstrate broad expression, with FATP4 being the most abundant isoform in the small intestine (45). Previous studies have identified mRNA transcripts for CD36, as well as for FATP1, FATP3 and FATP4 in the murine GLUTag L cell line (24). The GLUTag cells have been extensively validated as an L cell model, demonstrating appropriate GLP-1 secretion in response to a wide-variety of known secretagogues (4; 15; 19; 23; 26; 37). Furthermore, the GLUTag cells have been shown to take up the fatty acid analog, C_1-Bodipy-C_{12} (24), consistent with an ability of these cells to internalize fatty acids. We therefore hypothesized that one or more of the intestinal L cell fatty acid transport proteins plays a role in OA-uptake and subsequent GLP-1 secretion.

114 **Methods**

115 **Cells.** Murine GLUTag cells were grown in media (Dulbecco's Modified Eagle Medium

116 (DMEM; Gibco Invitrogen, Burlington, ON, Canada) containing 25 mM glucose and 10% foetal

117 bovine serum. Cells were plated in 6- or 24-well plates coated with poly-D-lysine (Sigma

118 Chemical Co., St. Louis, MO, USA) and allowed to recover for 24 hr for uptake assay or 48 h for

119 immunoblot, transfection and secretion experiments. Cell viability following treatment with OA

120 was assessed by uptake of neutral red during the last hour of a 2-hr incubation (36).

121

122 **Small interfering (si) RNA transfection.** Preliminary attempts to knockdown FATP4 were

123 conducted using siRNA from Ambion (Austin, TX, USA). However, this led to a maximum 20%

124 reduction in FATP4 protein levels despite numerous attempts to optimize the approach (data not

125 shown). Subsequent studies were therefore performed by transfection of cells using Smartpool

126 siRNA, a mixture of 4 targeted FATP4 siRNA sequences (SMARTpool; Dharmacon, Lafayette,

127 CO, USA), or scrambled control, in Opti-MEM I media (Gibco Invitrogen) (16). The

128 SMARTpool siRNA approach is designed to reduce 'off-target' effects by up to 90%, by

129 reducing the concentration of each of the individual siRNA sequences. After optimization of the

130 approach, based upon protein expression levels, all experiments were conducted in cells that

131 were incubated with the siRNA (50 nM with 2.25 µL DharmaFECT-4 transfection reagent;

132 Dharmacon) for 5 h, washed twice and allowed to recover for 48 h.

133

134 **Immunoblot.** Cells or mouse duodenum (positive control) were collected into

135 radioimmunoprecipitation assay buffer. One-hundred µg of protein (measured by Bradford

136 assay; Bio-Rad, Hercules, CA, USA) was run on a 10% gel, transferred onto a polyvinyl

137 difluoride membrane, and probed with rabbit anti-FATP1, 3 or 4 (1:1000), rabbit anti-CD36
138 (1:1000; Cayman Chemicals, Ann Arbor, MI, USA), and rabbit anti-actin (1:4000; Sigma
139 Chemical Co.), followed by detection using horseradish peroxidise-linked goat anti-rabbit IgG
140 (1:2000; Cell Signaling Technology, Beverly, MA, USA) and electrochemiluminescence
141 Western blotting detection reagent (Amersham GE Healthcare, Baie d'Urfe, QC, Canada).
142
143 **^{3}H-Oleic acid uptake assay.** Cells were starved in serum-free medium overnight. ^{3}H-OA (3.0
144 μCi/mL; specific activity 1.96 x 10^{12} Bq/mmol) and ^{14}C-mannitol (0.6 μCi/mL; specific activity
145 2.04 x 10^{9} Bq/mmol; Moravek Biochemicals Inc., Brea, CA, USA) were added to $CaCl_2$-free
146 medium containing 0.5% fatty acid-free bovine serum albumin (Sigma Chemical Co.). In some
147 experiments, 500 μM or 1000 μM unlabeled-OA (100 mM stock solution in ethanol; Sigma
148 Chemical Co.), 200 μM phloretin (a non-specific inhibitor of carrier-mediated transport (49); 20
149 mM stock solution in ethanol; Sigma Chemical Co.), or 400 μM sulfo-N-succinimidyl oleate
150 (SSO; a CD36 inhibitor (6); 0.4 M stock solution in DMSO; Toronto Research Chemicals,
151 North York, ON, Canada) was added. The maximum final concentrations of ethanol and DMSO
152 in the media were 1.6% and 0.1%, respectively. Lastly, $CaCl_2$ was added back to the medium to
153 a final concentration of 1.8 mM. Cells treated with phloretin (200 μM) or SSO (400 μM) were
154 pre-incubated with medium containing only phloretin or SSO, respectively, for 30 min at 37 C
155 prior to the start of the uptake assay.
156 Immediately preceding the assay, cells were briefly washed twice with 500 μL of Hank's
157 Balanced Salt Solution before receiving 130 μL of treatment media and incubation at 37 C. As
158 preliminary uptake assays revealed that 62% of the total uptake observed over 120 min occurred
159 during the first 60 min, and we have previously shown that 70% of GLP-1 secretion occurs

160 within the same timeframe (27), all further uptake studies were conducted using a 60 min
161 incubation period. Hence, at various time points between t = 5 and t = 60 min, the media was
162 removed and the cells were briefly washed twice with Hank's Balanced Salt Solution containing
163 0.5% fatty acid-free bovine serum albumin to remove any tracer bound non-specifically to the
164 cell membrane. Ice-cold 1.0 M KOH (Sigma Chemical Co.) was then added to the cells and an
165 aliquot used to measure radioactivity in a beta counter with the isotope windows set at ^{3}H = 0-8
166 keV and ^{14}C = 35-156 keV to avoid signal overlap, as determined in preliminary studies. The
167 remaining sample was used to determine protein concentration by Bradford assay.
168
169 **GLP-1 secretion assay.** All treatments were made up in $CaCl_2$-free DMEM media (Gibco
170 Invitrogen) containing 0.5% fatty acid-free bovine serum albumin (Sigma Chemical Co.), and
171 $CaCl_2$ was then added at a final concentration of 1.8 mM. Cells were washed twice with Hank's
172 Balanced Salt Solution and then treated with media containing 1 µM phorbol 12-myristate 13-
173 acetate (PMA; 100 µM stock solution in ethanol; positive control; Sigma Chemical Co.), 150-
174 1000 µM OA (from a 40 mM stock solution in 0.5 M NaOH; Sigma Chemical Co.) or vehicle
175 alone (negative control). Some cells were pre-treated for 30 min with 200 µM phloretin (20 mM
176 stock solution in ethanol; Sigma Chemical Co.), 400 µM SSO (0.4 M stock solution in DMSO;
177 Toronto Research Chemicals), or for 48 h with siRNA (or scrambled control; as above). Cells
178 were then incubated with treatments for 2 h, including phloretin or SSO in the media, as
179 appropriate. At the end of the incubation period, the media was collected into 1% trifluoroacetic
180 acid, while cells were scraped into 1N hydrochloric acid containing 5% formic acid, 1%
181 trifluoroacetic acid and 1% sodium chloride. Peptides from both media and cell samples were
182 collected by reversed-phase extraction using C18 Sep-Pak cartridges (Waters Associates,

