Role of Fatty Acid Transport Proteins in Oleic Acid-Induced Secretion of Glucagon-Like Peptide-1

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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ABSTRACT OF THESIS

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Master of Science 2011
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Glucagon-like peptide-1 (GLP-1) is an anti-diabetic intestinal L cell hormone. The monounsaturated fatty acid, oleic acid (OA), is an effective GLP-1 secretagogue that crosses the cell membrane by an unknown mechanism. Immunoblotting demonstrated the presence of fatty acid transport proteins (CD36 and FATP1, 3 and 4) in the murine GLUTag L cell model. The cells demonstrated specific $^3$H-OA uptake, which was dose-dependently inhibited by unlabeled-OA. Phloretin and SSO, inhibitors of carrier-mediated transport and CD36, respectively, also significantly decreased $^3$H-OA uptake, as did knocking down FATP4 by transfection of siRNA. OA dose-dependently increased GLP-1 secretion in GLUTag cells, while phloretin and FATP4 knockdown, but not SSO, decreased this response. OA injected directly into the ileum of wild-type mice increased plasma GLP-1 levels; in contrast, preliminary findings suggest decreased GLP-1 levels in FATP4 null mice at 60 min. Collectively, these findings indicate a role for FATP4 in OA-induced GLP-1 secretion.
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# TABLE OF CONTENTS

ABSTRACT OF THESIS..............................................................................................................ii

ACKNOWLEDGEMENTS...........................................................................................................iii

TABLE OF CONTENTS...........................................................................................................iv

LIST OF FIGURES................................................................................................................vi

LIST OF ABBREVIATIONS.......................................................................................................vii

1. INTRODUCTION ..............................................................................................................1

1.1 Rationale .......................................................................................................................1

1.2 Glucagon-like peptide-1 ...............................................................................................2

1.2.1 Synthesis ..................................................................................................................2

1.2.2 The GLP-1 receptor and GLP-1 actions in the body ............................................4

1.2.3 Metabolism and clearance .....................................................................................7

1.2.4 Secretion ..................................................................................................................8

1.2.4.1 Indirect mechanisms regulating GLP-1 secretion ......................................10

1.2.4.2 Direct mechanisms regulating GLP-1 secretion ........................................13

1.3 Cellular mechanisms of fatty acid action ..................................................................15

1.3.1 G protein-coupled receptors ..............................................................................15

1.3.2 PKCζ .....................................................................................................................17

1.4 Transport of fatty acids ..............................................................................................20

1.4.1 Fatty acid transport proteins ..............................................................................20

1.4.2 Subcellular localization .........................................................................................24

1.5 Hypothesis and aims ...................................................................................................27

2. METHODS ......................................................................................................................28

2.1 In vitro cell models .....................................................................................................28

2.2 Immunoblot ................................................................................................................29

2.3 ³H-Oleic acid uptake assay .........................................................................................29

2.4 GLP-1 secretion assay .................................................................................................31
2.5 FATP4 null mouse model..................................................................................32
2.6 Statistical analysis............................................................................................33

3. RESULTS..................................................................................................................34
3.1 Expression of fatty acid transport proteins in the L cell.................................34
3.2 OA dose-dependently stimulates GLP-1 secretion in murine GLUTag, but not in human NCI-H716, cells..................34
3.3 OA competitively inhibits $^3$H-OA uptake in murine GLUTag cells..............37
3.4 SSO decreases $^3$H-OA uptake but does not significantly decrease OA-induced GLP-1 secretion in GLUTag cells.........37
3.5 Phloretin decreases $^3$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells..................................................41
3.6 FATP4 knockdown decreases $^3$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells.............................................41
3.7 Possible decrease in OA-induced GLP-1 levels in FATP4 null mice..................45

4. DISCUSSION............................................................................................................47

5. REFERENCES...........................................................................................................57
LIST OF FIGURES

1.1 Proglucagon processing ................................................................................................................................................... 3
1.2 Indirect and direct mechanisms regulating GLP-1 secretion ................................................................................................. 9
1.3 Fatty acid transport proteins in cellular fatty acid uptake .................................................................................................. 19

3.1 Murine GLUTag and human NCI-H716 L cells express FATP1, 3, 4 and CD36 fatty acid transport proteins .................................................................................................................................................................................. 35
3.2 OA dose-dependently stimulates GLP-1 secretion in murine GLUTag, but not in human NCI-H716, cells .......................................................................................................................................................................................................................... 36
3.3 OA competitively inhibits $^{3}$H-OA uptake in murine GLUTag cells ............................................................................................... 39
3.4 SSO decreases $^{3}$H-OA uptake but does not significantly decrease OA-induced GLP-1 secretion in GLUTag cells ................................................................. 40
3.5 Phloretin decreases $^{3}$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells .......................................................................................................................................................................................................................... 42
3.6 First FATP4 knockdown decreases $^{3}$H-OA uptake but does not significantly decrease OA-induced GLP-1 secretion in GLUTag cells .......................................................................................................................................................................................................................... 43
3.7 Second FATP4 knockdown decreases $^{3}$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells .......................................................................................................................................................................................................................... 44
3.8 Possible decrease in OA-induced GLP-1 levels in FATP4 null mice ............................................................................................... 46
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>4-DAMP</td>
<td>4-Diphenylacetoxy-N-methylpiperidine</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACH</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACS</td>
<td>Acyl CoA-synthetase</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DIRKO</td>
<td>Double incretin receptor knockout</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPP4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
</tr>
<tr>
<td>FAF</td>
<td>Fatty acid-free</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FRIC</td>
<td>Fetal rat intestinal cell culture</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Glucagon-like peptide-2</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRP</td>
<td>Gastrin-releasing peptide</td>
</tr>
<tr>
<td>HBMEC</td>
<td>Human brain microvessel endothelial cells</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long-chain fatty acid</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>M1R</td>
<td>Muscarinic receptor 1</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>OEA</td>
<td>Oleylethanolamide</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidylglycine alpha-amidating monoxygenase</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone convertase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
</tbody>
</table>
siRNA  Small interfering ribonucleic acid
SSO   Sulfo-N-succinimidyl oleate
STC-1 Secretin tumour cell line
T2D   Type 2 diabetes
VLACS Very long-chain acyl-CoA synthetase
VLCFA Very long-chain fatty acid
WT    Wild-type

Symbols and Units

g        Gram
h        Hour
kDa      Kilodalton
L        Litre
m        Milli
M        Molar
min      Minute

α        Alpha
β        Beta
δ        Delta
µ        Micro
ζ        Zeta
1. INTRODUCTION

1.1 Rationale

In light of the looming type 2 diabetes (T2D) epidemic taking hold of the Western world, alternative ways to control hyperglycemia in patients is becoming a necessity. The hormone glucagon-like peptide-1 (GLP-1) is released from the intestine postprandially and stimulates insulin secretion from the pancreatic β cell in a glucose-dependent manner. Although GLP-1 mimetics are already being used in the clinic to treat T2D patients, increasing endogenous GLP-1 secretion in lieu of administrating exogenous GLP-1 is an enticing notion. The beneficial health effects of unsaturated fatty acids have already been observed and arguments have even been made for the advantages of a Mediterranean diet, rich in the monounsaturated fatty acid (MUFA) oleic acid (OA). The concept that specific nutrients, such as OA, can stimulate endogenous GLP-1 secretion from the enteroendocrine L cell is exciting and determining how to maximize L cell GLP-1 secretion could be utilized to help treat chronic hyperglycemia. It is thus crucial to further understand the pathways that lead to GLP-1 secretion to achieve this end. The goal of this study was to better understand how OA stimulates the L cell to release GLP-1 and to identify components that comprise this signaling pathway. The findings of this study delineate a cellular mechanism by which OA enters the intestinal L cell to increase GLP-1 secretion.
1.2 Glucagon-like peptide-1

1.2.1 Synthesis

GLP-1 is a 30-amino acid intestinal hormone derived from the proglucagon gene and released from the enteroendocrine L cell. Found predominately in the distal intestine and colon, the L cell is an ‘open-type’ epithelial cell that comes into contact with nutrients in the gastrointestinal lumen (1). The proglucagon transcript is expressed in the intestinal L cell but also in the endocrine pancreas α cell and in selected neurons in the brain (2;3). Posttranslational processing by tissue-specific prohormone convertases (PCs) leads to the release of different peptides from proglucagon (Fig. 1.1). In the intestine, PC1/3 allows for the release of GLP-1 and GLP-2, as well as the glucagon-containing peptides, glicentin and oxyntomodulin (4-6). GLP-1 is further truncated to produce two equipotent biologically active forms, GLP-1\(^{7-37}\) and GLP-1\(^{7-36NH_2}\) (7). Briefly, GLP-2 is an intestinal growth factor that helps to increase the surface area of the intestinal epithelium (8), whereas oxyntomodulin has been shown to both decrease gastric acid secretion and increase satiety (9;10). A biological role for glicentin has not yet been characterized (11). Proglucagon processing in the brain liberates the same peptides as released from the intestinal L cell (3). In contrast, in the α cell, proglucagon processing by PC2 produces glucagon, the counter-regulatory hormone to insulin, as well as glicentin-related pancreatic peptide and the major proglucagon fragment, which have unknown functions (4;11).

The carboxyterminal glycine residue of GLP-1\(^{7-37}\) is necessary for the posttranslational amidation of the preceding arginine residue by the enzyme peptidylglycine alpha-amidating monooxygenase (PAM), producing GLP-1\(^{7-36NH_2}\) (12;13). The majority of GLP-1 secreted from the intestine in humans is in the amidated form (14). Though amidated and non-amidated forms of GLP-1 appear to have similar abilities in terms of increasing glucose-dependent insulin
Figure 1.1 Proglucagon processing. Posttranslational processing by tissue-specific prohormone convertases (PCs) leads to the release of different peptides from proglucagon. In the intestinal L cell, PC1/3 allows for the release of GLP-1 and GLP-2, as well as the glucagon-containing peptides, glicentin and oxyntomodulin. GLP-1 is further truncated to produce two equipotent biologically active forms, GLP-17-37 and GLP-17-36NH2. Proglucagon processing in the brain liberates the same peptides as released from the intestinal L cell. In contrast, in the pancreatic α cell, proglucagon processing by PC2 produces glucagon as well as glicentin-related pancreatic peptide (GRPP) and the major proglucagon fragment (MPGF).
secretion and present comparable metabolism and clearance rates (7), one study does suggest that the amidation of GLP-1 may slightly increase the half-life of GLP-1 in the plasma (15).

### 1.2.2 The GLP-1 receptor and GLP-1 actions in the body

The receptor for GLP-1 was first cloned from a cDNA library constructed from rat islets (16). This receptor is composed of 463 amino acids and displays high homology with the glucagon, GLP-2 and GIP (glucose-dependent insulintropic peptide) receptors, also known as the Class B group of G protein-coupled receptors (GPCRs) (16-18). As a GPCR, the GLP-1 receptor contains seven transmembrane domains and activates heterotrimeric G proteins to transmit signals through second messenger pathways. Specifically, the third intracellular loop has been demonstrated to be crucial for coupling to G-proteins (19;20). This receptor has been shown to link to Gαs, Gαq, Gαi and Gαo, and is thus capable of activating the adenylyl cyclase (AC)-protein kinase A (PKA) pathway and the phospholipase C (PLC)-protein kinase C (PKC) pathway, among others (19-21). The GLP-1 receptor has a wide distribution in the body, including the α, β and δ cells in pancreatic islets, the heart, lungs, kidneys, intestines, stomach, vagus nerve and various regions in the brain (22-27). Due to this widespread distribution of the GLP-1 receptor, this hormone induces a diverse array of effects on the body.