183 Milford, MA, USA), as previously validated (4; 9; 23; 26; 37). Samples were then subjected to a
184 radioimmunoassay using an antibody that recognized the carboxy terminal of GLP-$1^{7\text{-}36NH2}$
185 (Enzo Life Sciences, Farmingdale, NY, USA) (4; 9; 23; 26; 37). GLP-1 secretion was calculated
186 as the amount of GLP-1 detected in the media, normalized to total GLP-1 in the media and cells
187 combined, and expressed as percent of negative control, as previously reported (4; 9; 23; 26; 37).
188 Total GLP-1 cell content (media plus cells) of cells treated with vehicle was 381 ± 60 pg/mL (n
189 = 10) and did not differ with any of the treatments.
190
191 **Immunocytochemistry**
192 Cells were grown on glass coverslips until 80% confluent, and then treated for one hr with
193 vehicle or oleic acid, as above. Cells were then rinsed, incubated overnight at 4 C with rabbit
194 anti-mouse/human FATP4 antiserum (1/500; Abnova/Cedarlane Laboratories, Burlington, ON,
195 Canada) followed by Cy3-coupled donkey anti-rabbit IgG (1/400; Jackson
196 ImmunoResearch/Cedarlane Laboratories) for 1 hr at 20 C, rinsed and mounted with DAPI for
197 visualization using a Zeiss AxioPlan microscope with AxioPlan software (Carl Zeiss Canada, Don
198 Mills, ON, Canada). Images along the z-axis were taken at 1 μm intervals.
199
200 ***In vivo* experiments.** All animal protocols were approved by the Animal Care Committee at the
201 University of Toronto. $Fatp4^{-/-}$;Ivl-$Fatp4^{tg/+}$ (FATP4 null) mice (30), were on a mixed
202 129/B6/CBA background. Transgenic re-expression of FATP4 in the skin of the $FATP4^{-/-}$ mice,
203 via Ivl-$Fatp4^{tg/+}$, is required to prevent the neonatal lethality of the whole-body FATP4 knockout.
204 These mice have been reported to display no compensatory upregulation of other FATP isoforms
205 in the intestine (42). Both $Fatp4^{+/-}$;Ivl-$Fatp4^{tg/+}$ mice and $Fatp4^{+/-}$mice were used as control mice
206 and the results were combined as they did not differ between genotypes (data not shown). The

207 study was conducted using female and male littermates at 9-24 weeks of age and the results were
208 combined. CD36$^{-/-}$ mice, a generous gift from Dr. Kevin Kain (University of Toronto, Toronto,
209 ON, Canada) and originally derived by Dr. Maria Febbraio (13), were on a C57BL/6
210 background. FATP4 levels are not altered in the small intestine of these animals (42). Age-
211 matched C57BL/6 mice (Charles River, St. Constant, QC, Canada) were used as controls. The
212 study was conducted using both female and male mice at 8-25 weeks of age and the results were
213 combined.

214 Following an overnight fast, mice were anesthetized with isofluorane and blood samples
215 (50-100 µL) were obtained from the saphenous vein. After a laparotomy, 200 µL of 125 mM OA
216 in 125 mM Tween-80 (Sigma Chemical Co.) was injected directly into the lumen of the ileum in
217 an oral direction (24); Tween-80 was used to solubilize the OA rather than bile acids due to the
218 ability of bile acids to directly stimulate GLP-1 release (47), whereas we have previously shown
219 that Tween-80 does not affect GLP-1 release from the rat ileal L cell in vivo (24; 26). Blood
220 samples were then obtained via a cardiac puncture at either 15 or 60 min. All blood samples were
221 collected into a 10% volume of trasylol:EDTA;diprotin A (5,000 kallikrein inhibitory units:0.03
222 M:0.1 M), and plasma was stored at -80 C. Plasma GLP-1 levels were determined using the
223 Total GLP-1 Assay Kit (Meso Scale Discovery, Gaithersburg, MD, USA), with a detection limit
224 of 0.98 pg/mL. Ileal tissue sections (2 cm) were collected into radioimmunoprecipitation assay
225 buffer, protein concentrations were measured by Bradford assay, and levels of FATP4 or CD36
226 were determined by immunoblot, as described above.

227

228 **Statistical analysis.** All results are expressed as mean ± SEM. Statistical analysis was performed
229 with SAS software (SAS Institute, Cary, NC, USA) using Student's t test or one- or two-way

230 ANOVA followed by Student's t test or one-way ANOVA, as appropriate. Some data were

231 log10 transformed to normalize variances. Significance of data was assumed at $P < 0.05$.

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239 **Results**

240 **GLUTag cells express fatty acid transport proteins.** To confirm expression of the fatty acid
241 transport proteins, CD36, FATP1, FATP3 and FATP4 in the murine GLUTag L cell model,
242 immunoblot was carried out using mouse duodenum as a control (Fig. 1). Bands were
243 consistently detected for all 4 proteins. However, interestingly, although there was a clear band
244 of CD36 immunoreactivity at approximately 55 kDa, consistent with intracellular localization of
245 CD36, little-to-no expression of the heavily-glycosylated, high-molecular weight cell-surface
246 form of CD36 was detected in either the cells or the tissue.
247

248 **OA is taken up by GLUTag cells and stimulates GLP-1 secretion.** GLUTag cells
249 demonstrated uptake of ^{3}H-OA for up to 60 min (Fig. 2A). No significant uptake of the cell-
250 integrity control, ^{14}C-mannitol, was observed in any of the treatment groups. Uptake of ^{3}H-OA
251 was competitively-inhibited in a dose-dependent manner by 500 μM and 1000 μM unlabeled-
252 OA, to 60 ± 2% and 37 ± 2% of control levels at t = 60 min with ($P < 0.001$ versus control; $P <$
253 0.01 versus each other), respectively. Independent experiments that included an additional time
254 point between 45 and 60 min (i.e. t = 52 min) confirmed the linearity of the response between 45
255 and 60 min ($R^2 = 0.999$, n=8; data not shown). The absolute uptake of ^{3}H-OA by the GLUTag
256 cells over 60 min was 3.4×10^{-12} nmol/min-cell. Furthermore, a combination of the vehicle only
257 (control) data from multiple experiments (including the data shown in Fig. 2A, 3A and 4A, as
258 well as additional studies to make n = 7, with each experiment conducted in at least triplicate)
259 revealed that the slope of the line of the ^{3}H-OA uptake curve increased at t = 45 min, from 0.52 ±
260 0.06 (at t = 0 - 45 min) to 1.60 ± 0.22 (at t = 45-60 min; $P < 0.01$). This increase in slope was not
261 observed in paired cells treated with 1000 μM unlabeled-OA, which demonstrated a straight line
262 from t = 0 – 60 min (slope = 0.27 ± 0.03; $R^2 = 0.99$).

Treatment of GLUTag cells with increasing concentrations of OA also led to a dose-dependent increase in GLP-1 secretion, such that 500 µM and 1000 µM OA increased GLP-1 release to 124 ± 9% and 159 ± 9% of control cells, respectively ($P < 0.05$-0.001 vs. control, $P < 0.01$ vs. each other; Fig. 2B). The positive control PMA increased GLP-1 secretion to 154% ± 9% of controls ($P < 0.001$). Importantly, cell viability assay demonstrated no effect of incubation with the highest concentration of OA for 2 hr (Fig. 2D).

Phloretin decreases OA uptake and GLP-1 secretion by GLUTag cells. Treatment of GLUTag cells with the non-specific inhibitor of carrier-mediated transport, phloretin, decreased the uptake of ^{3}H-OA, by 38 ± 4% at t = 15 min ($P < 0.001$) and by 14 ± 4% at t = 60 min ($P < 0.05$; Fig. 3A). As in previous uptake assays, unlabeled-OA (1000 µM) decreased ^{3}H-OA uptake at t = 5 - 60 min ($P < 0.01$-0.001), while no significant uptake of ^{14}C-mannitol was observed in any of the treatment groups. As found previously, incubation of the cells with OA (1000 µM) increased GLP-1 secretion, by 137 ± 21% (Fig. 3B). Pre- and co-incubation with phloretin (200 µM) markedly reduced OA-induced GLP-1 secretion, by 67 ± 14%, but did not abrogate the effect of OA ($P < 0.01$). Basal secretion in the presence of phloretin alone was 85.3 ± 10.2% of control values (P = NS). Control experiments demonstrated no effect of phloretin treatment on PMA-induced GLP-1 release (secretion was 104.9 ± 22.7% that found in the absence of phoretin, P = NS; data not shown).

CD36 plays a role in the L cell *in vitro* but not *in vivo*. Treatment of GLUTag cells with the CD36 inhibitor, SSO, reduced ^{3}H-OA uptake, by 36 ± 8% at t = 60 min ($P < 0.001$; Fig. 4A). As observed previously, unlabeled-OA (1000 µM) decreased ^{3}H-OA uptake at t = 15-60 min (P <

286 0.05-0.001), and no significant uptake of ^{14}C-mannitol was observed in any of the treatment
287 groups. Basal secretion in the presence of SSO alone was increased to 221.9 ± 43.5% of control
288 ($P < 0.05$). However, SSO treatment decreased OA-induced GLP-1 secretion by GLUTag cells,
289 from 243 ± 37% of control values to 151 ± 15% ($P < 0.05$; Fig. 4B). Control experiments
290 demonstrated no effect of SSO treatment on PMA-induced GLP-1 release (secretion was 80.7 ±
291 12.5% that found in the absence of SSO; P = NS). The role of CD36 was therefore examined *in*
292 *vivo* using the CD36 null mouse. Immunoblotting confirmed the absence of CD36 in the ileum of
293 $CD36^{-/-}$ mice (Fig. 4C). To determine the effect of OA on plasma GLP-1 levels, 125 mM OA was
294 injected directly into the ileum of anesthetized control and CD36 null mice, and blood samples
295 were collected at t = 0 and 15 min or at t = 0 and 60 min in a paired fashion for determination of
296 total plasma GLP-1 levels. The CD36 null mice were found to have increased basal GLP-1
297 plasma levels, by 61.3 ± 19.4% as compared to control animals ($P < 0.01$; Fig. 4D). However, no
298 differences in GLP-1 plasma levels were observed between the two groups of mice at t = 15 or
299 60 min following intraileal injection of OA, when either absolute values were compared (Fig.
300 4D) or following determination of the change from basal levels (data not shown).
301

302 **FATP4 plays a role in the L cell *in vitro* and *in vivo*.** As there is no specific inhibitor for
303 FATP4, this protein was knocked down in the GLUTag cells using FATP4-targeting siRNA,
304 leading to a maximum 27 ± 6% reduction in protein levels ($P < 0.05$; Fig. 5A). Nonetheless,
305 knockdown of FATP4 reduced ^{3}H-OA uptake at t = 60 min, by 28 ± 7% ($P < 0.05$) (Fig. 5B).
306 Basal GLP-1 secretion in the presence of FATP4 siRNA alone was not different from control
307 values (146.1 ± 21.3% of control; $P>0.05$). In contrast, FATP4 knockdown completely abrogated
308 OA-induced GLP-1 secretion, decreasing release from 220 ± 29% to 121 ± 21% of control

309 values ($P < 0.05$; Fig. 5C). Control experiments showed no effect of FATP4 knockdown on
310 PMA-induced GLP-1 release (secretion was 101.9. ± 7.8% that found for PMA with the
311 scrambled control, P = NS; data not shown). Immunocytochemistry for FATP4
312 immunoreactivity revealed a lack of membrane localization, with the majority of the staining
313 appearing in the cytoplasm and/or perinuclear area of both vehicle- and oleic acid-treated cells
314 (Fig. 5D).