The major biological actions of GLP-1 are anti-diabetic in nature, mediated most notably via direct effects on the pancreatic islets. This includes enhancement of glucose-dependent insulin secretion as well as increasing β cell proliferation and anti-apoptotic pathways (11;28-32). GLP-1 also replenishes insulin stores by increasing insulin gene transcription and mRNA translation (29). Moreover, GLP-1 inhibits glucagon secretion from
the α cell and increases the secretion of somatostatin from the δ cell (11;33), all of which aid in controlling blood glucose levels.

GLP-1 is one of the two known intestinal incretin hormones, the other being GIP. The incretin effect is described as a significantly greater increase in plasma insulin levels in response to an oral glucose load as compared to the insulin levels secreted in response an isocaloric glucose load administered intravenously. Both incretin hormones have the ability to increase glucose-dependent insulin secretion from pancreatic β cells (34). The GLP-1 receptor knockout mouse displays only a slight disturbance in the control of blood glucose levels, most probably due to compensation by the second incretin (35). Indeed, one study demonstrated the upregulation of GIP secretion and action in the GLP-1 receptor knockout mouse (36). Similarly, the GIP receptor knockout mouse also displays only mild glucose intolerance (37). As such, the double incretin receptor knockout (DIRKO) mouse was derived and these mice display moderate hyperglycemia (38). A more recent study has shown that DIRKO mice are protected from high fat diet-induced obesity, partially explained by increased locomotor activity and energy expenditure. Moreover, DIRKO mice do not show increased islet insulin content on a high fat diet, which is observed in wild-type mice. At the same time, DIRKO mice preserve insulin sensitivity in peripheral tissues, compensating for lower levels of stored insulin (39).

In addition to its effects on islet hormones, GLP-1 exerts effects on the liver and peripheral tissues. For example, GLP-1 decreases endogenous glucose production and increases glycogen synthesis in the liver (40;41). In adipose tissue and muscle, GLP-1 enhances the uptake and storage of glucose (40-42). However, the presence of the GLP-1 receptor, as found
in pancreatic β cells, is still controversial in these extra-pancreatic tissues and the effects of GLP-1 in these tissues may therefore be mediated indirectly.

Neurons in the nucleus of the solitary tract of the brainstem are the major source of GLP-1 in the brain and the GLP-1 receptor, in turn, is found in regions known to regulate energy balance, including the hypothalamus and the arcuate and paraventricular nuclei (3). Although GLP-1 has the ability to cross the blood-brain barrier (BBB) (43), it can also stimulate the brain by activating peripheral receptors on vagal afferent nerves (25). Whether administered peripherally or centrally, GLP-1 acts as an anorexic hormone that plays a role in satiety. Studies have shown that centrally administered GLP-1 decreases food intake in fasted rats and the GLP-1 receptor agonist, exendin-4, decreases food intake and body weight in rats when injected peripherally (44;45). Central nervous system (CNS) actions of GLP-1 also include effects on gastric motility and cardiovascular function, as discussed below (11).

In the stomach, GLP-1 decreases gastric acid secretion and slows gastric emptying of food contents into the small intestine, a phenomenon known as the ‘ileal brake’ (46). Although the GLP-1 receptor is additionally found in the stomach, it has been shown that the CNS plays a crucial role in these effects, by way of the GLP-1 receptor on vagal afferent neurons (47). By slowing gastric emptying and gastrointestinal motility, the absorption of nutrients from the lumen of the intestine is delayed, giving the body more time to control blood glucose levels as well as to digest nutrients such as fats (48;49).

There is evidence of GLP-1 receptor expression in the human and rodent heart (26;27). Peripheral or central administration of GLP-1 increases mean arterial blood pressure and heart rate in rodents. The addition of the GLP-1 receptor antagonist exendin-4\textsuperscript{9-39} blocks these effects of GLP-1, pointing to a role for both peripheral and central GLP-1 receptors in mediating
effects on the cardiovascular system (50). GLP-1 additionally plays a role in cardioprotection, as seen in models of cardiac injury and heart failure. In dogs with dilated cardiomyopathy, GLP-1 improves glucose uptake and left ventricular function (51), whereas in studies using isolated rodent hearts, GLP-1 protects the tissue against ischemia/reperfusion injury (27;52).

Due to its potent anti-diabetic actions, GLP-1 mimetics are currently in use in the clinic to treat hyperglycemia in patients with T2D. Common drugs include exenatide (GLP-1 receptor agonist; synthetic form of exendin-4) and liraglutide (long-acting GLP-1 analog) (53;54). These drugs are administered subcutaneously and have been shown to decrease glycosylated hemoglobin levels, an indicator of plasma glucose concentration, as well as reduce body weight in T2D patients, with the main adverse side effect being nausea (53;54).

1.2.3 Metabolism and clearance

GLP-1 is metabolized and cleared very rapidly from the body. After release from the intestinal L cell, GLP-1 is a target for the enzyme dipeptidyl peptidase-4 (DPP4). DPP4 cleaves off the first two N-terminal amino acids of GLP-1 \(^{7-37}\) and GLP-1 \(^{7-36\text{NH}_2}\), producing the biologically inactive forms of GLP-1 \(^{9-37}\) or GLP-1 \(^{9-36\text{NH}_2}\), respectively (55). It is estimated that only about 25% of secreted GLP-1 enters the portal circulation intact, owing to the fact that DPP4 is found on the intestinal brush border and on endothelial cells of capillaries within the lamina propria (56). Further degradation of approximately 40-50% occurs upon reaching the liver, and therefore only about 10-15% of secreted GLP-1 enters the systemic circulation in the biologically-active form (57;58). Once in the systemic circulation, DPP4 continues to target GLP-1 and is responsible for the short GLP-1 half-life of approximately 1-2 min (58).
Metabolized GLP-1 is cleared mainly by the kidneys. The metabolite too has a relatively short half-life of about 4-5 min before being removed from the body (59).

Due to the major role DPP4 plays in the inactivation of GLP-1, DPP4 inhibitors are currently being used to treat T2D patients. Three DPP4 inhibitors currently available include sitagliptin, saxagliptin and vildagliptin. These drugs help decrease blood glucose levels and are weight neutral, neither increasing nor decreasing body weight in patients (60). A recent study has indicated an increased incidence of pancreatitis and pancreatic cancer in patients taking sitagliptin or the GLP-1 receptor agonist, exenatide (61). These findings, however, are controversial (62) and need to be furthered to draw more significant conclusions about the use of these compounds. Hence, an alternative to the currently available T2D treatments would be to increase endogenous GLP-1 secretion, in combination with DPP4 inhibitors.

1.2.4 Secretion

The secretion of GLP-1 in response to nutrients occurs in a biphasic manner in which the first rapid peak of secretion is observed 15-30 min postprandially and the second more prolonged phase of secretion occurs at 90-120 min (63;64). Termed the indirect and direct phases of secretion, respectively, the indirect phase mediates nutrient-induced GLP-1 secretion via vagal nerve stimulation in the proximal intestine. In the direct phase, luminal nutrients directly interact with the L cell in the distal intestine (Fig. 1.2) (65;66).
Figure 1.2 Indirect and direct mechanisms regulating GLP-1 secretion. The secretion of GLP-1 in response to nutrients occurs in a biphasic manner in which the first rapid peak of secretion is observed 15-30 min postprandially (indirect phase) and the second more prolonged phase of secretion occurs at 90-120 min (direct phase). The indirect phase mediates nutrient-induced GLP-1 secretion through a proximal-distal loop. Nutrient ingestion increases GIP secretion from the proximal K cell in rodents (CCK secretion from the I cell in humans) that then activates the vagus nerve and leads to GLP-1 secretion from the L cell in the distal intestine. Signaling occurs through the release of acetylcholine (Ach) and the cholinergic receptor M1 (M1R). In the direct phase, luminal nutrients (especially fats) directly interact with the L cell in the distal intestine to increase GLP-1 secretion.
1.2.4.1 Indirect mechanisms regulating GLP-1 secretion

The initial rapid rise of GLP-1 following a meal occurs before nutrients reach the L cell in the distal ileum and colon (1,67), indicating an indirect signaling pathway in the proximal intestine. Studies in a rat model demonstrated that placing fat into the duodenum while preventing nutrient flow into the distal intestine stimulates L cell secretion. Moreover, removal of the entire intestine distal to the fat-infused duodenum prevents any response, supporting the notion of a proximal-distal signaling loop (68,69). Nutrients in the proximal intestine additionally increase the level of GIP, secreted from the proximal intestinal K cell, such that increasing GIP levels correlate with increasing L cell secretion. An infusion of GIP in the absence of nutrients is similarly able to increase L cell secretion in a rat model and treating fetal rat intestinal cell cultures (FRICs) with GIP also stimulates L cell secretion (69,70). Briefly, FRICs are a heterogeneous primary L cell model produced by enzymatically dispersing fetal rat intestines. The cells can be grown in culture for up to several days and have been shown to synthesize and secrete proglucagon-derived peptides (70-72). Further work identified the vagus nerve in regulating this neuroendocrine loop, such that a vagotomy eliminates the GLP-1 response to fat placed in the duodenum (73). GIP was also further implicated in the regulation of the proximal-distal loop in rats by demonstration of the requirement for an intact vagus nerve in its actions on the L cell. The early phase of nutrient-induced GLP-1 secretion is therefore regulated by GIP and the vagus nerve in rodent models (73). In humans, it is cholecystokinin (CCK) secreted from the intestinal I cell that regulates this enteroendocrine loop (74).

Further studies of the proximal-distal loop demonstrated that the cholinergic agonists bethanechol and carbachol stimulate GLP-1 secretion in FRICs as well as in the murine
GLUTag and human NCI-H716 L cell models, supporting the involvement of the neurotransmitter acetylcholine (70;75;76). In brief, the GLUTag L cell line was derived from an enteroendocrine tumour that arose after placing the SV40 large T antigen under the control of the proglucagon promoter in transgenic mice. The tumour cells were then passaged through nude mice and single-cell cloned to produce an immortalized cell line (77;78). In contrast, the human NCI-H716 L cell model was originally obtained from cells contained in ascites fluid from a patient with a poorly-differentiated adenocarcinoma in the cecum and is presumed to be heterogeneous (79;80). The presence of cholinergic muscarinic receptors (e.g. M1, M2 and M3) has been demonstrated in rat ileal sections, FRIC L cells, paraffin-embedded human small intestinal sections and human NCI-H716 L cells (76;81). Additionally, pirenzepine (M1 antagonist) and gallamine (M2 antagonist) block bethanechol-stimulated GLP-1 secretion in FRIC cultures and human NCI-H716 cells (76;81). In a rat model, pirenzepine, but not gallamine, also reduces fat-induced GLP-1 secretion (76). Hence, the muscarinic receptor M1 plays a role in vagal control of GLP-1 secretion in human and both fetal and adult rat L cell models, whereas the M2 receptor seems to play a role in human and fetal rat cells (76;81). The M3 antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) is not able to inhibit fat-induced GLP-1 secretion in the rat model, nor does it inhibit bethanechol-stimulated GLP-1 secretion in human NCI-H716 cells. However, 4-DAMP increases GLP-1 secretion in FRIC cultures, pointing to a potential role for the M3 receptor in fetal rat L cells (76;81). Studies have also supported the involvement of second neural regulator, gastrin-releasing peptide (GRP), released from neurons in the enteric nervous system. Infusing GRP into anesthetized rats increases GLP-1 but not GIP levels and administration of a GRP antagonist attenuates the L cell response to fat-infusion into the duodenum (82). Similarly, GRP receptor null mice
display impaired GLP-1 and insulin levels after gastric glucose administration (83). Taken together, therefore, the proximal-distal loop in rodents is activated upon nutrient ingestion, which increases GIP secretion from the proximal intestinal K cell (CCK from the I cell in humans). GIP then activates the vagus nerve, leading to GLP-1 secretion from the L cell in the distal intestine. Signaling occurs through the release of acetylcholine and the cholinergic receptor M1, as well as though the locally-released neuropeptide GRP (65).