315 To further explore the role of FATP4 in OA-induced GLP-1 secretion, the FATP4 null
316 mouse model was utilized. Immunoblotting confirmed the absence of FATP4 in the ileum of
317 FATP4 null mice (Fig. 5E). As for the CD36 null mice, 125 mM OA was injected directly into
318 the ileum of anesthetized wild-type and FATP4 null mice. Although no differences were seen
319 between the two groups of animals at t = 0 and 15 min, plasma GLP-1 levels were markedly
320 lower in the FATP4 null mice at t = 60 min, by 72.3 ± 4.8% as compared to control animals (p
321 <0.05; Fig. 5F).
322
323

324 **Discussion**

325 The anti-diabetic hormone, GLP-1, is released from the intestinal L cell upon nutrient
326 ingestion. Fatty acids and OA, in particular, are potent GLP-1 secretagogues, exerting direct
327 effects on the intestinal L cell *in vitro*, as well as increasing GLP-1 release in both humans and
328 rodents (2; 23; 24; 35; 37). However, the mechanism underlying OA-induced GLP-1 secretion
329 has not been fully elucidated, with the only essential component identified to date being the
330 isozyme PKCζ (23; 24). The results of the current study demonstrate that the L cell specifically
331 takes up OA via a carrier-mediated process, and that FATP4 plays a key role in OA-induced
332 GLP-1 secretion, both *in vitro* and *in vivo*.

333 In keeping with a requirement for FATP4 in OA uptake by the intestinal L cell, FATP4
334 mRNA [10] and protein are expressed in GLUTag cells. Furthermore, both phloretin treatment
335 and a 27% knockdown of FATP4 reduced OA uptake, by up to 28%, consistent with reports of
336 parallel decreases in protein levels and OA uptake in enterocytes after FATP4 knockdown (45).
337 However, our findings in the GLUTag cells differ from those made in endothelial cells, in which
338 a 50% knockdown of FATP4 completely abrogated the ability of vascular endothelial growth
339 factor B to induce OA uptake (16), and from adipocytes and brain microvessel endothelial cells,
340 in which 50-90% knockdown did not alter OA uptake at all [28;40;41]. Hence, the response to
341 modulation of FATP4 expression appears to be highly cell-specific, with the intestinal L cell
342 behaving in a similar fashion to the gut absorptive cells, rather than to the unrelated adipocytes
343 and endothelial cells.

344 Consistent with a role for FATP4 in the regulation of OA-induced GLP-1 secretion, both
345 phloretin treatment and FATP4 knockdown markedly reduced stimulated GLP-1 release in vitro,
346 and knockout of FATP4 completely abrogated the intestinal L cell secretory response to OA in

347 vivo. However, somewhat unexpectedly, the effects of both phloretin treatment and FATP4
348 knockdown to reduce OA uptake by the GLUTag cells were relatively modest as compared to
349 their ability to prevent OA-induced GLP-1 release. A similar dissociation between OA uptake
350 and insulin secretion has been noted in mouse insulinoma, MIN6, cells, such that knockdown of
351 the adipose differentiation-related protein which coats lipid droplets decreases OA uptake by
352 17% but reduces insulin secretion by over 50% (12). This discrepancy was attributed to impaired
353 lipid metabolism, although the exact mechanisms were not investigated. We have also observed
354 in GLUTag cells that, despite only 23% knockdown of the receptor, GPR119 there was a much
355 greater effect (e.g. 45% decrease) on GLP-1 secretion induced by the OA derivative,
356 oleoylethanolamide [20]. Hence, even relatively low levels of knockdown of proteins that
357 mediate fat handling by the intestinal L cell appear sufficient to impair GLP-1 release.
358 Interestingly, immunocytochemistry for FATP4 in the GLUTag cells revealed a
359 predominance of staining in the cytoplasm and/or perinuclear region under both basal and OA-
360 stimulated conditions, with no immunoreactivity detectable in the cell membrane. We have
361 previously reported that PKCζ is required for the effects of OA on GLP-1 secretion, and we have
362 found that this enzyme translocates to the cell membrane upon stimulation with OA (23; 24).
363 However, preliminary data has indicated that the majority of the PKCζ immunoreactivity under
364 basal conditions is localized to the perinuclear compartment of the GLUTag cell (unpublished
365 observations). These findings are consistent with the demonstration that, in addition to its
366 reported plasma membrane expression (45), FATP4 also localizes to the endoplasmic reticulum,
367 where its expression drives fatty acid uptake through its ability to act as long-chain acyl-CoA
368 synthetase (29). Hence, intracellular compartmentalization of this enzyme in the L cell could
369 explain the relatively late effect of FATP4 knockdown on fatty acid uptake (e.g. at 60 min only),

370 as compared to that of phloretin (e.g. at both 5 – 30 min and 60 min). Finally, the absolute uptake
371 of ^{3}H-OA by the GLUTag cells over 60 min was found to be 3.4 x 10^{-12} nmol/min-cell. Although
372 markedly lower than the uptake of OA reported for enterocytes (1.2 x 10^{-7} nmol/min-cell,
373 determined over 4 min), FATP4 accounts for ~50% of the uptake in enterocytes, the major
374 function of which is absorption of ingested fatty acids (45). When taken together, therefore, the
375 results of the present study indicate that FATP4 plays a key role in both OA-uptake and OA-
376 induced GLP-1 secretion in the enteroendocrine L cell in vitro and in vivo, likely through
377 delivery of the fatty acid to its effector, PKCζ.

378 In contrast to the findings on FATP4, a role for CD36 in GLP-1 secretion was found *in*
379 *vitro* but not *in vivo*. Hence, the CD36 inhibitor, SSO, decreased both ^{3}H-OA uptake and OA-
380 induced GLP-1 release in the GLUTag cells, whereas CD36 knockout did not prevent the effects
381 of OA on GLP-1 secretion *in vivo*. SSO has been reported to be a specific CD36 inhibitor,
382 reducing fatty acid uptake by up to 70% in a wide-variety of tissues (6). However, a recent report
383 showing that SSO also inhibits complex III of the mitochondrial respiratory chain has called the
384 specificity of SSO into question (7). Such a role for CD36 would be consistent with the finding
385 of only the lower-molecular weight, intracellular form of this protein (22) in the GLUTag cells.
386 Hence, the finding that CD36 null mice exhibit a normal GLP-1 secretory response to
387 intraluminal OA suggests that CD36 does not a play an essential role in the effects of OA on the
388 intestinal L cell, whereas it appears to be relatively more important in the immortalized GLUTag
389 cells. Nonetheless, the finding of higher basal GLP-1 levels in the null animals, as compared to
390 control mice, implies either that CD36 plays a minor role in the regulation of GLP-1 release
391 under fasting conditions, which seems unlikely, or that the mice have undergone compensatory
392 responses to the global loss of CD36.

393 Interestingly, the effects of both FATP4 knockdown and CD36 inhibition on OA uptake
394 by the GLUTag cells were found to occur at t = 60 min only, as compared to the inhibition of
395 uptake caused by phloretin as well as by unlabelled OA, at early (t = 5 – 30/45 min) as well as
396 late (t = 60 min) time points. These findings are also consistent with the observation of a change
397 in the rate of OA uptake at t = 45 – 60 min, although studies of a longer duration may be useful
398 in further examining this phenomenon. Nonetheless, these findings support the notion that
399 multiple uptake mechanisms may be taking place in the L cell over the course of the 60 min
400 assay, including possible roles for FATP1 and 3. Additionally, transport proteins including CD36
401 and FATP1 are known to translocate to the plasma membrane from subcellular locations (5; 44).
402 It is therefore possible that there is an upregulation of plasma membrane fatty acid transport
403 proteins after t = 45 min, which would explain the increased rate of OA uptake at this time point.
404 Further studies will clearly be necessary to elucidate the specific roles of all of these proteins in
405 the intestinal L cell.
406 Finally, increasing concentrations of OA increased GLP-1 secretion from the murine
407 GLUTag L cell model in a dose-dependent manner. Although there is evidence that
408 accumulation of free fatty acids in tissues can lead to lipotoxicity and cell dysfunction (38), the
409 highest dose used in this current study (1000 μM OA) is well below the physiological
410 concentration of OA reached in the ileum (approximately 105 mM), as determined by
411 measurement of the OA concentration in chyme following oral gavage of olive oil (24).
412 Furthermore, exposure of the GLUTag cells to 1000 μM OA had no effect on cell viability,
413 consistent with our previous findings using 500 μM OA (23). However, as the luminal
414 concentration of fat, as well as the aboral distance transited by ingested fat, are dependent upon

415 the load of fat ingested (28), the absolute concentration of OA to which the intestinal L cell is
416 exposed will vary depending upon the meal.

417 The findings of the present study indicate a role for fatty acid transport proteins, and
418 specifically FATP4, in OA-induced GLP-1 secretion by the intestinal L cell. Endogenous GLP-1
419 production has been shown to be elevated upon stimulation with MUFAs, such as OA (24; 37),
420 and has been implicated in the improved glycemic control observed in insulin-resistant patients
421 placed on a MUFA-rich diet (34). Although FATP4 plays a role in mediating the effects of OA
422 on the L cell, FATP4 is not likely to be a therapeutic target, due to its wide-spread distribution
423 throughout the body, including the enterocytes and skin (13; 45). Instead, this signalling
424 pathway, including the essential isozyme PKCζ (23; 24), should be further explored to identify
425 suitable therapeutic targets that could be manipulated to increase endogenous GLP-1 secretion in
426 patients with type 2 diabetes. Finally, mutations in FATP4 were recently described in patients
427 with ichthyosis prematurity syndrome, a condition characterized by premature birth with the
428 infant covered in thick, caseous skin and having respiratory complications, followed by lifelong
429 dry, thick skin (25). Whether these patients exhibit reduced GLP-1 release and a subsequent
430 impairment in glycemic control has not been explored. Nonetheless, an essential role for FATP4
431 has been established in the skin, in these patients as well as in FATP4 null mice, and it now
432 appears that FATP4 additionally plays a key role in mediating OA-induced GLP-1 secretion
433 from the intestinal L cell.
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601 **Figure Legends**

602

603 **Fig. 1. Expression of fatty acid transport proteins in the L cell.** Immunoblot for (**A**) CD36

604 (55 kDa - non-glycosylated, intracellular form; 88 kDa – glycosylated, membrane form), (**B**)

605 FATP1 (63 kDa), (**C**) FATP3 (72 kDa) and (**D**) FATP4 (72 kDa) in murine GLUTag L cells (n =

606 3). Actin (42 kDa) was used as the loading control, and murine duodenum as a positive (+ve)

607 control.

608

609 **Fig. 2. OA uptake in the L cell and the effect of OA on GLP-1 secretion. (**A) GLUTag cells

610 were incubated with ^{3}H-OA and treated with vehicle control (*solid line*) or 500 µM (*dashed line*)

611 or 1000 µM (*dotted line*) unlabeled-OA, followed by determination of ^{3}H-OA uptake (*black*

612 *circles*). ^{14}C-Mannitol was used as a cell integrity control in each treatment group *(black*

613 *triangles*). Counts per minute (CPM) were normalized to total protein (inset: expanded scale) (n

614 = 6). (**B**) GLUTag cells were treated with vehicle (control) or increasing concentrations of OA

615 for 2 hr and secretion of GLP-1 was determined by radioimmunoassay (n = 6-11). Basal

616 secretion was 8.6 ± 0.8% of total cell content. (**C**) GLUTag cells were treated for 2 hr with

617 vehicle alone (control), 5 mM H_2O_2 or 1000 µM OA for 2 hr, followed by determination of

618 viability using neutral red uptake, as assessed by optical density (O.D.) at 540 nm (n=8). * P <

619 0.05, ** P < 0.01, *** P < 0.001 versus control, ## P < 0.01, ### P < 0.001 for 500 µM versus

620 1000 µM unlabeled-OA, or as indicated.