Hormones such as insulin and leptin have also been shown to regulate GLP-1 secretion. Insulin treatment leads to the phosphorylation of Akt and ERK, both known to participate in the insulin signaling pathway, and increases GLP-1 secretion in GLUTag, NCI-H716 and FRIC cultures. Furthermore, in conditions of insulin resistance, insulin-induced GLP-1 secretion is reduced (84). The MKR insulin-resistant mouse displays both higher fasting plasma insulin and GLP-1 levels but also has an impaired early (e.g. 10 min) GLP-1 response to oral glucose. The results are suggestive of a positive-feedback loop, in which GLP-1 increases glucose-dependent insulin secretion from the β cell and, in turn, insulin increases GLP-1 secretion from the L cell (84). The adipocyte hormone leptin, known to play a critical role in energy homeostasis, also increases GLP-1 release in the same three in vitro L cell models (85). Furthermore, leptin resistant, obese mice display impaired early (e.g. 10 min) responses to orally-administered glucose (85). A decrease in L cell responsiveness in conditions of both leptin and insulin resistance may help explain the decreased GLP-1 levels that have been observed in obesity and T2D, respectively (86;87). Together, therefore, these hormones appear to play a permissive role in the first phase of GLP-1 release following nutrient ingestion.
1.2.4.2 Direct mechanisms regulating GLP-1 secretion

The second or later phase of GLP-1 release occurs as a result of direct nutrient stimulation of the intestinal L cell. Nutrients such as sugars, peptides and fats have been shown to increase the level of GLP-1 secretion (65). Placement of glucose directly into the ileal lumen stimulates L cell secretion in rodents (69). Furthermore, in murine GLUTag L cells, both glucose and fructose increase cellular electrical activity, associated with membrane depolarization and the closure of $K_{ATP}$ channels, leading to GLP-1 granule exocytosis (88;89). Sodium-induced depolarization is also associated with sodium-glucose co-transport in these cells (89). Peptones increase GLP-1 release \textit{ex vivo} in the isolated perfused rat intestine and \textit{in vitro} in the secretin tumour cell line (STC-1). Treating STC-1 cells with peptones additionally increases proglucagon mRNA levels (90). STC-1 cells are a heterogeneous enteroendocrine cell line derived from a mouse expressing two oncogenes under the control of the rat insulin promoter. Specifically, the cells for the STC-1 cell line were collected from a small intestinal endocrine tumour and have been shown to secrete proglucagon-related peptides as well as secretin, GIP and somatostatin, among other hormones (91). Meat hydrolysate also stimulates GLP-1 secretion from human NCI-H716 cells in a dose-dependent manner (80). Moreover, a recent study in NCI-H716 cells showed that essential branched-chained amino acids, such as those found at high concentrations in dairy products, exert an anti-obesity effect by increasing GLP-1 secretion and decreasing the mRNA levels of genes involved in fatty acid and cholesterol absorption and synthesis (92). Hydrolysate from the corn protein zein also greatly enhances GLP-1 secretion in rats, either directly when placed in the ileum, or indirectly when infused into the duodenum. Underlining the direct and indirect phases of GLP-1 secretion,
treating the vagus nerve with capsaicin abolishes the duodenal but not the ileal effect of zein hydrolysate in this in vivo model (93).

Although placing nutrients, such as glucose and zein, directly into the lumen of the ileum increases GLP-1 levels (69;93), fat has been suggested to be a more physiological, direct regulator of GLP-1 release since it reaches the distal intestine in high concentrations, as opposed to glucose and peptones (67;94;95). Previous in vitro studies using the FRIC L cell model demonstrated the ability of long-chain monounsaturated fatty acids (MUFAs), such as oleic acid (OA), to stimulate L cell secretion, whereas saturated fatty acids elicit no response (96). Furthermore, the requirement for a free carboxy end was discovered when the methyl ester of OA had no significant effect on increasing L cell secretion. The L cell response to fat is also chain length-dependent, requiring 16 carbons or greater (96). The murine GLUTag L cell model has also been shown to respond to MUFAs (97). Moreover, MUFAs improve glycemic tolerance in vivo by increasing the secretion of GLP-1 (98). Consistent with these experimental findings, a study in humans showed improved postprandial glucose and GLP-1 levels in insulin-resistant individuals fed a MUFA-rich diet (99).
1.3 Cellular mechanisms of fatty acid action

1.3.1 G protein-coupled receptors

Several GPCRs have been reported to be regulated by free fatty acids. GPR41 and GPR43 are activated by short-chain fatty acids in the colon and induce colonic smooth muscle contraction (100;101). GPR84 is regulated by medium-chain fatty acids and is located on immune cells, participating in lipopolysaccharide-initiated immune responses (102). Furthermore, TGR5, expressed in brown adipose tissue and muscle, is stimulated by bile acids to increase energy expenditure. Recent studies have also shown the presence of this receptor in the enteroendocrine L cell such that stimulation of NCI-H716 and STC-1 cells with bile acids or the TGR5 agonist INT-777 increases GLP-1 secretion and intracellular cAMP levels (103;104). Furthermore, transgenic mice over-expressing TGR5 display improved glucose tolerance on a high fat diet, and a test meal challenge to stimulate bile acid release from the gallbladder produces robust GLP-1 and insulin secretory responses in these mice. TGR5 null mice, in turn, display impaired glucose tolerance (104). In contrast, GPR40, GPR120 and GPR119 have been particularly implicated as long-chain fatty acid receptors on the L cell (105-107). GPR40 and GPR120 respond to saturated fatty acids (105) and polyunsaturated fatty acids (PUFAs), respectively (107). Both of these GPCRs are Gαq-coupled receptors and signal through the PLC-dependent PKC-calcium pathway (108). GPR120 mRNA is present in human and mouse intestinal tracts as well as in the endocrine STC-1 cell line. Long-chain unsaturated fatty acids, especially the PUFA α-linolenic acid, increase GLP-1 secretion from STC-1 cells, while medium-chain and saturated fatty acids elicit no response (107). Both calcium and ERK are known mediators of the GPR120 signaling pathway. ERK phosphorylation is significantly increased in α-linolenic acid-treated STC-1 cells, and incubation in calcium-free media inhibits
α-linolenic acid-stimulated release. Interestingly, the α-linolenic acid methyl ester displays a reduced effect on GLP-1 release, underlining again the importance of a free-carboxy end in the initiation of GLP-1 secretion (107). Another fatty acid receptor, GPR40, is also present in enteroendocrine cells such that staining for GPR40 shows co-localization with GLP-1 in the mouse intestine. GPR40 null mice administered a high fat diet exhibit reduced GLP-1 and insulin levels as well as impaired glucose clearance, suggesting a role for GPR40 in saturated fatty acid-induced GLP-1 secretion (105). GPR40 is additionally known to be located on the pancreatic β cell and is involved in free fatty acid-mediated insulin release (109).

Unlike GPR40 and GPR120, GPR119 is a Gαs-coupled receptor that signals through the AC-cAMP-PKA pathway (108). This receptor is present on the pancreatic β cell and stimulation of GPR119 with the agonist AR231453 enhances glucose-dependent insulin release (110). In addition to the pancreas, GPR119 mRNA was detected in three validated L cell models and in rodent and human intestinal sections, indicating the presence of this receptor on the L cell (106;111). GLP-1 levels increase in murine GLUTag cells following treatment with AR231453 and AR231453-treated mice demonstrate increased plasma GLP-1 after an oral glucose dose. When treated with the GLP-1 receptor antagonist, exendin-4^9-39, mice no longer display improved glucose tolerance, indicating the necessity of the GLP-1 receptor in this response (111). The endogenously-occurring fatty acid derivative oleoylethanolamide (OEA) is a more physiologically relevant GPR119 ligand that also increases GLP-1 secretion via GPR119. OEA treated GLUTag cells show an increase in both GLP-1 and cAMP levels, whereas PKA inhibition or GPR119 siRNA silencing diminishes these OEA-stimulated effects. In the intestine, OEA is degraded by the enzyme fatty acid amide hydrolase into OA and ethanolamido. Prevention of OEA degradation by the fatty acid amide hydrolase inhibitor,
URB597, further increases the GLP-1 secretory response. When administered directly into the rat intestine, OEA increases plasma GLP-1, as well as insulin levels when rats are subjected to a hyperglycemic clamp (106). This OA derivative has also been linked to satiety in previous studies, and OEA levels are known to decrease during fasting and to increase upon re-feeding (112). As GLP-1 is known signal of satiety, it is possible that OEA-induced satiety is partially mediated through GPR119 and GLP-1 secretion.

Thus, while the MUFA OA stimulates GLP-1 secretion from the intestinal L cell, whether it functions through a GPCR is still unknown. However, OA-induced GLP-1 secretion is not associated with increased intracellular calcium levels or activation of Akt or ERK, suggesting GPR40 or GPR120 are not involved in the L cell response to OA (97).

### 1.3.2 PKCζ

The PKC family of proteins is composed of 12 isozymes that are grouped into the classes of classical (cPKC: -α, -βI, -βII, -γ), novel (nPKC: -δ, -ε, -θ, -η), atypical (aPKC: -ζ, -ι/λ) and PKC-related proteins (-µ, -ν) (113;114). Studies specifically examining the atypical isozyme PKCζ have shown that PKCζ is activated by mono- and polyunsaturated fatty acids but not by saturated fatty acids. In addition, this PKC isoform does not respond to calcium or diacylglycerol/phorbol ester activation, as do classical and novel isoforms (115). Importantly, in the L cell, the actions of the OA have been shown to be mediated via PKCζ (94;97). The murine GLUTag cell line expresses PKCζ mRNA, and PKCζ protein is found in the cytoplasm of GLUTag and FRIC cells, as well as in mouse and rat intestinal sections (97). GLP-1 secretion from the L cell is increased when treated with OA, whereas both the PKCζ/λ inhibitor ZI and siRNA-mediated knockdown of PKCζ eliminate this effect (97). In addition, inhibition
of classical and novel PKC isoforms does not abrogate OA-induced GLP-1 secretion (96;97).

Finally, PKCζ also mediates the effects of OA on the L cell in vivo. OA administration directly into the rat ileum and colon increases GLP-1 levels in the plasma, and this effect is inhibited by pre-treatment with ileocolonic adenoviral siRNA targeting PKCζ (94). However, although OA enters the distal intestine in sufficient quantities to stimulate PKCζ-mediated GLP-1 secretion (94), it is not yet known whether OA stimulates PKCζ directly in the L cell or indirectly through an unknown mechanism. Nonetheless, the murine GLUTag L cells have the ability to take up C1-Bodipy-C12, an OA analog, in a saturable manner, suggesting that OA is internalized by the L cell (94). To determine whether this uptake process is passive or active was the purpose of this current study.
Figure 1.3 Fatty acid transport proteins in cellular fatty acid uptake. Long-chain fatty acids (LCFAs) may interact either with fatty acid transport proteins (FATPs) or CD36 on the plasma membrane to be transported into the L cell in a carrier-mediated fashion. Once in the cell, LCFAs may bind to fatty acid binding proteins (FABPs) and/or be coupled to a CoA group by an acyl-CoA synthetase (ACS). Alternatively, GPR40/120 and GPR119 (GPCRs) have been implicated as fatty acid and fatty acid derivative receptors, respectively, activating the Gaq (GPR40 and GPR120) or Gas (GPR119) intracellular signaling pathway.
1.4 Transport of fatty acids

1.4.1 Fatty acid transport proteins

Though the topic of fatty acid transport has remained controversial, it is generally believed that the predominant mechanisms of fatty acid uptake consist of passive diffusion as well as a saturable protein-mediated process (116;117). Passive diffusion includes the use of a ‘flip-flop’ mechanism that occurs independently of any transport proteins. In this proposed transport process, fatty acids must dissociate from a carrier molecule, bind to the plasma membrane and flip over into the interior of the cell (118).

Supporting the alternate mechanism of active uptake, several candidates for protein-mediated transport have been identified, specifically intracellular fatty acid binding proteins (FABPs) (119;120), the family of plasma membrane associated fatty acid transport proteins (FATPs) (116;121), and CD36/fatty acid translocase (122;123) (Fig. 1.3).

The FABP family is comprised of intracellular proteins approximately 15 kDa in size, with the ability to bind long-chain fatty acids (LCFAs). These proteins have been implicated in tissue-specific functions that are linked to fatty acid uptake, trafficking and metabolism (119;120). In the GLUTag L cell specifically, the intestinal isoform of FABP has been shown to be present at both the mRNA and protein level (P.L.Brubaker, unpublished data; and (94)), though its role in L cell metabolism has yet to be investigated.

In contrast, identified by examining fluorescent fatty acid uptake, FATP1 was the first FATP family member discovered and described as a plasma membrane protein that increases the uptake of fatty acids when stably expressed in cell lines (124). Additionally, FATP1 is the major FATP isoform expressed in adipose tissue (116;124;125). A large family of homologous LCFA transporters was subsequently discovered and termed FATP 2-6 (121). The major
distributions of the FATP homologues has been described as follows: FATP2 in the liver and
kidney (126); FATP3 in the adrenal cortex, lungs, ovaries and testis (125); FATP4 in the
intestine and skin (127;128); FATP5 in the liver (129); and FATP6 as the major cardiac FATP
(130). FATPs are approximately 70 kDa integral transmembrane proteins with at least one
membrane-spanning domain (131). Moreover, these proteins possess two identifying sequence
motifs – an ATP/AMP motif required for ATP binding and the FATP/VLACS (very long-chain
acyl-CoA synthetase) motif, found exclusively in FATP family members. A recent study also
identified a 73 amino acid region between the ATP/AMP and FATP/VLACS domains,
common to both FATP1 and FATP4, that is thought to be involved in fatty acid transport (132).
The FATP proteins additionally exhibit acyl-CoA synthetase activity, catalyzing the
esterification of coenzyme A with fatty acids, with specificity for long-chain or very long-chain
fatty acids (133-135).

Null mouse models for several of the fatty acid transport proteins have been developed.
FATP1 null mice have no visual phenotype distinguishing them from wild-type littermates.
These mice, however, are protected from developing insulin resistance and muscle
accumulation of fatty acyl-CoA when placed on a high fat diet (136). Fatty acid uptake and
triglyceride synthesis patterns are also altered post-prandially in FATP1 null mice, wherein
fatty acid accumulation decreases in adipose and skeletal muscle and increases in the liver
(137). In contrast, the FATP2 knockout mouse shows decreased hepatic peroxisomal VLACS
activity but otherwise displays normal liver and kidney histology (138). Further studies
revealed that FATP2 is a multifunctional protein with both hepatic peroxisomal VLACS and
fatty acid uptake activities (126). A knockout mouse model for FATP4 has also been created.
FATP4 is the most abundant FATP isoform in the small intestine (127), but also plays a role in
skin homeostasis (128). A spontaneous, autosomal recessive mutation in exon 3 in \textit{Slc27a4} (the FATP4 encoding gene) produced a ‘wrinkle-free’ phenotype, such that homozygous mice were born with thick, tight, shiny skin and a defective skin barrier, dying shortly after birth (128). This phenotype was later confirmed in mice with a targeted disruption of the FATP4 gene (139). Mutations in FATP4 have also recently been described in patients with ichthyosis prematurity syndrome, a condition characterized by premature birth with the infant covered in thick, caseous, desquamating skin and respiratory complications, followed by lifelong dry, thick skin (140). The lethality of the FATP4 null mouse phenotype and most of its skin defects can be rescued by skin keratinocyte-specific transgenic expression of FATP4, driven by the involucrin promoter (141). These \textit{Fatp4\textsuperscript{−/−};Ivl-Fatp4\textsuperscript{tg/+}} FATP4 null mice seem to display no compensatory upregulation of other FATP isoforms and, additionally, show that FATP4 is dispensable for lipid absorption in intestinal enterocytes (142). FATP5, in turn, is the FATP isoform expressed in the liver. FATP5 knockout mice demonstrate a significant decrease in the uptake of LCFAs and as well as impaired bile acid conjugation in hepatocytes (129;143). Interestingly, FATP5 null mice fail to gain weight on a high-fat diet despite showing normal fat absorption, suggesting a further role for FATP5 in body weight regulation (143). Null mouse models for the remaining FATP isoforms, FATP3 and FATP6, have not yet been developed.

CD36, also known as fatty acid translocase, is found in two molecular weight species; a heavily glycosylated 88 kDa form localized on the plasma membrane and a 54 kDa cytosolic form (144;145). CD36 demonstrates broad expression that includes the heart, skeletal muscle, intestinal enterocytes, capillary endothelium, and monocytes/macrophages. Its ligands too encompass many different and varying molecules, most notably LCFAs and oxidized low-density lipoproteins (LDLs). CD36 is a Class B scavenger receptor and plays a major role in
the development of atherosclerosis. For example, CD36 aids in the internalization of oxidized LDL in macrophages, leading to the formation of foam cells (145). It was shown that CD36 null mice (146) are relatively well-protected from forming atherosclerotic plaques, and the macrophages derived from these animals are deficient in oxidized LDL uptake and foam cell formation (147). CD36 has also been reported to be required for the enterocyte absorption of saturated very long-chain fatty acids (VLCFAs), providing evidence of protein-facilitated fatty acid uptake in the intestine (122). In a separate study, CD36 was demonstrated to be necessary for the absorption of fatty acids and cholesterol in the proximal but not distal intestine (123). Interestingly, the intestinal hormone GLP-2 increases intestinal lipid absorption and chylomicron production in a CD36-dependent manner (148). Though necessary for fatty acid absorption in the intestinal enterocytes, a role for CD36 in enteroendocrine cells has not yet been defined.

Since the identification of the FATPs and CD36, studies have been investigating the prospect of carrier-mediated fatty acid transport. A recent study has implicated FATP1, FATP4 and CD36 in the transport of fatty acids, including OA, across the blood-brain barrier (BBB). Using a monolayer of human brain microvessel endothelial cells (HBMEC), $^{14}$C-oleate transport has been detected that decreases upon treatment with phloretin, a non-specific inhibitor of carrier-mediated transport (149). Knockdown of FATP1, FATP4 and CD36 with siRNA reduces the transport of fatty acids across the HBMEC monolayer. FATP1 and FATP4 appear to be quite selective, such that siRNA silencing mainly affects the transport of LCFAs, including OA. In contrast, CD36 is a more general transport protein, with knockdown in HBMEC cells decreasing the transport of short-, medium- and long-chain saturated and unsaturated fatty acids. Overall, unsaturated fatty acids are able to across the HBMEC
monolayer much more efficiently than saturated fatty acids with a similar chain length (149;150). Notably, transport was detected both in the apical to basolateral direction and in the basolateral to apical direction, although the transport co-efficient is more than doubled in the former as compared to the latter direction. As the ratio of the two co-efficients does not equal one, these findings indicate that there is a net influx of OA into the basolateral media and suggests that activate protein-mediated transport is involved in lieu of simple, passive diffusion (150).

1.4.2 Subcellular localization

Though initially thought to be plasma membrane proteins, FATP isoforms have also been shown to be present in various subcellular localizations. Studies in adipocyte cells have demonstrated that FATP1 is present in the perinuclear compartment and that insulin treatment induces translocation to the plasma membrane, resulting in increased fatty acid uptake (151). Consistent with these findings, adipocytes from FATP1 null mice show no decrease in basal fatty acid uptake, but fail to increase uptake in response to insulin. Similar findings were made in skeletal muscle of FATP1 null mice (137). Interestingly, another group demonstrated that FATP1 also localizes to the mitochondria in cultured skeletal myotubes and that overexpression of FATP1 increases the production of carbon dioxide, suggesting an effect on glucose metabolism (152). In hepatocytes, FATP2 localizes both to the plasma membrane and to peroxisomes, indicative of being a multifunctional protein (126). FATP3, in turn, appears to co-localize with the endoplasmic reticulum (ER) and, partially, with the mitochondrial fraction. Interestingly, in MA-10 Leydig cells, knockdown of FATP3 decreases acyl-CoA synthetase activity but not fatty acid uptake (135). Furthermore, expression of FATP3 in yeast lacking the
endogenous ability to take up fatty acids only weakly increases uptake. In contrast, expressing murine FATP1, FATP2 and FATP4 significantly improves fatty acid uptake. FATP3 does, however, promote an increase in acyl-CoA synthetase activity in these yeast cells (153). FATP4 is the major FATP isoform found in the small intestine (116;127) and was initially thought to be localized on the plasma membrane on the apical side of enterocytes, based upon immuno-staining of mouse small intestine (127). However, this view has recently been challenged, most notably in a study localizing FATP4 to the ER-membrane where, despite its ER localization, FATP4 still increases the rate of fatty acid uptake, attributed to its acyl-CoA synthetase activity (154). Isoform FATP5 is localized to the cellular plasma membrane in isolated mouse hepatocytes and in mouse liver sections, and this is consistent with its proposed roles in hepatic fatty acid uptake and bile acid conjugation (129;143). The last FATP family member, FATP6, is the predominant isoform found in the heart. The majority of FATP6 mRNA is found in cardiac myocytes and immunofluorescence microscopy in murine heart localizes FATP6 to the sarcolemma, with increased expression in regions adjacent to microvasculature. Co-localization with CD36 has also been noted (130). A separate study showed that electrostimulation of isolated rat cardiac myocytes increases the uptake of palmitate. This uptake process proved to be CD36-dependent such that inhibition of CD36 with the CD36-specific inhibitor sulfo-N-succinimidyl oleate (SSO) abolishes the uptake response (155). Like FATP1, CD36 also displays the ability to translocate from intracellular compartments to the plasma membrane. Its translocation in cardiac and skeletal muscle is regulated by both insulin and muscle contraction (156). Notably, CD36 translocation to the plasma membrane requires this receptor to be glycosylated to form its 88 kDa isoform (144). In insulin-resistant muscle, some of the CD36 protein is permanently found on the plasma
membrane, thus continually increasing fatty acid uptake (156). A more recent study using murine hindlimb muscle cells has confirmed that insulin and muscle contraction induce CD36 and FAPT1 translocation to the plasma membrane in an additive fashion. Additionally, the study showed that FATP4, expressed in muscle at lower levels, can also translocate, while isoform FATP6 does not (157).