621

622 **Fig. 3. Effect of phloretin on L cell OA uptake and GLP-1 secretion. (**A) GLUTag cells were

623 incubated with ^{3}H-OA and treated with vehicle control (*solid line*) or 1000 µM unlabeled-OA

624 (*dotted line*) or 200 µM phloretin (*dashed line*), followed by determination of ^{3}H-OA uptake

625 (*black circles*). ^{14}C-Mannitol was used as a cell integrity control in each treatment group *(black*
626 *triangles*). Counts per minute (CPM) were normalized to total protein (inset: expanded scale) (n
627 = 6). (**B**) GLUTag cells were treated with either vehicle control or 200 µM phloretin and further
628 incubated with or without 1000 µM OA. GLP-1 secretion was determined by radioimmunoassay
629 (n = 11-12). Basal secretion was 8.5 ± 1.4% of total cell content. * $P < 0.05$, ** $P < 0.01$, *** $P <$
630 0.001 versus respective control, ## $P < 0.01$ as indicated.
631
632 **Fig. 4. Role of CD36 in L cell OA uptake and GLP-1 secretion.** (**A**) GLUTag cells were
633 incubated with ^{3}H-OA and treated with vehicle control (*solid line*) or 1000 µM unlabeled-OA
634 (*dotted line*) or 400 µM SSO (*dashed line*), followed by determination of ^{3}H-OA uptake (*black*
635 *circles*). ^{14}C-Mannitol was used as a cell integrity control in each treatment group (*black*
636 *triangles*). Counts per minute (CPM) were normalized to total protein (n = 6). (**B**) GLUTag cells
637 were treated with either vehicle control or 400 µM SSO and were further incubated with or
638 without 1000 µM OA. GLP-1 secretion was determined by radioimmunoassay (n = 11-12). Basal
639 secretion was 8.1 ±0.8% of total cell content. (**C**) Immunoblot for CD36 and actin (loading
640 control) in the ileum of control (CO) and CD36 null (KO) mice (representative of n = 5-6). (**D**)
641 OA (125 mM in 125 mM Tween-80) was injected directly into the ileum of control (solid line)
642 and CD36 null (dashed line) mice and blood samples were collected at t = 0 and 15 min or at t =
643 0 and 60 min. Total GLP-1 levels were determined in the collected plasma using a sandwich-
644 immunoassay (n = 9-19). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus respective control, # $P <$
645 0.05 as indicated.
646

647 **Fig. 5. Role of FATP4 in L cell OA uptake and GLP-1 secretion.** (**A**) GLUTag cells were
648 treated with scrambled or FATP4 siRNA and FATP4 and actin (loading control) levels were
649 detected by immunoblot (representative of n = 3). (**B**) ^{3}H-OA uptake was determined in GLUTag
650 cells treated with scrambled (*solid line*) or FATP4 siRNA (*dashed line*), followed by
651 determination of ^{3}H-OA uptake (*black circles*). ^{14}C-Mannitol was used as a cell integrity control
652 in each treatment group *(black triangles*). Counts per minute (CPM) were normalized to total
653 protein (n = 4-5). (**C**) GLUTag cells were treated with scrambled or FATP4 siRNA and were
654 further incubated with or without 1000 μM OA. GLP-1 secretion was determined by
655 radioimmunoassay (n = 7-8). Basal secretion was 6.7 ± 0.5% of total cell content. (**D**)
656 Immunocytochemistry for FATP4 (red) in vehicle- (a, c) and oleic acid- (b, d) treated GLUTag
657 cells; nuclei = blue (a, b). Panels a and b represent whole cell images, whereas the sections
658 shown in panels c and d were taken from 3-D 'z-stack' analyses at approximately the mid-cell
659 level. (**E**) Immunoblot for FATP4 (72 kDa) and actin (42 kDa; loading control) in the ileum of
660 wild-type (WT) and FATP4 null (KO) mice (representative of n = 5). (**F**) OA (125 mM in 125
661 mM Tween-80) was injected directly into the ileum of wild-type (solid line) and FATP4 null
662 (dashed line) mice and blood samples were collected at t = 0 and 15 min or at t = 0 and 60 min.
663 Total GLP-1 levels were determined in the collected plasma using an immunoassay system (n =
664 7-22). * $P < 0.05$, ** $P < 0.01$ versus respective control, # $P < 0.05$ as indicated.
665
666