### 1.5 Hypothesis and aims

OA is known to increase GLP-1 release via PKCζ (97). The murine intestinal L cell has also been shown to take up an OA analog, although the mechanism underlying this phenomenon (i.e. diffusion or protein-mediated transport) is not known (94). Previous studies have demonstrated mRNA transcript expression for the fatty acid transport proteins CD36 and FATP1, 3 and 4 in murine GLUTag L cells (94). Whether any of these L cell fatty acid transport proteins are involved in OA-induced GLP-1 secretion remains to be established. The hypothesis for the current study states that intestinal L cell fatty acid transport proteins play a role in the regulation of OA-induced GLP-1 secretion. Aims for this project included:

i) To confirm that OA induces GLP-1 secretion in the L cell.

ii) To establish that OA is taken up by the L cell.

iii) To determine whether this uptake process is protein-mediated.

iv) To determine whether inhibiting fatty acid transport proteins, either indirectly or directly, decreases the uptake of OA, and subsequently, decreases GLP-1 secretion.

v) Based on results from aims i-iv, to determine whether FATP4 null mice demonstrate lower plasma levels of GLP-1 following intraileal OA administration, as compared to wild-type mice.

These aims were addressed using the validated murine GLUTag and human NCI-H716 L cell models, as well as the FATP4 null mouse model.
2. METHODS

2.1 In vitro cell models

Murine GLUTag L cells (77;78) were cultured in Dulbecco’s modified eagle medium (DMEM; Gibco Invitrogen, Burlington, ON) which contained 25 mM glucose and 10% fetal bovine serum (FBS). The medium was changed every 2-3 days. Cells were trypsinized (Sigma-Aldrich, St. Louis, MO) to passage and seeded at a dilution of 1:3. Human NCI-H716 L cells (ATCC, Manassas, VA) were cultured in suspension in RPMI-1640 media (Gibco Invitrogen) which contained 25 mM glucose and 10% FBS. The medium was changed every 3-4 days and the cells were seeded at a dilution of 1:3 when passaged. Both the GLUTag and NCI-H716 cells have been previously validated as L cell models in terms of appropriate GLP-1 secretion responses to known secretagogues (75;80).

In preparation for siRNA transfection, GLUTag cells were plated with DMEM + 10% FBS in 24-well plates coated with poly-D-lysine hydrobromide (Sigma-Aldrich) and allowed to recover for 48 h. Cells were transfected using either scrambled control siRNA or siRNA targeting FATP4 in Opti-MEM I media (Gibco Invitrogen). The siRNA for the first knockdown (1 siRNA sequence; Ambion, Austin, TX) was used at a concentration of 100 nM along with 1.25 µL of Lipofectamine 2000 transfection reagent (Invitrogen) in a final volume of 0.5 mL. The siRNA for the second knockdown (Smartpool siRNA – mixture of 4 siRNA sequences; Dharmacron, Lafayette, CO) was used at a concentration of 50 nM along with 2.25 µL of DharmaFECT 4 transfection reagent (Dharmacon) in a final volume of 0.5 mL. Cells were incubated with the siRNA for 5 h, washed twice with DMEM + 10% FBS and allowed to recover for 48 h post-transfection.
2.2 Immunoblot

Non-transfected GLUTag cells were grown in 6-well plates coated with poly-D-lysine hydrobromide (Sigma-Aldrich) and NCI-H716 cells were grown in 6-well plates coated with Matrigel (Becton Dickinson, Bedford, MA). GLUTag cells transfected with siRNA were grown in 24-well plates coated with poly-D-lysine hydrobromide (Sigma-Aldrich). All cells were allowed to recover for 48 h after plating and transfection. Cells were scraped and collected into RIPA buffer and lysed by sonication. Protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA) and 100 µg of protein per sample was loaded onto a 10% gel. The protein samples were transferred overnight at 30 V onto Immun-Blot PVDF membrane (Bio-Rad). The immunoblot was probed with rabbit anti-FATP1, 3 or 4 (1:1000; gifts from Dr. Andreas Stahl, University of California Berkeley, Berkeley, CA), rabbit anti-CD36 (1:1000; Cayman Chemicals, Ann Arbor, MI), and rabbit anti-actin (1:4000; Sigma-Aldrich), followed by detection using horseradish peroxidise-linked goat anti-rabbit IgG (1:2000; Cell Signaling Technology, Beverly, MA) and Amersham ECL Western blotting detection reagent (Amersham GE Healthcare, Baie d’Urfe, QC).

2.3 ³H-Oleic acid uptake assay

GLUTag cells were plated with DMEM + 10% FBS in 24-well plates coated with poly-D-lysine hydrobromide (Sigma-Aldrich) and allowed to recover for 24 h. Cells were then serum-starved by replacing the media with FBS-free DMEM and allowed to recover overnight. To prepare the treatment media, ³H-OA (3.0 µCi/mL; specific activity 53 Ci/mmol) and ¹⁴C-mannitol (0.6 µCi/mL; specific activity 55 mCi/mm) (Moravek Biochemicals Inc., Brea, CA) were added to CaCl₂-free DMEM media (Gibco Invitrogen) containing 0.5% fatty-acid free
(FAF)-bovine serum albumin (BSA) (Sigma-Aldrich). In some experiments, 500 µM or 1000 µM unlabeled-OA (100 mM stock in EtOH; Sigma-Aldrich), 200 µM phloretin (20 mM stock in EtOH; Sigma-Aldrich), or 400 µM sulfo-N-succinimidyl oleate (SSO; 0.4 M stock in DMSO; Toronto Research Chemicals, North York, ON) was added to the treatment media. The maximum final concentrations of EtOH and DMSO in the media were 1.6% and 0.1%, respectively. Lastly, CaCl$_2$ (0.5 M stock solution) was added back to the media with gentle shaking at room temperature at a final concentration of 1.8 mM. Cells treated with phloretin or SSO were pre-incubated with media containing only 200 µM phloretin or 400 µM SSO, respectively, for 30 min at 37 C prior to start of the uptake assay.

Immediately preceding the assay, cells were briefly washed twice with 500 µL of Hank’s balanced salt solution (HBSS) before receiving 130 µL of treatment media and incubated at 37 C. At t = 5, 15, 30, 45 and 60 min, the media was removed and the cells were briefly washed twice with 400 µL of HBSS containing 0.5% FAF-BSA. The time course of the assay was later extended to include the time points of t = 30, 45, 53, 60, 90 and 120 min. Following this, 200 µL of ice cold 1.0 M KOH (Sigma-Aldrich) was added to dissociate the cells from the plate. An aliquot of 100 µL of each sample was used to measure radioactivity. Each 100 µL aliquot received 3 mL of Ultima Gold XR scintillation fluid (Perkin Elmer, Woodbridge, ON) and was counted using a beta counter. The isotope windows were set at $^3$H = 0-8 eV and $^{14}$C = 35-156 keV. The remaining 100 µL of each sample was sonicated to lyse the cells and the protein concentration was measured by Bradford assay (Bio-Rad).

The $^3$H-OA uptake assay required optimization in order to generate reproducible and accurate data. The following are key points of the optimization process. FAF-BSA (0.5%) was added to the final HBSS wash to help remove any $^3$H-OA adsorbed to the surface of the cells,
thus avoiding artificially increasing CPM values. The isotope windows were optimized and set to $^3\text{H} = 0-8$ keV and $^{14}\text{C} = 35-156$ keV to prevent any overlap in isotope counting, thus again avoiding any artificial increase in CPM values. The more ideal solvent for $^3\text{H}$-OA was determined to be pure EtOH since 0.5 M NaOH appeared to interfere with basal levels of $^3\text{H}$-OA uptake. The GLUTag cells tolerated EtOH up to a final concentration of 3%. Uptake in GLUTag cells was also greater when using in DMEM at 37°C in the incubator, as opposed to HBSS at room temperature, for the duration of the 60 min assay. Lastly, $^3\text{H}$-OA was used in preference to the fluorescent fatty acid analog C$_1$-Bodipy-C$_{12}$, as the analog is not identical to OA.

2.4 GLP-1 secretion assay

GLUTag cells were grown in 24-well plates coated with poly-D-lysine hydrobromide (Sigma-Aldrich) and NCI-H716 cells were grown in 24-well plates coated with Matrigel (Becton Dickinson). Cells were allowed to recover for 48 h prior to the secretion assay. All treatments were made up in CaCl$_2$-free DMEM media (Gibco Invitrogen) containing 0.5% FAF-BSA (Sigma-Aldrich). CaCl$_2$ (0.5 M stock solution) was added back to media at a final concentration of 1.8 mM prior to treating the cells. Cells were washed twice with HBSS and then treated with media containing 1 μM phorbol 12-myristate 13-acetate (PMA; 100 μM stock in EtOH; positive control; Sigma-Aldrich), 150-1500 μM OA (Sigma-Aldrich) or DMEM media alone (negative control). OA stock solutions were prepared in 0.5 M NaOH (40 mM stock), pure EtOH (100 mM stock) or Krebs-Ringer bicarbonate buffer containing 10% FAF-BSA (8 mM stock), as dictated by the experiment. Some cells were pre-treated for 30 min with 200 μM phloretin (20 mM stock in EtOH; Sigma-Aldrich) or 400 μM SSO (0.4 M stock in
DMSO; Toronto Research Chemicals) prior to the start of the secretion assay. These same compounds were added to the treatment media, as appropriate. Cells were incubated with treatments for 2 h. At the end of the incubation period, the media was collected, centrifuged at 1300xg for 10 min, and 1% trifluoroacetic acid was added to the supernatants. Cells were scraped into extraction buffer (1N hydrochloric acid containing 5% formic acid, 1% trifluoroacetic acid and 1% sodium chloride) and lysed by sonication. Peptides from both media and cell samples were eluted by reversed-phase extraction using C18 Sep-Pak cartridges (Waters Associates, Milford, MA). Samples were then subjected to a radioimmunoassay (RIA), as previously described (84;97), where GLP-1 was detected using an antibody that recognizes the carboxy terminal of GLP-1<sup>7-36NH<sub>2</sub></sup> (Enzo Life Sciences, Farmingdale, NY). GLP-1 secretion was calculated as the amount of GLP-1 detected in the media, normalized to total GLP-1 in the media and cells combined, and expressed as percent of negative control. Total GLP-1 content, an indicator of cell viability, did not significantly differ between the various treatments. In the GLUTag cells, total GLP-1 cell content (media plus cells) of cells treated with vehicle control (DMEM + 0.5 M NaOH) was 381 ± 60 pg/mL (n = 10) and of those treated with 1000 µM OA was 423 ± 62 pg/mL (n = 9). In NCI-H716 cells, total cell content of cells treated with vehicle control (DMEM + 0.5 M NaOH) was 917 ± 43 pg/mL (n = 8) and of those treated with 1000 µM OA was 958 ± 95 pg/mL (n = 8).

2.5 FATP4 null mouse model

All animal protocols were approved by the Animal Care Committee at the University of Toronto. Fatp4<sup>−/−</sup>;Ivl-Fatp4<sup>tg/+</sup> mice obtained from Dr. Jeff Miner (Washington University, St. Louis, MO) (141) were on a mixed 129/B6/CBA strain background. Re-expression of
transgenic FATP4 in only the skin of null mice was required to prevent the neonatal lethality of the whole-body FATP4 knockout. Both Fatp4<sup>+/−</sup>;Ivl-Fatp4<sup>tg/+</sup> mice and Fatp4<sup>+/−</sup> mice (lacking the Ivl-Fatp4 transgene) were used as control mice. Studies were conducted using both female and male paired littermates at 12-22 weeks of age and the results were combined. Following an overnight fast, mice were anesthetized with isofluorane and blood samples (50-100 µL) were obtained from the saphenous vein. A laparotomy exposed the intestines and 200 µL of 125 mM OA (technical grade 90%; Sigma-Aldrich) in 125 mM Tween-80 (Sigma-Aldrich) was injected directly into the lumen of the ileum in an oral direction. Blood samples (200-500 µL) were then obtained via a cardiac puncture at either 15 or 60 min. All blood samples were collected into a 10% volume of TED (50 mL trasylol, 1.2 g EDTA, 3.4 mg diprotin-A, 50 mL H<sub>2</sub>O), spun down to collect the plasma, and stored at -80 C. Total GLP-1 levels were measured in the collected plasma using the Total GLP-1 (ver. 2) Assay Kit (Meso Scale Discovery, Gaithersburg, MD). The detection limit of this kit was 0.98 pg/mL. Ileal tissue sections (2 cm) were collected into RIPA buffer, sonicated and spun down. Protein concentrations of the supernatants were measured by Bradford assay (Bio-Rad) and levels of FATP4 proteins were determined via immunoblot, as described above.

### 2.6 Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using SAS software (SAS Institute, Cary, NC) using one- or two-way ANOVA, followed by a Student’s t test or one-way ANOVA post hoc analysis, as appropriate. A Student’s t test was used to compare the levels of protein in the siRNA knockdown studies. Some of the data was log10 transformed to normalize variances. Significance of data was assumed at P < 0.05.
3. RESULTS

3.1 Expression of fatty acid transport proteins in the L cell.

Expression of mRNA transcripts for fatty acid transport proteins FATP1, FATP3, FATP4 and CD36 has previously been reported in the murine GLUTag L cell model (94). Expression of these transport proteins has now also been confirmed at the protein level in both murine GLUTag L cells (Fig. 3.1A) and human NCI-H716 L cells (Fig. 3.1B), as determined by immunoblot. The presence of these transport proteins suggests that they may play a role in OA uptake, consistent with previous findings demonstrating the ability of the L cell to take up a fluorescent OA analog (94).

3.2 OA dose-dependently stimulates GLP-1 secretion in murine GLUTag, but not in human NCI-H716, cells.

Treatment of GLUTag cells with increasing concentrations of OA led to a dose-dependent increase in GLP-1 secretion. More specifically, 500 µM and 1000 µM OA increased GLP-1 secretion by 24 ± 9% and 59 ± 9%, respectively (P < 0.05-0.001), as compared to vehicle (0.5 M NaOH)-treated control cells (Fig. 3.2A). In contrast to the findings in GLUTag cells, treatment of human NCI-H716 cells with increasing concentrations of OA in 0.5 M NaOH elicited no GLP-1 response (Fig. 3.2B). Similarly, using different OA solvents (Krebs-Ringer bicarbonate buffer – Fig. 3.2C; pure EtOH – Fig. 3.2D) also did not increase GLP-1 secretion in NCI-H716 cells. These cells did, however, respond when treated with the positive control PMA (in pure EtOH; P < 0.001) (Fig. 3.2D), indicating that they are capable of secreting GLP-1 in response to some stimuli. NCI-H716 cells were therefore not utilized for any future experiments and 0.5 M NaOH was used as the vehicle for all subsequent GLUTag secretion studies.
Figure 3.1 Murine GLUTag and human NCI-H716 L cells express FATP1, 3, 4 and CD36 fatty acid transport proteins. Immunoblot for FATP1 (63 kDa), FATP3 (72 kDa), FATP4 (72 kDa), CD36 (54 kDa - non-glycosylated intracellular form; 88 kDa - glycosylated membrane form) and actin (42 kDa; loading control) in murine GLUTag L cells (A; n = 3) and human NCI-H716 L cells (B; n = 3). Mouse duodenal tissue was used as a positive (+ve) control.
Figure 3.2 OA dose-dependently stimulates GLP-1 secretion in murine GLUTag, but not in human NCI-H716, cells. GLUTag cells were treated with increasing concentrations of OA dissolved in 0.5 M NaOH and secretion of GLP-1 was determined by radioimmunoassay (A; n = 6-11). NCI-H716 cells were treated with OA dissolved in 0.5 M NaOH (B; n = 8), Krebs-Ringer bicarbonate buffer (C; n = 3-4) or pure EtOH (D; n = 3-7). Vehicle alone (control) and PMA (1 µM) were used as negative and positive controls, respectively. GLP-1 secretion is expressed as percent of control. * P < 0.05, *** P < 0.001 versus control, ## P < 0.01 as indicated.
3.3 OA competitively inhibits $^3$H-OA uptake in murine GLUTag cells.

GLUTag cells incubated with $^3$H-OA demonstrated uptake for up to 60 min (Fig. 3.3A; black circles). $^{14}$C-Mannitol served as a cell integrity control; no significant uptake of $^{14}$C-mannitol was observed in any of the treatment groups (Fig. 3.3A; black triangles), confirming that the L cell can specifically take up OA. Uptake of $^3$H-OA was competitively-inhibited in a dose-dependent manner by 40 ± 2% and 63 ± 2% at t = 60 min with 500 µM and 1000 µM unlabeled-OA (P < 0.001 versus control; P < 0.01 versus each other), respectively, suggestive of a protein-mediated process. Furthermore, different mechanisms appear to be involved in OA uptake by the L cell, as indicated by an increase in the slope of the line of the control uptake curve at t = 45 min (t = 0-45 min: 0.55 ± 0.06; t = 45-60 min: 1.77 ± 0.17; P < 0.001) (Fig. 3.3B; black circles). This same increase in slope was not observed when cells were treated with 1000 µM unlabeled-OA (Fig. 3.3B; white circles). To confirm linear $^3$H-OA uptake between t = 45-60 min, the uptake assay was extended to 120 min and an additional time point was added at t = 53 min (Fig. 3.3B inset).

3.4 SSO decreases $^3$H-OA uptake but does not significantly decrease OA-induced GLP-1 secretion in GLUTag cells.

Sulfo-N-succinimidyl oleate (SSO) is a specific inhibitor of CD36 (155). Treatment of GLUTag cells with 400 µM SSO reduced their ability to take up $^3$H-OA by 36 ± 8% at t = 60 min only (P < 0.001). As observed previously, OA (1000 µM) decreased $^3$H-OA uptake at t = 15-60 min (P < 0.05-0.001) (Fig. 3.4A; black circles). No significant uptake of the cell integrity control $^{14}$C-mannitol was observed in any of the treatment groups (Fig. 3.4A; black triangles). The ability of OA to induce GLP-1 secretion in GLUTag cells was not, however, significantly decreased by treatment with SSO (Fig. 3.4B). These findings suggest that, while
CD36 may play a role in late OA uptake in the L cell, it is not required for OA-induced GLP-1 secretion.
Figure 3.3 OA competitively inhibits $^3$H-OA uptake in murine GLUTag cells.
GLUTag cells were incubated with $^3$H-OA and treated with vehicle (control) or 500 µM or 1000 µM unlabeled-OA to determine competitive inhibition of $^3$H-OA uptake (black circles). $^{14}$C-Mannitol was used as a cell integrity control in each treatment group (black triangles) (A; n = 6). The slopes of the line for the control curve (black circles) and the 1000 µM OA curve (white circles) were calculated from 0-45 min and from 45-60 min (B; n = 18). Inset: the uptake assay was extended to 120 min for both $^3$H-OA (black circles – control; white circles – 1000 µM OA) and $^{14}$C-mannitol (black triangles) (n = 8). Counts per minute (CPM) were normalized to total protein. ** P < 0.01, *** P < 0.001 versus control or as indicated. ## P < 0.01, ### P < 0.001 for 500 µM versus 1000 µM OA. N.S. (Not significant).
Figure 3.4 SSO decreases $^3$H-OA uptake but does not significantly decrease OA-induced GLP-1 secretion in GLUTag cells. GLUTag cells were incubated with $^3$H-OA and treated with vehicle (control) or 1000 µM unlabeled-OA or 400 µM SSO, a specific inhibitor of CD36 (*black circles*). $^{14}$C-Mannitol was used as a cell integrity control in each treatment group (*black triangles*). Counts per minute (CPM) were normalized to total protein (A; n = 6). GLUTag cells were treated with either vehicle (control) or 400 µM SSO and were incubated with or without 1000 µM OA. GLP-1 secretion was determined by radioimmunoassay (B; n = 11-12). * P < 0.05, *** P < 0.001 versus respective control.
3.5 Phloretin decreases $^{3}$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells.

Phloretin is a non-specific inhibitor of carrier-mediated transport (149;158). Treating GLUTag cells with 200 µM 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). As in previous assays, OA (1000 µM) decreased $^{3}$H-OA uptake at t = 5-60 min (P < 0.01-0.001) (Fig. 3.5A; black circles) while no significant uptake of the cell integrity control $^{14}$C-mannitol was observed in any of the treatment groups (Fig. 3.5A; black triangles). Incubation of the cells with OA (1000 µM) in the presence of phloretin (200 µM) reduced OA-induced GLP-1 secretion from 137 ± 21% to 46 ± 14% above control values, as compared to cells incubated with OA in the absence of the inhibitor (P < 0.01) (Fig. 3.5B). Thus, taken together, phloretin may decrease GLP-1 release by affecting OA uptake in the L cell.