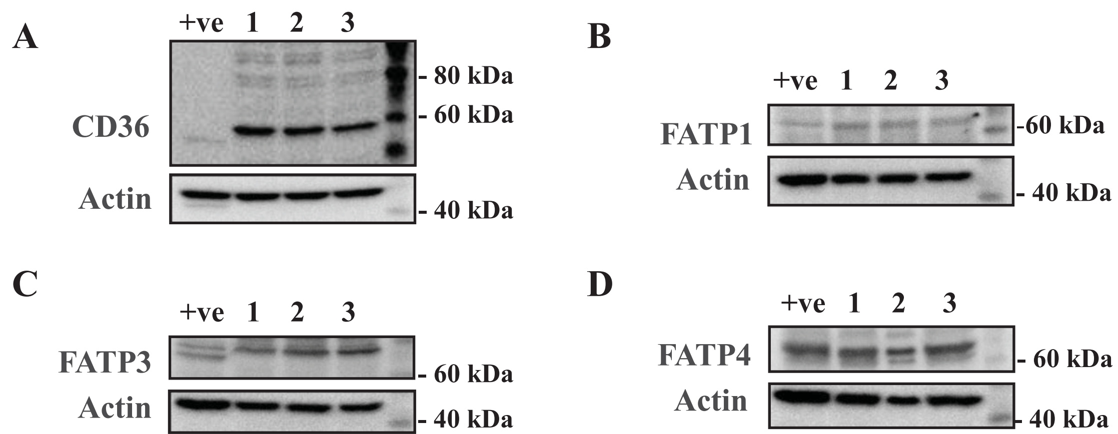


Figure 1

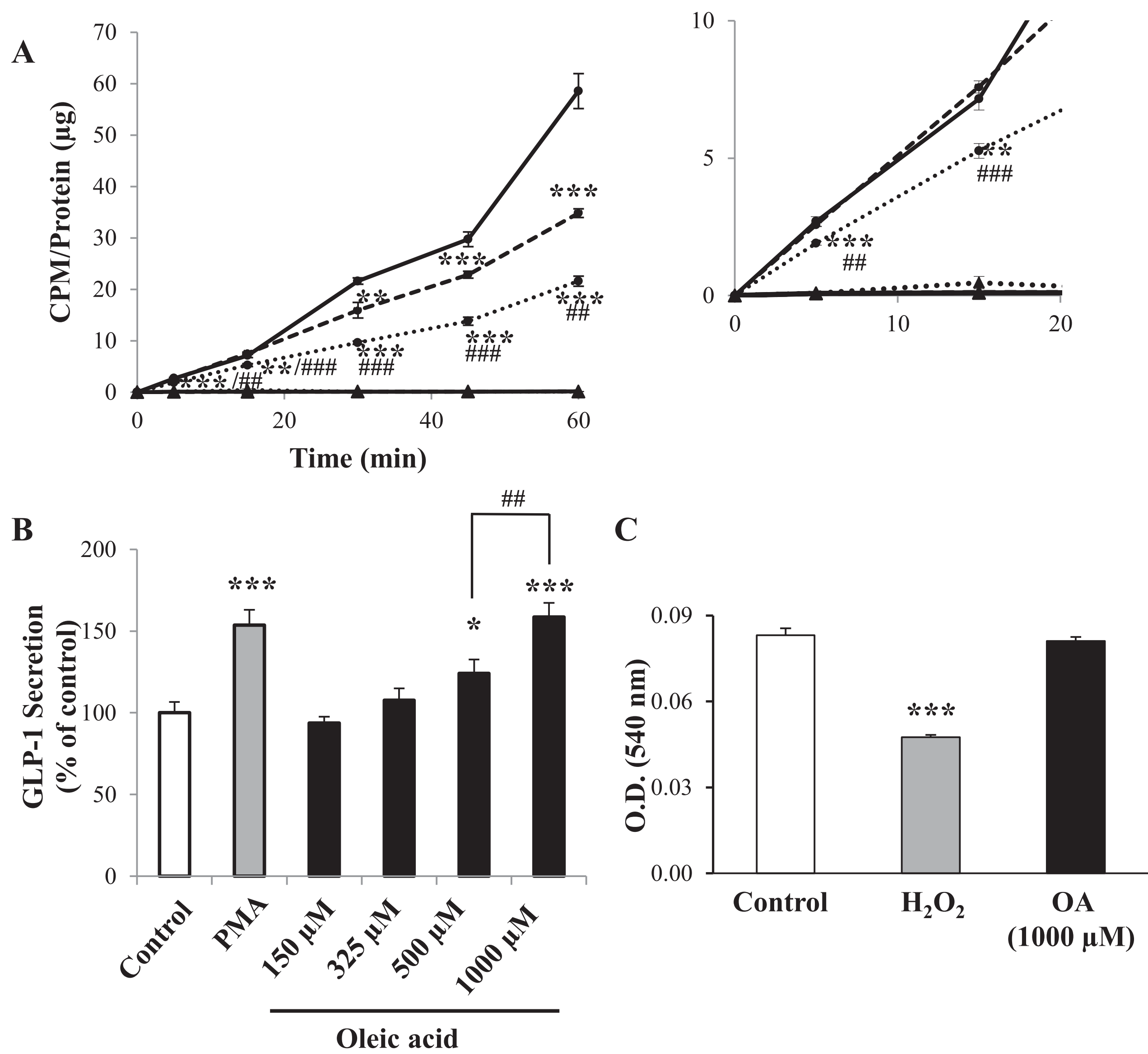
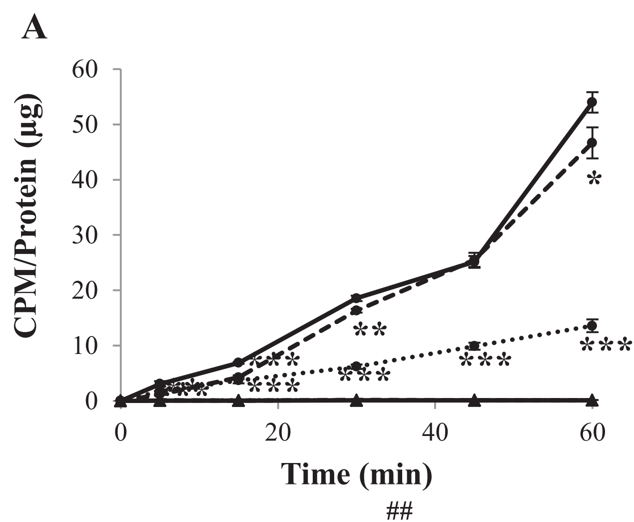
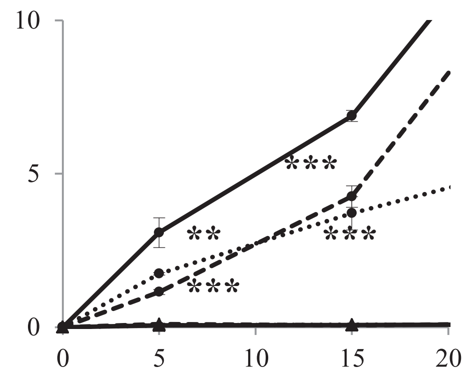


Figure 2





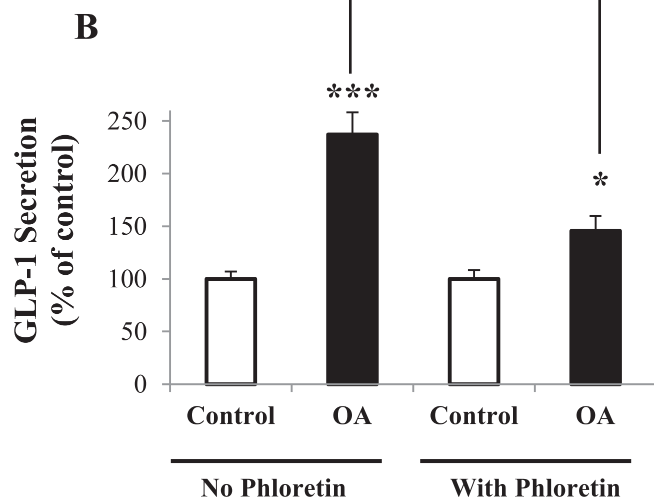
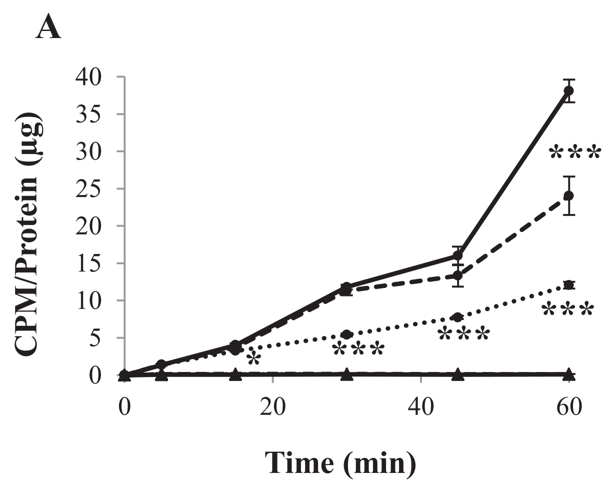
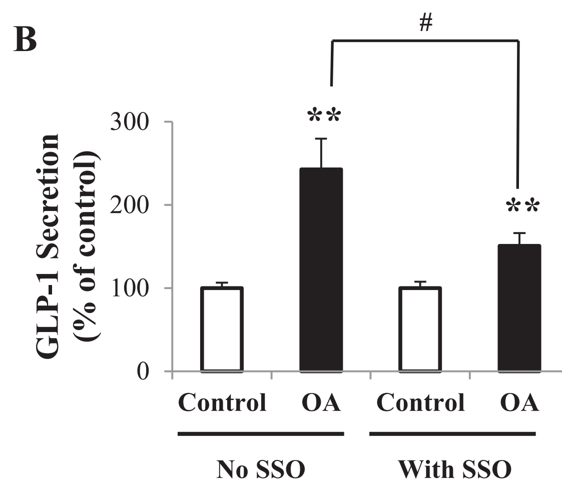
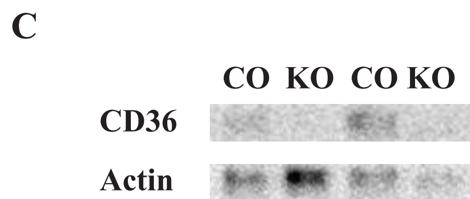