3.6 FATP4 knockdown decreases $^{3}$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells.

The most abundant FATP isoform in the small intestine is FATP4 (127). Hence, FATP4 was knocked down in GLUTag cells using FATP4-targeting siRNA. Two different siRNA sequences and approaches were taken to optimize the knockdown, leading to 20 ± 3% (first knockdown; P < 0.01) (Fig. 3.6A) and 27 ± 6% (second knockdown; P < 0.05) (Fig. 3.7A) reductions in FATP4 protein. Both approaches reduced $^{3}$H-OA uptake at t = 60 min by 32 ± 5% (P < 0.05) (Fig.3.6B) and 28 ± 7% (P < 0.05) (Fig. 3.7B), respectively. However, only the greater knockdown (i.e. 27 ± 6%) reduced OA-induced GLP-1 secretion, as compared to the scrambled siRNA control, decreasing release from 120 ± 29% to 21 ± 21% above control values (P < 0.05) (Fig. 3.6C and 3.7C).
Figure 3.5 Phloretin decreases $^3$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells. GLUTag cells were incubated with $^3$H-OA and treated with vehicle (control) or 1000 µM unlabeled-OA or 200 µM phloretin, a non-specific inhibitor of carrier-mediated transport (black circles). $^{14}$C-Mannitol was used as a cell integrity control in each treatment group (black triangles). Counts per minute (CPM) were normalized to total protein (inset: expanded scale) (A; n = 6). GLUTag cells were treated with either vehicle (control) or 200 µM phloretin and were incubated with or without 1000 µM OA. GLP-1 secretion was determined by radioimmunoassay (B; n = 11-12). * P < 0.05, ** P < 0.01, *** P < 0.001 versus respective control, ## P < 0.01 as indicated.
Figure 3.6 First FATP4 knockdown decreases $^3$H-OA uptake but does not significantly decrease OA-induced GLP-1 secretion in GLUTag cells. GLUTag cells were treated with scrambled or FATP4 siRNA and FATP4 (72 kDa) and actin (42 kDa; loading control) levels were detected by immunoblot. A representative blot is shown (A; n = 4). $^3$H-OA uptake was determined in GLUTag cells treated with scrambled or FATP4 siRNA (black circles). 14C-Mannitol was used as a cell integrity control (black triangles). Counts per minute (CPM) were normalized to total protein (B; n = 8-12). GLUTag cells were treated with scrambled or FATP4 siRNA and were incubated with or without 1000 µM OA. GLP-1 secretion was determined by radioimmunoassay (C; n = 6-8). * P < 0.05, ** P < 0.01, *** P < 0.001 versus respective control.
Figure 3.7 Second FATP4 knockdown decreases $^3$H-OA uptake and OA-induced GLP-1 secretion in GLUTag cells. GLUTag cells were treated with scrambled or FATP4 siRNA and FATP4 (72 kDa) and actin (42 kDa; loading control) levels were detected by immunoblot. A representative blot is shown (A; n = 3). $^3$H-OA uptake was determined in GLUTag cells treated with scrambled or FATP4 siRNA (black circles). $^{14}$C-Mannitol was used as a cell integrity control (black triangles). Counts per minute (CPM) were normalized to total protein (B; n = 4-5). GLUTag cells were treated with scrambled or FATP4 siRNA and were incubated with or without 1000 µM OA. GLP-1 secretion was determined by radioimmunoassay (C; n = 7-8). * P < 0.05, ** P < 0.01 versus respective control, # P < 0.05 as indicated.
3.7 Possible decrease in OA-induced GLP-1 levels in FATP4 null mice.

To further explore the role of FATP4 in OA-induced GLP-1 secretion, a FATP4 null mouse model was utilized. These mice lack FATP4 globally (Fatp4−/−) but have been engineered to specifically re-express FATP4 in only the skin (Fatp4−/−;Ivl-Fatp4tg/+). This is necessary to rescue the mice from the lethality of the FATP4 whole body knockout (141). These Fatp4−/−;Ivl-Fatp4tg/+ FATP4 null mice have been reported to display no compensatory upregulation of other FATP isoforms in the proximal intestine (142). Immunoblotting confirmed the absence of FATP4 in the ileum of FATP4 null mice (Fig. 3.8A). To determine the effect of OA on plasma GLP-1 levels, 125 mM OA was injected directly into the ileum of anesthetized wild-type and FATP4 null mice. Blood samples were collected at t = 0 and 15 min or at t = 0 and 60 min in a paired fashion, and total plasma GLP-1 levels were determined. Although no differences were seen between the groups at t = 0 and 15 min, there was an observed trend towards lower plasma GLP-1 levels in the FATP4 null mice at t = 60 min (Fig. 3.8B). While this data is supportive of a role for FATP4 in OA-induced GLP-1 secretion from the intestinal L cell, further in vivo studies in the FATP4 null mouse model are required to statistically confirm this finding.
Figure 3.8 Possible decrease in OA-induced GLP-1 levels in FATP4 null mice.
Immunoblot for FATP4 (72 kDa) and actin (42 kDa; loading control) in the ileum of wild-type (WT) and FATP4 null (KO) mice (representative of n = 5) (A). 125 mM OA in 125 mM Tween-80 was injected directly into the ileum of wild-type and FATP4 null mice and blood samples were collected at t = 0 and 15 min or at t = 0 and 60 min. Total GLP-1 levels were determined in the collected plasma using an immunoassay system (B; n = 5-15).
4. DISCUSSION

The hormone GLP-1 is a known insulin secretagogue that is released from the intestinal L cell upon nutrient ingestion. The ability of certain nutrients, like the fatty acid OA, to elicit a secretion response has been well studied in cell and animal models (69;80;92-94;97;98). Most notably, OA has also been shown to increase GLP-1 levels in human studies (74;99). The mechanism behind OA-induced GLP-1 secretion, however, has not yet been fully elucidated and the only component known to be necessary is the isozyme PKCζ (94;97). The current study demonstrates that the L cell can specifically take up OA via a carrier-mediated process. Moreover, it shows for the first time the involvement of fatty acid transport proteins, specifically FATP4, in the OA-induced GLP-1 secretion response.

A MUFA-rich diet has been shown to improve glycemic control in subjects with insulin resistance (99). Furthermore, improved glycemic control has been linked to the secretion of GLP-1 from the intestinal L cell (98). The importance of understanding how MUFAs, and especially OA, interact with the L cell to elicit GLP-1 release is vital to understanding this pathway. The ability of fatty acids to activate GPCRs has already been confirmed. Polyunsaturated and saturated fatty acids are known to activate GPR120 (107) and GPR40 (105), respectively, culminating in L cell GLP-1 secretion. The endogenous fatty acid derivative OEA, in turn, increases GLP-1 release through GPR119 (106;111). Although the ability of OA to stimulate a GPCR has not yet been completely ruled out, no increase in intracellular calcium is observed nor are Akt or ERK activated upon stimulation of the L cell with OA. These pathways are linked to Gαq-coupled GPCR activation and the data therefore indicates that GPCRs such as GPR40 and GPR120 are not involved in the OA response (97). In contrast to a cell surface receptor, mRNA transcripts for cell surface fatty acid transport
proteins FATP1, FATP3, FATP4 and CD36 have been reported in the murine GLUTag L cells (94). The current study confirms the presence of these transporters at the protein level in both the murine GLUTag and the human NCI-H716 L cell models, suggesting that they play a role in fatty acid uptake in the L cell and, thus, in the effects of OA on GLP-1 secretion.

The L cell response to fatty acids has been shown to be specific, such that unsaturated fatty acids require a minimum chain length of 16 carbons and saturated fatty acids elicit no GLP-1 secretory response (96). Additionally, non-esterified fatty acids in the circulation have been shown to either decrease or have no effect on GLP-1 secretion, highlighting the importance of luminal fatty acids (159;160). OA is a potent stimulator of L cell GLP-1 release (96;97) and this has been confirmed in the current study. Increasing concentrations of OA increased GLP-1 secretion from the murine GLUTag L cell in a dose-dependent manner. Although there is evidence that an accumulation of free fatty acids in tissues can lead to lipotoxicity and cell dysfunction (161), the highest dose used in this current study (1000 µM OA) is well below the physiological concentration of ileal OA (approximately 105 mM), as per measurement in the rat ileum following an oral gavage of olive oil (94). The dose of 1000 µM OA was used throughout the study and consistently increased GLP-1 secretion by 59-164% above basal levels. In contrast, human NCI-H716 L cells did not prove responsive to OA. Initially, treatment of NCI-H716 cells with increasing doses of OA dissolved in 0.5 M NaOH, as per the GLUTag cell protocol, did not increase GLP-1 secretion above basal levels and indeed, the vehicle alone abolished the response to the positive control PMA. Changing the OA solvent to pure EtOH also had no effect on increasing the OA response, although it did appear to restore the response to PMA. Interestingly, OA-induced GLP-1 secretion in the NCI-H716 cells has previously been reported, wherein 800 µM OA nearly doubled GLP-1 secretion (80).
Replicating this protocol, in which OA was dissolved in Krebs-Ringer bicarbonate buffer, still resulted in a lack of response to OA. Thus, it is possible that the cells used in the current study may have de-differentiated so as to no longer be responsive to OA. As such, all further studies in this project utilized only the murine GLUTag L cell model. However, using only one cell model, and that being an L cell line rather than primary L cells, does impose limitations on the conclusions drawn from the collected data. Using a second species of cells would be ideal to confirm that all observed effects are not species-specific. An alternative to the use of the NCI-H716 cells would be to extend these studies into FRIC cultures to observe whether the same effects are seen in primary rat cells, although it must be noted that these are fetal-derived cells and may therefore also differ from the normal adult L cell. Alternatively, a transgenic mouse model has been previously described that expresses the yellow-fluorescent protein Venus in proglucagon-expressing cells (162). By using primary intestinal cultures that clearly contain Venus-labeled L cells, it would thus be possible to study the effects of OA on primary adult mouse L cells, as an alternative to using FRIC cultures.

It was previously reported that the L cell can take up the fluorescent OA analog C₁-Bodipy-C₁₂ (94). The present study has shown, for the first time, that the L cell demonstrates specific uptake for OA for up to 60 min. Additionally, radiolabeled ³H-OA uptake was competitively inhibited in a dose-dependent manner by unlabeled-OA. Unlabeled-OA was used at a dose of 1000 µM in all uptake studies and consistently decreased ³H-OA uptake by 63-73%, as compared to control uptake. These findings are supportive of a carrier-mediated process in which transport proteins may be involved. Competitive inhibition of fatty acid uptake has been reported in other cell models as well. One study in particular demonstrated that C₁-Bodipy-C₁₂ uptake is competitively decreased in 3T3-L1 adipocytes such
that treatment with palmitate, at 0.1 to 100 µM, dose-dependently decreased C1-Bodipy-C12 uptake, as determined following a 10 min incubation (163). Thus, protein-mediated uptake of fatty acids potentially occurs not only in the L cell but in other cell types as well.

To explore the possibility of CD36 involvement in OA-uptake and GLP-1 secretion, the CD36-specific inhibitor SSO was utilized (155). SSO decreased 3H-OA uptake at 60 min in GLUTag cells by 36 ± 8%. This is consistent with a study conducted in salmon hepatocytes, wherein SSO also decreases OA uptake, though only by about 8% (164). SSO-reduced LCFA uptake has also been reported in rat cardiac myocytes (by up to 65%) (165) and in rat adipocytes (by up to 70%) (166). However, despite decreasing OA uptake at 60 min in the present study, SSO did not significantly decrease OA-induced GLP-1 secretion. Notwithstanding, these findings do not preclude the involvement of CD36 in L cell GLP-1 secretion and need to be extended to draw more significant conclusions. Hence, the use of siRNA to knockdown CD36 in GLUTag cells is one possible option. A study conducted in HBMEC cells, a model of the blood-brain barrier, showed that the knockdown of CD36 with siRNA reduces OA transport across the monolayer (150). This finding supports the notion of using CD36 siRNA to further study the role of CD36 in the L cell. Alternatively, the CD36 null mouse model has been developed (146) and may be another approach to further explore the role of CD36 in OA uptake and in OA-induced GLP-1 secretion.

To study the involvement of fatty acid transport proteins in general, the non-specific inhibitor of carrier-mediated transport phloretin has been used in a number of studies (149;164;165). In the present study, phloretin decreased OA uptake at both early and late time points during the assay by 38 ± 4 and 14 ± 4%, respectively. A similar phloretin effect was previously observed in salmon hepatocytes, wherein phloretin reduced OA uptake by
approximately 28% (164). Transport of OA across HBMEC cells is also reduced upon phloretin treatment (149). In the present study, phloretin treatment also affected OA-induced GLP-1 release, an event that is presumed to be downstream of OA uptake. This data suggests that carrier-mediated transport is necessary for OA uptake and its downstream effects on GLP-1 secretion. A study in cardiac myocytes demonstrated that phloretin affects not only palmitate uptake but also subsequent palmitate oxidation (165). A limitation of phloretin is that it is a non-specific inhibitor (158) and, therefore, it was additionally necessary to use more specific approaches to target individual fatty acid transport proteins to further establish their role in OA uptake.

The most abundant FATP isoform in the small intestine is FATP4 (127). Although it is not known whether this is the case in the L cell, which would require a detailed proteomic analysis of isolated L cells, two different approaches were taken to knocking down FATP4 with siRNA, resulting in a 20 ± 3% (first knockdown) and 27 ± 6% (second knockdown) decrease in FATP4 protein levels, respectively. Even after numerous rounds of optimization, that included both different siRNA sequences and multiple transfection reagents, all in varying ratios of RNA to carrier (data not shown), the decrease in FATP4 protein was not very profound and is thus a limitation of this experiment. Future studies will include measuring mRNA levels to determine whether the siRNA is effectively decreasing FATP4 mRNA transcript levels. Nonetheless, both the first and second knockdown decreased OA uptake at 60 min in the GLUTag cells, consistent with a reduction in functional FATP4. In a similar study conducted in HBMEC cells, it was shown that knockdown of both FATP1 and FATP4, by 41% and 39%, respectively, decreases the transport of OA across the monolayer (149;150). Additionally, a previous study in GLUTag cells reported that, despite a low level of GPR119 knockdown
(23%), there was a resultant 45% decrease in OEA-induced GLP-1 secretion (106). Thus, low levels of protein knockdown have previously been shown to produce downstream effects in the GLUTag L cell model. The same FATP4 siRNA sequence used to induce the second knockdown in the present study has previously been used to examine FATPs and LCFA transport in endothelial cells. This study linked the vascular endothelial growth factor-B to the upregulation of FATPs, allowing for increased LCFA transport across the endothelial cell layer. Using the FATP4 siRNA sequence in these cells resulted in decreased C1-Bodipy-C12 accumulation. Moreover, transfecting these cells with expression plasmids for FATP3 and FATP4 further increased C1-Bodipy-C12 uptake and this uptake was abolished by an excess of unlabeled-OA (167). These studies support the findings of the current project, wherein both unlabeled-OA and FATP4 knockdown decreased the uptake of \(^3\)H-OA.

In contrast to the consistent effects of FATP4 knockdown on OA uptake by the L cell, the second FATP4 knockdown additionally abrogated OA-induced GLP-1 secretion, whereas the first knockdown did not. It is possible that a certain threshold concentration of OA must enter the L cell before GLP-1 secretion is initiated and that the smaller knockdown failed to abolish OA uptake sufficiently. It must also be mentioned that there may be compensation for OA uptake by other FATP isoforms, either in their basal state or in an upregulated state due to FATP4 knockdown. Further studies will involve immunoblotting for other FATP isoforms following FATP4 knockdown to determine whether compensation is occurring.

A FATP4 null mouse model was utilized to further study the role of FATP4 in the L cell. These mice lack FATP4 globally (Fatp4\(^{-/-}\)) but have been engineered to specifically re-express FATP4 in the skin (Fatp4\(^{-/-}\); lvl-Fatp4tg/\(^{+/-}\)). This is necessary to rescue the mice from the lethality of the FATP4 whole body knockout (141). OA injected directly into the ileum of wild-
type mice increased plasma GLP-1 levels over the time course of 60 min. Although no difference was observed between wild-type and FATP4 null mice at t = 0 and 15 min, a trend suggested decreased GLP-1 levels in FATP4 null mice at t = 60 min, as compared to wild-type mice. These preliminary studies need to be continued in order to completely validate this conclusion and future studies include increasing the n value for this set of experiments. Of note, previous studies using the same FATP4 null mouse model showed that these mice do not display compensatory upregulation of other FATP isoforms in the proximal intestine and additionally proved that FATP4 is not necessary for intestinal lipid absorption (142), although the role of this protein in the intestinal L cell has not previously been determined. Future studies using this mouse model will also include determining whether there is altered expression of CD36 or any other FATP isoforms in the distal intestinal of the FATP4 null animals. Additionally, GLP-1 levels in the ileum of both wild-type and null mice will be measured to establish whether there is any compensation. Taken together, the data suggests that FATP4 is not necessary for normal lipid absorption in the enterocyte but appears to play a role in both OA-uptake and OA-induced GLP-1 secretion in the enteroendocrine L cell.

The subcellular localization of FATP4 has recently come into question. FATP4 was initially thought to be localized to the plasma membrane on the apical side of enterocytes, based upon immuno-staining of the mouse small intestine (127). In contrast, a more recent study localized FATP4 to the ER membrane where, despite its intracellular localization, it still increased the rate of fatty acid uptake. The ability to affect fatty acid uptake from an intracellular position was attributed to its acyl-CoA synthetase activity, such that the addition of a CoA group onto incoming fatty acids decreases intracellular free fatty acid concentrations, thus promoting further uptake of free fatty acids (154). Additional studies are necessary to
resolve the true localization and role of FATP4 in the intestine. More specifically, the subcellular localization of FATP4 in the intestinal L cell needs to be investigated to determine whether its potential role in OA-induced GLP-1 secretion is as a cell membrane transport protein, directly allowing for the uptake of OA into the cell, or whether it drives the uptake of OA through its acyl-CoA synthetase activity by decreasing intracellular free fatty acid concentrations. Future studies will include co-staining for FATP4 and GLP-1 in mouse, rat and human intestinal ileal sections, as well as in subcellular fractions of the GLUTag cells, to attempt to determine its true localization.

Another possible explanation of the current findings is the existence of multiple OA uptake mechanisms in the L cell, due to the presence of several fatty acid transport proteins. Supporting this notion is the observed change in the slope of the line of the control $^3$H-OA uptake curve after $t = 45$ min. The slope of the line significantly increased at $t = 45-60$ min, as compared to the slope of the line at $t = 0-45$ min. This supports the idea that multiple uptake mechanisms may be taking place over the course of the 60 min assay, such that different mechanisms are responsible for OA uptake at early versus late time points. Transport proteins such as CD36 and FATP1 are known to translocate to the plasma membrane from subcellular locations (151;156). It is therefore possible that there is an upregulation of plasma membrane fatty acid transport proteins after $t = 45$ min, which would explain the further increase in OA uptake. Subcellular fractionation experiments over the course of the uptake assay would be necessary to prove that such translocations are occurring. Specifically, exploring whether FATP4 has the ability to translocate to the plasma membrane could help explain why differences in OA uptake and GLP-1 secretion were only observed at $t = 60$ min in the FATP4 knockdown studies and FATP4 null mice, respectively. A limitation of this observation is that
the slope of the line between \( t = 45-60 \) min is composed of only two points. Thus, it would also be necessary to extend the uptake assay past 60 min and/or to add additional time points between \( t = 45-60 \) min to observe what occurs to \(^3\)H-OA uptake and whether this affects the slope of the line. Preliminary data showed that the addition of a time point at \( t = 53 \) min supported the trend of linear \(^3\)H-OA uptake between the time points of \( t = 45-60 \) min.

Additionally, the OA uptake studies were run over the course of 60 min, whereas the \textit{in vitro} GLP-1 secretion studies were completed after a 2 h incubation period. Although this appears as a discrepancy between the uptake and secretion data in terms of the time course, previous work in the GLUTag cells has shown that approximately 60\% of GLP-1 secretion occurs in the first hour of treatment, both in basal and stimulatory conditions (168). Therefore, a decrease in OA uptake, as seen during the 60 min uptake assay, still affects the majority of GLP-1 secretion.

Previous studies exploring the OA-induced GLP-1 secretory pathway have demonstrated a requirement for the isozyme PKC\(\zeta\) (94;97). It is not known whether OA activates PKC\(\zeta\) in the L cell directly or indirectly, although the ability of unsaturated fatty acids, including OA, to activate purified PKC\(\zeta\) has already been established (115). PKC\(\zeta\) was found to be present in the membrane, cytosolic and particulate fractions in GLUTag cells, with the majority of the protein found in the particulate fraction (97). Upon incubation with 500 \( \mu \)M OA, PKC\(\zeta\) levels in the membrane fraction were increased 2-fold, with no effect on the cytosolic and particulate fractions (97). How this affects PKC\(\zeta\) interaction with OA or other components of this pathway is currently unknown. The components downstream and upstream of PKC\(\zeta\) additionally remain to be established. Further studies should address how OA interacts with PKC\(\zeta\) and how its actions lead to the subsequent exocytosis of GLP-1 granules. However, interestingly, a study in enterocytes demonstrated that PKC\(\zeta\) is required for formation of pre-
chylomicron transport vesicles, necessary to transport chylomicrons from the ER to the Golgi body (169). Whether PKCζ plays a role in GLP-1 granule formation is only one of the questions that could be explored in the OA-induced GLP-1 secretory pathway in future studies.

In conclusion, the findings of the present study indicate a role for fatty acid transport proteins, and specifically FATP4, in OA-induced GLP-1 secretion in the intestinal L cell. Endogenous GLP-1 production has been shown to be elevated upon stimulation with MUFAs, such as OA (94;98), and has been implicated in improving glycemic control in insulin resistant patients placed on a MUFA-rich diet (99). Although FATP4 seems to play a role in mediating the effects of OA on the L cell, FATP4 does not appear as a plausible therapeutic target due to its wide-spread distribution in the intestinal enterocytes (127). Instead, this signaling pathway should be furthered explored to find more suitable therapeutic targets that could be manipulated to increase endogenous GLP-1 secretion. Interestingly, mutations in FATP4 were recently described in patients with ichthyosis prematurity syndrome, a condition characterized by premature birth with the infant covered in thick, caseous, desquamating skin and respiratory complications, followed by lifelong dry, thick skin (140). Whether these patients exhibit impaired glycemic control was not explored, nor where GLP-1 levels determined. Nonetheless, an essential role for FATP4 has been established in the skin, as seen in these patients and in FATP4 null mice and it now appears that FATP4 additionally plays a role in mediating OA-induced GLP-1 secretion in the intestinal L cell.
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