Figure 3







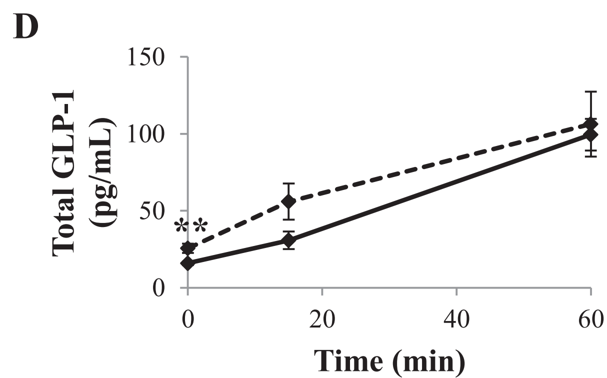
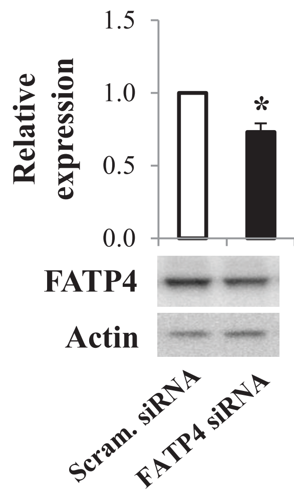
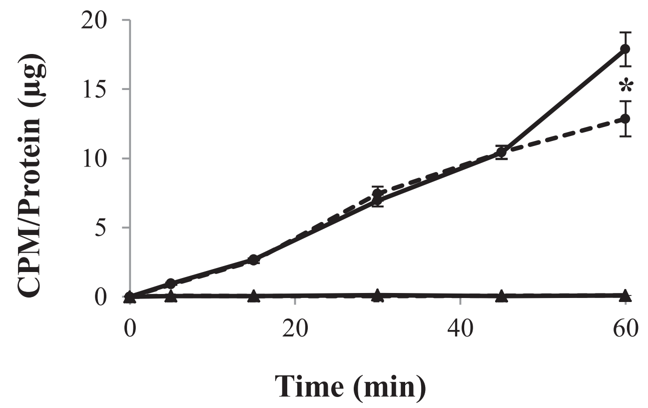
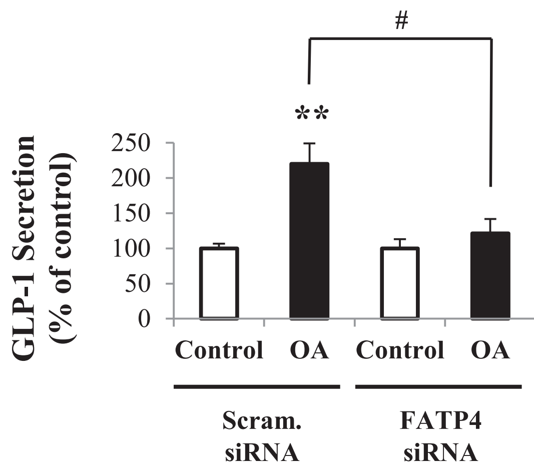
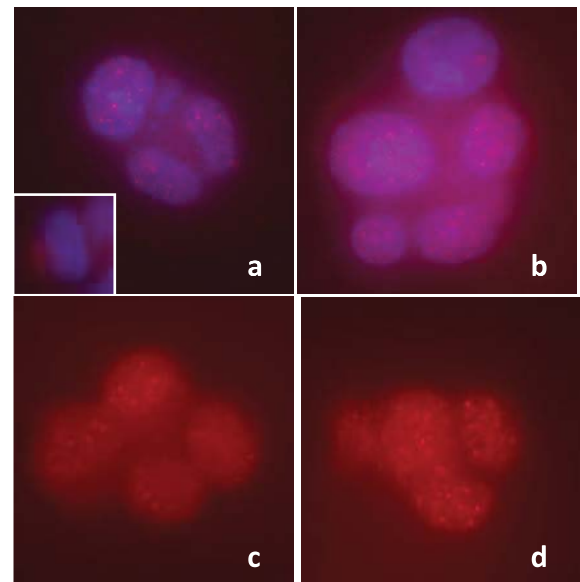


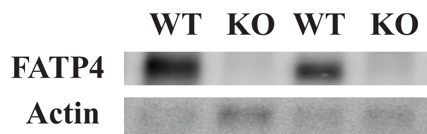
Figure 4











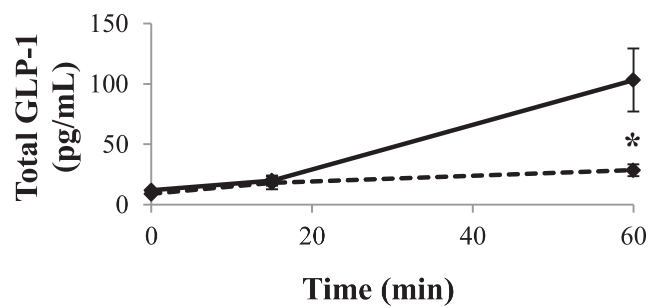


Figure 5